does not differ significantly from the normal in the 6- to 11- and 12- to 36-m intervals, and differs from it in the −4- to 5-m interval only on account of one sample's low score, according to Filliben's [*Technometrics* **17**, 111 (1975)] test. The distributions' means do not differ even at the .4 level. Only the 12- to 36-m distribution differs from others in variance, having a slightly but, at the .05 level, significantly higher one.
21. For the nine transects (*16*), $r_{AD.L}$ (*12*) indicates significant ($P < .1$) clinal variation in the 3rd and 36th ($P < .001$), 15th ($P < .01$), and possibly 2nd and 8th ($P < .1$) meters; $r_{AE.L}$ (*12*) indicates significant clinal variation in the 2nd, 8th, and 14th ($P < .05$), 36th ($P < .001$), and possibly 15th ($P < .1$) meters. Discrepancies as to indications of clines between the two environmental measures (*D* and *E*) (*15*, *16*) likely are due to a small number of specimens per transect, and possibly to changes in environment or clines within meter intervals. Results are indeterminate ($.1 < P < .9$) as to clines along other transects (*16*).
22. S. Ekman, *Zoogeography of the Sea* (Sidgwick & Jackson, London, 1953), pp. 244–263.
23. J. L. Cisne, J. Molenock, B. D. Rabe, *Lethaia* **13**, 47 (1980).
24. C. S. Elton, *The Ecology of Invasions by Animals and Plants* (Chapman and Hall, London, 1958), pp. 117–124; also see (*5*), pp. 152–178.
25. D. J. Futuyma, *Evolutionary Biology* (Sinauer, Sunderland, Mass., 1979), pp. 424–426.
26. We thank B. Grimes and D. J. Solomon, who helped with statistical analyses, and W. L. Brown, Jr., J. E. Oliver, R. J. Ross, Jr., F. H. T. Rhodes, J. W. Wells, and anonymous reviewers for constructive criticism. Supported by NSF grants DEB-7509651 and DEB-7803179.

4 January 1980; revised 15 May 1980

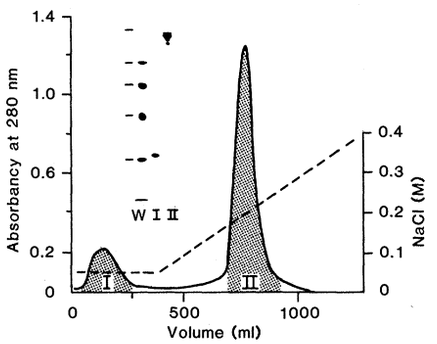
Binding and Factor XIII$_a$–Mediated Cross-Linking of a 27-Kilodalton Fragment of Fibronectin to *Staphylococcus aureus*

Abstract. *A 27-kilodalton tryptic fragment, derived from the amino terminus of the 200-kilodalton fibronectin subunit, inhibited binding of intact fibronectin to* Staphylococcus aureus *and could be cross-linked to* Staphylococcus aureus *by blood coagulation Factor XIII$_a$. Interactions of fibronectin with* Staphylococcus aureus *via this fragment may be important for bacterial opsonization and attachment.*

Fibronectin is a high-molecular-weight glycoprotein that is found in a soluble form in blood and other body fluids; it is found in an insoluble form in connective tissues and is associated with basement membranes (*1*). In cell culture, fibronectin is secreted into the media of cultured adherent cells and is organized into an extracellular fibrillar matrix (*2*). Transformed cells generally lack a fibronectin matrix (*1*). Fibronectin binds to *Staphylococcus aureus* (*3*) and opsonizes this organism for neutrophils (*4*). Fibronectin also binds to collagen, sulfated proteoglycans, hyaluronic acid, fibrin, and gangliosides and is a substrate for blood coagulation Factor XIII$_a$ (plasma transglutaminase) (*1*). As a result of these multiple interactions, fibronectin probably functions as an adhesive and opsonic protein (*1*).

