Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 174-181

A NOVEL OXIDATION OF THE CARCINOGEN N-HYDROXY-N-2-FLUORENYLACETAMIDE CATALYZED BY PEROXIDASE/H_2O_2/Br^-

Clare L. Ritter and Danuta Malejka-Giganti

Department of Laboratory Medicine and Pathology, University of Minnesota and Laboratory for Cancer Research, Veterans Administration Medical Center, (151B), 54th St and 48th Ave S, Minneapolis, Minnesota 55417

Received May 28, 1985

SUMMARY: N-Hydroxy-N-2-fluorenylacetamide, a proximate carcinogenic metabolite of N-2-fluorenylacetamide, is oxidized largely to 2-nitrosofluorene by lactoperoxidase or extract of peroxidative activity of rat uterus in an H_2O_2- and Br^--dependent reaction. Evidence is presented that the oxidizing species includes OBr^- (HOBr). This novel oxidation may be involved in carcinogenesis by N-arylhydroxamic acids.

Mammalian peroxidases, found in the blood and other tissues, are vital to the body's defense systems and to hormone synthesis. However, because of their strong oxidizing potential, they may also activate foreign compounds to mutagenic and carcinogenic agents. Activation is crucial for N-substituted aromatic compounds which may be industrial and environmental pollutants, model carcinogens or drugs. We are especially interested in the oxidation of N-arylacetohydroxamic acids, proximate mammary gland (MG)[1] carcinogens in the rat (1,2). As shown in equation 1, HRP catalyzed le^- oxidation of N-OH-2-FAA to N-$\dot{O}$-2-FAA yielding equimolar 2-NOF and N-AcO-2-FAA (3-5).

$$2\ \text{N-OH-2-FAA} \xrightarrow{\text{HRP}/H_2O_2} 2\ \text{N-}\dot{O}\text{-2-FAA} \longrightarrow \text{2-NOF} + \text{N-AcO-2-FAA} \qquad (1)$$

By contrast, extracts of rat UT or MG, with peroxidative activity solubilized with the cationic detergent Cetab, converted N-OH-2-FAA to large amounts of 2-NOF and little or no N-AcO-2-FAA in the presence of H_2O_2 (6,7). No N-$\dot{O}$-2-FAA was detectable. Since 2-NOF was shown to be the most mutagenic of the N-2-fluorenyl compounds tested in the Ames assay (8,9) and was also

[1]Abbreviations: MG, mammary gland; UT, uterus; HRP, horseradish peroxidase; LPO, lactoperoxidase; G.U., guaiacol unit; Cetab, cetyltrimethylammonium bromide; Cetac, cetyltrimethylammonium chloride; N-OH-2-FAA, N-hydroxy-N-2-fluorenylacetamide; N-$\dot{O}$-2-FAA, nitroxyl free radical of N-OH-2-FAA; 2-NOF, 2-nitrosofluorene; N-AcO-2-FAA, N-acetoxy-N-2-fluorenylacetamide; 2-FAA, N-2-fluorenylacetamide; N-OH-2-FA, N-hydroxy-N-2-fluorenamine; TME, tetramethylethylene; HPLC, high performance liquid chromatography; R_T, retention time; le^-, one electron.

0006-291X/85 $1.50

carcinogenic (10,11), this novel oxidation of N-OH-2-FAA to 2-NOF may be significant in tumorigenesis. In this report, we present evidence for its Br^--dependency and suggest a mechanism.