Thrombin (*5*, *6*), plasmin (*7*), and low concentrations of trypsin (*6*) cleave the 200-kilodalton (kd) subunit of human plasma fibronectin into 27-kd and 160- to 180-kd fragments. The 27-kd fragment contains the site or sites of Factor XIII$_a$ transamidation (*6*, *7*) and mediates cross-linking to collagen (*6*). The 160- to 180-kd fragments contain separate sites that mediate binding to collagen (*5*, *6*, *8*), heparin (*9*), and eukaryotic cells (*10*). Sequence analysis places the 27-kd fragment at the NH_2-terminus and the 160- to 180-kd fragments toward the COOH-terminus of the 200-kd fibronectin subunit (*5*). We now report that the binding site for *S. aureus* is in the 27-kd fragment and that both intact fibronectin and the 27-kd fragment can be cross-linked to *S. aureus* by Factor XIII$_a$.

Two-dimensional electrophoretic analysis [isoelectric focusing followed by discontinuous slab polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (*11*)] revealed that the 27-kd fragment of early tryptic digests is the only sizable basic fragment in either early or late tryptic digests of fibronectin; the other fragments are acidic. Therefore, chromatography of the early tryptic digest on diethylaminoethyl (DEAE)-cellulose efficiently separated the 27-kd fragment from 160- to 180-kd fragments and traces of a 31-kd fragment (Fig. 1). The 27-kd fragment could be distinguished from the 31-kd fragment by three criteria in addition to migration in gels: (i) the 27-kd fragment contained the



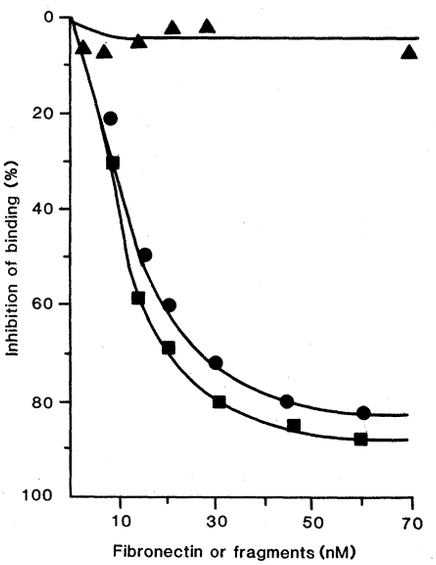


Fig. 1 (left). Chromatographic separation of fragments of lightly trypsinized fibronectin. Plasma fibronectin was purified from a fibrinogen- and fibronectin-rich side fraction of Factor VIII preparation (*6*). The purified protein (6.4 mg/ml) was incubated in tris-buffered saline, *p*H 7.4, with trypsin (1 μg/ml) at 37°C. After 15 minutes, soybean trypsin inhibitor (5 μg/ml) was added. The mixture was diluted threefold with water and applied to a column (5 by 6 cm) of DEAE-cellulose, equilibrated and eluted with a buffer of 0.01*M* tris-chloride and 0.05*M* sodium chloride (*p*H 7.4). The pass-through peak, I, contained the 27-kd fragment (inset). The 160- to 180-kd fragments, along with traces of a 31-kd fragment, were eluted in peak II by a 1-liter, linear (0.05*M* to 0.40*M* sodium chloride) gradient. The fractions were concentrated by precipitation with 90 percent saturated ammonium sulfate, dissolved in and dialyzed against tris-buffered saline, and stored at −70°C. Peak fractions were analyzed after reduction by discontinuous slab polyacrylamide gel electrophoresis (inset). The migration of the fragments was compared to the migration of size standards (*W*): (from top to bottom) fibronectin, 200 kd; phosphorylase, 93 kd; albumin, 68 kd; ovalbumin, 43 kd; and chymotrypsinogen, 24.5 kd. Fig. 2 (right). Inhibition of binding of ^{125}I-fibronectin to *S. aureus* by fibronectin fragments. Varying concentrations of 27-kd or 160- to 180-kd fibronectin fragments or intact fibronectin were incubated for 2 hours at 22°C with lyophilized *S. aureus* (0.4 mg/ml; approximately 0.8×10^8 bacteria per milliliter) in tris-buffered saline (*p*H 7.4) containing 0.1 percent albumin. ^{125}I-Labeled fibronectin (3 n*M*) was added, the mixtures were incubated for an additional 2 hours at 22°C, and bound and unbound ^{125}I-labeled fibronectin were separated by centrifugation ($10^4 g$ for 2.5 minutes) in a Microfuge. The labeling in both the supernatant and the top of the Microfuge tube, which contained the bacteria, was determined in a gamma counter. In the absence of unlabeled fibronectin or the 27-kd fragment, 10 percent of the ^{125}I-labeled fibronectin bound to the bacteria. Concentrations of intact fibronectin and fibronectin fragments were calculated from absorbance of 280 nm, based on the extinction coefficient of intact fibronectin (*16*). (●) Intact fibronectin; (■) 27-kd fragment; and (▲) 160- to 180-kd fragments.

0036-8075/80/0822-0927$00.50/0

site (or sites) of transamidation (*6*); (ii) the 31-kd fragment stained heavily for carbohydrate; and (iii) the 27-kd fragment appeared slightly earlier than the 31-kd fragment during sequential treatment with trypsin (*6*). By the standards shown in Fig. 1, the apparent size of the 27-kd fragment was 24 kd when analyzed by polyacrylamide electrophoresis in sodium dodecyl sulfate without prior reduction, and 27 kd when analyzed by gel permeation chromatography on Sephacryl S-200 in physiological saline.

The 27-kd fragment was as effective as intact fibronectin at inhibiting binding of ^{125}I-labeled fibronectin to *S. aureus*, whereas the mixture of 31-kd and 160- to 180-kd fragments had no inhibitory activity (Fig. 2). Of the fragments of lightly trypsinized ^{125}I-labeled fibronectin the iodinated 27-kd fragment, but not the iodinated 160- to 180-kd and 31-kd fragments, bound to *S. aureus* (Fig. 3). In an experiment like that shown in Fig. 2, we could show no inhibition of binding of ^{125}I-labeled fibronectin (60 ng/ml) to *S. aureus* (0.4 mg/ml) by heparin (2 to 20 μg/ml) or cyanogen bromide fragment α1(I)-CB7 of type I collagen (2 to 20 μg/ml). Trypsinogen and cytochrome c, two basic proteins of approximately the same size as the 27-kd fragment, also did not inhibit binding of ^{125}I-labeled fibronectin to *S. aureus*.

Bound intact ^{125}I-labeled fibronectin and the iodinated 27-kd fragment both could be cross-linked by Factor $XIII_a$ (Fig. 3). In slots containing bacteria from a complete reaction mixture, labeled protein migrated at the interface of the 3 percent stacking gel and the 10 percent separating gel (size, ~ 500 kd) and at the top of the 3 percent stacking gel (size, > 2000 kd). No cross-linking was seen if Factor XIII or calcium ion was omitted from the reaction mixture. The cross-linking observed when thrombin was omitted from the reaction mixture can be attributed to small amounts of thrombin-activated zymogen in the Factor XIII preparation. In addition to cross-linking of the bound proteins, cross-linked fibronectin dimers were seen in the slot containing unbound ^{125}I-labeled fibronectin from the complete reaction mixture (Fig. 3).