MATERIALS AND METHODS

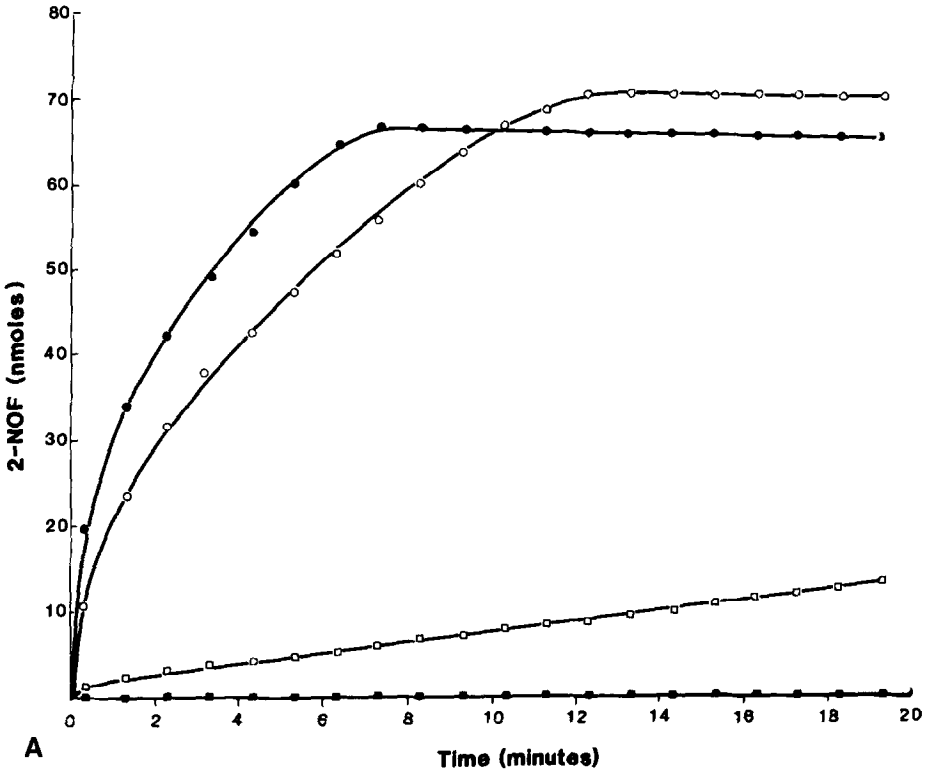
Bovine milk LPO (Sigma Chemical Co., St. Louis, MO) and extracts of peroxidative activity from UT of 56- to 70-day old Sprague-Dawley rats (specific pathogen free) from Harlan Sprague-Dawley, Inc., Madison, WI were used. All buffers were cooled in ice and all procedures were carried out at 4°C unless specified otherwise. Excised UT were freed of fat and mesentery, minced with scissors, thoroughly rinsed in 0.05 M Tris-HCl buffer, pH 7.4, with 0.154 M KCl, homogenized with a Polytron homogenizer (type PT 10, Kinematica GMBH, Lucerne, Switzerland) in 0.01 M Tris-HCl buffer, pH 7.2, or 5 mM Na phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA, and centrifuged at 105,000g. Resuspension of the pellets in 1 mM Tris-HCl or 0.25 M NaCl in 0.1 M Na phosphate buffer, pH 7.4, removed hemoglobin. Extraction with 0.5 M $CaCl_2$ in 0.01 M Tris-HCl buffer, pH 7.2 (12), or 4% Cetab in 0.05 M Na phosphate buffer, pH 7.4 (13), solubilized peroxidative activity. Addition of 5% glycerol stabilized activity of $CaCl_2$ extracts. Cetab extracts were dialyzed 2 h at room temperature and overnight at 4°C against 0.1 M Na phosphate buffer, pH 6.3, and then 20 h against 0.08 M Na phosphate buffer, pH 6.3, containing 20% glycerol and 0.02% Cetab. Activities in both extracts were preserved with -80°C storage. Guaiacol (13 mM) served as hydrogen donor for routine determination of peroxidative activity. Assay of $CaCl_2$ extracts was in 0.01 M Tris-HCl buffer, pH 7.2, with 0.5 M $CaCl_2$ and of Cetab extracts in 0.1 M Na phosphate buffer, pH 7.4, at room temperature. After addition of 0.33 mM H_2O_2 to initiate the reaction, the increase in absorbance at 470 nm was measured for 1 min. A G.U. is the amount of enzyme giving a change of one absorbance unit per sec (13).