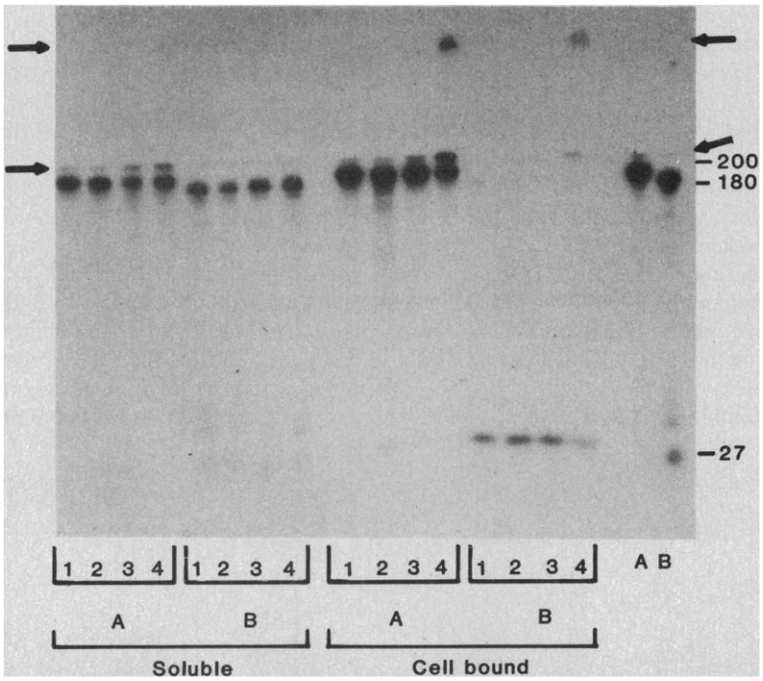


Fig. 3. Binding and cross-linking of ^{125}I-labeled fibronectin or ^{125}I-labeled 27-kd fibronectin fragment to *S. aureus*. Plasma fibronectin was iodinated in the presence of a minimal amount of chloramine-T, and the labeled protein was purified by affinity chromatography on gelatin-agarose (*6*). Labeled fibronectin (4 μg/ml; approximately 2.8×10^6 counts per minute per milliliter), was digested with trypsin (1.5 μg/ml) at 37°C for 5 minutes. Digestion was stopped by the addition of soybean trypsin inhibitor (7.8 μg/ml). Untrypsinized (*A*) or lightly trypsinized (*B*) ^{125}I-labeled fibronectin was incubated at 22°C for 60 minutes with lyophilized *S. aureus* in tris-buffered saline containing 0.1 percent albumin. The four incubation mixtures also contained various combinations of human Factor XIII purified (*6*), human thrombin purified (*6*), and calcium ion. Incubation mixture 4 contained Factor XIII (26 μg/ml), thrombin (1 U/ml), and 10 m*M* calcium ion. Incubation mixture 1 lacked calcium ion, incubation mixture 2 lacked Factor XIII, and incubation mixture 3 lacked thrombin. At the end of the incubations, soluble and cell-bound proteins were separated by centrifugation of the bacteria through silicone oil. Soluble and pellet proteins were incubated for 3 minutes at 95°C in a mixture of 2 percent sodium dodecyl sulfate and 2 percent 2-mercaptoethanol, and analyzed by polyacrylamide slab gel electrophoresis. The slab was stained for protein, dried, and subjected to autoradiography. The stacking and running gels consisted of 3 percent and 10 percent polyacrylamide, respectively; the upper arrow points to the top of the stacking gel, and the lower arrow points to the top of the running gel. The sizes of the radioactive bands were estimated in comparison to the same standards used in Fig. 1. Inspection of the gel after protein-staining (not shown) revealed distortion of the 27-kd region in lanes containing pellet proteins. This distortion causes the 27-kd radioactive band in the cell-bound fraction to appear larger than that in the trypsinized digest prior to binding (*B*) or to that in the soluble fraction (*B*, soluble).

Several conclusions follow from these results. First, binding to *S. aureus* is mediated by a site in the NH_2-terminal 27-kd fragment of fibronectin. Second, the binding site of fibronectin for *S. aureus* is distinct from the binding sites for collagen, heparin, and eukaryotic cells, all of which are in other parts of fibronectin (*5, 6, 8, 9, 10, 12*). Third, the bacterial macromolecule to which fibronectin can be cross-linked is apparently large and heterogeneous in size. It is likely that the cross-linking is between the Factor $XIII_a$-reactive glutaminyl residue or residues of fibronectin and primary amino groups of the bacterial macromolecule.

Binding and cross-linking of fibronectin to *S. aureus* may be an important determinant of the host-pathogen relationship. Factor $XIII_a$ would be activated in areas of inflammation and thus would be available to stabilize fibronectin–*S. aureus* complexes. Fibronectin can be cross-linked by Factor $XIII_a$ to fibrin (*13*) and collagen (*14*) and is thought to enhance removal of soluble fibrin and collagen by fixed macrophages (*15*). We find that fibronectin opsonizes *S. aureus* for neutrophils (*4*); this may be a mechanism for clearing *S. aureus* from the body prior to the development of antibody-mediated immunity. Binding and cross-linking of *S. aureus* to tissue fibronectin, however, could contribute to the pathogenicity of *S. aureus* by providing a mode of bacterial attachment.

DEANE F. MOSHER
RICHARD A. PROCTOR
Departments of Medicine and Medical Microbiology, University of Wisconsin, Madison 53706

References and Notes

1. A. Vaheri and D. F. Mosher, *Biochim. Biophys. Acta* **516**, 1 (1978); K. M. Yamada and K. Olden, *Nature (London)* **275**, 179 (1978); E. Pearlstein, L. I. Gold, A. Garcia-Pardo, *Mol. Cell. Biochem.* **29**, 103 (1980); T. M. Saba and E. Jaffe, *Am. J. Med.* **68**, 577 (1980); D. F. Mosher, *Prog. Hemostasis Thromb.* **5**, 111 (1980).

2. L. B. Chen, A. Murray, R. A. Segal, A. Bushnell, M. L. Walsh, *Cell* **14**, 377 (1978); E. Crouch, G. Balian, K. Holbrook, D. Duksin, P. Bornstein, *J. Cell Biol.* **78**, 701 (1978); L. T. Furcht, D. F. Mosher, G. Wendelschafer-Crabb, *Cell* **13**, 263 (1978); K. Hedman, A. Vaheri, J. Wartiovaara, *J. Cell Biol.* **76**, 748 (1978).
3. P. Kuusela, *Nature (London)* **276**, 718 (1978).
4. R. A. Proctor, E. Prendergast, D. F. Mosher, *Clin. Res.* **27**, 650A (1979).
5. M. B. Furie and D. B. Rifkin, *J. Biol. Chem.* **255**, 3134 (1980).
6. D. F. Mosher, P. E. Schad, J. M. Vann, *ibid.*, p. 255.
7. F. Jilek and H. Hörmann, *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1165 (1977).
8. D. D. Wagner and R. O. Hynes, *J. Biol. Chem.* **254**, 6746 (1979).
9. K. M. Yamada, K. Olden, L.-H. E. Hahn, in *The Cell Surface: Mediator of Developmental Processes*, S. Subtenly and N. K. Wessells, Eds. (Academic Press, New York, 1980), p. 43.
10. E. Ruoslahti and E. G. Hayman, *FEBS Lett.* **97**, 221 (1979); L.-H. E. Hahn and K. M. Yamada, *Cell* **18**, 1043 (1979).
11. L. Anderson and N. G. Anderson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5421 (1977).
12. E. Ruoslahti, E. G. Hayman, E. Engvall, in *Tumor Cell Surfaces and Malignancy*, R. O. Hynes and C. F. Fox, Eds. (Liss, New York, in press).
13. D. F. Mosher, *J. Biol. Chem.* **250**, 6614 (1975).
14. _____, P. E. Schad, H. K. Kleinman, *J. Clin. Invest.* **64**, 781 (1979).
15. T. M. Saba, F. A. Blumenstock, P. Weber, J. E. Kaplan, *Ann. N.Y. Acad. Sci.* **312**, 43 (1978); F. Jilek and H. Hörmann, *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1603 (1978); H. Hörmann and V. Jelinić, *ibid.*, in press.
16. M. W. Mosesson and R. A. Umfleet, *J. Biol. Chem.* **245**, 5728 (1970).
17. Supported by NIH grants HL 21644 and HL 24885, a grant from the University of Wisconsin Graduate School, and (to D.F.M.) an established investigatorship of the American Heart Association and its Wisconsin affiliate. We thank P. Olbranz, P. Schad, and R. Johnson for their assistance.

24 March 1980; revised 7 May 1980

Preferred Sites of Strand Scission in DNA Modified by *anti*-Diol Epoxide of Benzo[*a*]pyrene

Abstract. *The sites of DNA modification by the* anti-*diol epoxide of benzo*[a]*pyrene were investigated with the use of a DNA substrate of defined sequence. The modified DNA was labile to strand scission at alkaline and neutral* pH *at guanine, adenine, and cytosine positions.*

Benzo[*a*]pyrene, a potent mutagen and carcinogen is present in combustion products of gasoline and other fuels, and more than 1300 tons are liberated into the atmosphere of the United States per year (*1*, *2*). It requires metabolic conversion for activity in biological systems. One of the oxidative metabolites, the (±) *trans*-7β,8α-dihydroxy-9α,10α-epoxy-7, 8, 9, 10-tetrahydrobenzo[*a*]pyrene (*anti*-BP-diol epoxide) accounts for most of the benzo[*a*]pyrene DNA binding activity in animal feeding studies and in in vitro reactions (*3*, *4*). The *anti*-BP-diol epoxide is mutagenic and carcinogenic (*2*, *5*, *6*).

Incubation of DNA with *anti*-BP-diol epoxide results in breakage of the phosphodiester backbone of DNA (*7*). To determine whether DNA breakage occurred at a preferred nucleotide or nucleotide sequences, we incubated the *anti* isomer of *trans*-BP-diol epoxide with DNA fragments of defined sequence that were terminally labeled with ^{32}P. The reaction products were analyzed on high-resolution, denaturing polyacrylamide gels of the type used for DNA sequencing. As the DNA is labeled at only one end, and as the sequence of the DNA substrate is known, the lengths of the cleavage products should reveal the sites at which strand scission occurs.

A 30-minute incubation of the DNA fragment with *anti*-BP-diol epoxide modified the DNA so that the electrophoretic mobility was reduced (compare lanes 1 and 2 to lane 3 in Fig. 1). The higher the concentration of the *anti*-BP-diol epoxide (in the range from 10 to 100 μg/ml), the greater the observed reduction in the electrophoretic mobility of the DNA. Since the DNA was denatured prior to being layered on the gel, this experiment demonstrates that the phosphodiester bonds are not broken by this treatment.

In contrast, the DNA modified by exposure to *anti*-BP-diol epoxide (90 μg/ml) for 30 minutes was subject to strand scission on subsequent treatment at elevated *p*H. Treatment of the modified DNA with either 0.1*M* NaOH or 1*M* piperidine (Fig. 1, lanes 4 and 5, respectively) for 30 minutes at 90°C resulted in DNA products that migrated more rapidly than did unmodified DNA.

The length of the reaction products provides information regarding the sites of DNA cleavage. Comparison of the electrophoretic mobilities of the short DNA fragments that resulted from treatment of the DNA with *anti*-BP-diol epoxide to those produced in sequencing reactions shows that breakage occurred most frequently at guanine positions (Figs. 1 and 2). Cleavage products were also observed that would correspond to scission at cytosine and adenine and less frequently at thymine positions. The relative rates of strand scission at each nucleotide were determined by measurement of the amount of radioactivity in each band. The amount of radioactivity in bands corresponding to breakage at guanine positions was consistently three to four times higher than that observed for any other base.

Modification of DNA at lower concentrations (10 to 50 μg/ml) of *anti*-BP-diol epoxide for 30 minutes also resulted in lesions that caused strand breakage on alkaline hydrolysis and that were qualitatively similar to those observed at the higher concentration. In contrast, longer incubations (6 hours) with *anti*-BP-diol epoxide resulted in breakage of DNA that was not subject to subsequent alkaline hydrolysis (Fig. 2, lanes 7 and 8). Thus, the phosphodiester bonds were broken during the prolonged reaction. Again, the most frequent site of strand scission was at guanine positions. A similar pattern of DNA breakage was also observed in experiments in which DNA modified in 30-minute reactions with *anti*-BP-diol epoxide at 10.0 μg/ml was separated from the reactants by passage over a Sephadex G-100 column and then incubated for 6 hours more. These experiments demonstrated that modified DNA is labile to strand breakage at neutral *p*H in the absence of the aqueous hydrolysis products of the *anti*-BP-diol epoxide.

From these experiments we conclude that DNA modified by the *anti*-BP-diol epoxide is labile to strand scission both at alkaline and neutral *p*H. The question arises as to what are the modifications that result in the observed cleavage pattern. The principal DNA adduct of the *anti*-BP-diol epoxide is to the exocylic N^2-amino group of guanine (*3*, *4*, *8*, *9*). Formation of an adduct to the N-6 position of adenine has also been reported. Binding of benzo[*a*]pyrene to DNA at guanine and adenine probably accounts for the observed decrease in the electrophoretic mobility of the modified DNA, but it is not evident that such adducts would increase the lability of the phosphodiester bonds adjacent to the modified base. Osborne *et al.* (*10*) and King *et al.* (*11*) suggest that the *anti*-BP-diol epoxide forms an adduct at the N-7 position of guanine and that the lability of the corresponding glycosylic bond is greatly increased even at neutral *p*H. Such a lesion could result in the cleavage at the observed guanine positions. Adducts of the *anti*-BP-diol epoxide to adenine and cytosine residues of DNA have also been reported (*4*, *8*, *9*), but the site of modification is not known. We suggest that adducts at the N-3 position of cytosine and adenine could result in the cleavage pattern observed here. Gamper *et al.* (*7*) suggest that the *anti*-BP-diol epoxide may form phosphotriesters with

0036-8075/80/0822-0929$00.50/0