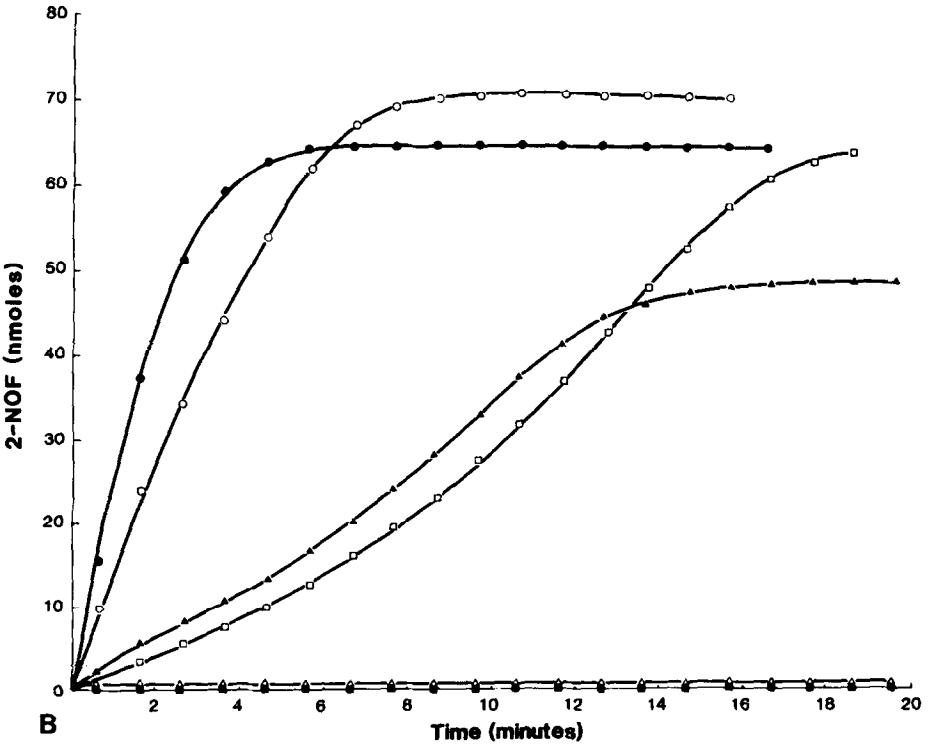
The fluorenyl compounds, N-OH-2-FAA (m.p. 150-1°C), N-OH-2-[1'-^{14}C]FAA (spec. act. 0.37 μCi/μmole), 2-NOF (m.p. 79-81°C), and N-AcO-2-FAA (m.p. 110-1°C), were prepared by the published procedures (14-16). 2-FAA from Aldrich, Milwaukee, WI, was recrystallized from ethanol:water (7:3) (m.p. 196-8°C). The compounds were found to be pure by HPLC.

The products of oxidation of N-OH-2-FAA by LPO and UT extracts were extracted from the incubation mixtures with methyl-*tert* butyl ether (3x). The ethereal extracts were combined, dried over Na_2SO_4, and evaporated under N_2 passed through Oxiclear (Pierce Chemical Co., Rockford, IL). The dried extracts were dissolved in methanol and analyzed with two reversed phase HPLC systems: 1) Zorbax C_8 column; methanol: 0.2 M Na acetate/1 M CH_3COOH, pH 3.8 (2:1), to separate 2-FAA (R_T 5.81), N-AcO-2-FAA (R_T 7.24) and 2-NOF (R_T 12.0); and 2) Golden Series Zorbax C_8 column; mobile phase (17), to separate N-OH-2-FAA (R_T 9.42), 2-FAA (R_T 11.3) and N-AcO-2-FAA (R_T 15.2). The identity and purity of the peaks were established with a photodiode array Hewlett-Packard 1040A Detection System. The compounds were quantified by peak area determination relative to standard curves and corrected for extraction losses.

RESULTS AND DISCUSSION

The dependencies of LPO and the $CaCl_2$ extract of rat UT on H_2O_2 and Br^- in converting N-OH-2-FAA to 2-NOF were similar as shown in Figure 1. With both enzyme systems in the presence of 4% Cetab, the extent of conversion of N-OH-2-FAA to 2-NOF was > 90%. On the other hand, in 4% Cetac the conversion was negligible, suggesting that Cl^- does not substitute for Br^- with either enzyme system. The addition of 4% Cetac together with 0.1 M KBr,


2-NOF (nmoles)
Time (minutes)
A


2-NOF (nmoles)
Time (minutes)
B

which approximates the Br^- content of 4% Cetab, gave about 100% conversion of N-OH-2-FAA to 2-NOF at a rate slightly slower than in the presence of 4% Cetab. In 0.1 M KBr without detergent, the rate of conversion to 2-NOF was decreased. This was not increased by the addition of 4% of the nonionic detergent Tween-20. With a constant concentration of Cetac and saturating levels of peroxidative activity of UT extract, the rate of conversion of N-OH-2-FAA to 2-NOF was dependent on the concentrations of Br^- (data not shown). These results indicated that the conversion to 2-NOF is Br^--dependent and is enhanced by the cationic detergents. Similar rates of generation of 2-NOF by equal G.U. units of LPO and UT extract, lack of conversion either with heat-denatured (boiled) enzyme or in the absence of H_2O_2 and complete inhibition by KCN (data not shown) indicated that the reaction is catalyzed by a peroxidative enzyme.

The products formed from N-OH-2-FAA in incubations containing LPO or UT extract were analyzed by HPLC (Table 1). LPO in the absence of Br^- gave low, but equimolar, amounts of 2-NOF and N-AcO-2-FAA (Expt. #1) implicating a minor amount of oxidation via $1e^-$ mechanism (Equation 1). N-OH-2-FAA was not metabolized by $CaCl_2$ extract of UT in the absence of Br^- (Expt. #4) when peroxidative activity (0.008 G.U.) equaled that of LPO in Expt. #1. However, with much larger amount of peroxidative activity (0.21 G.U.) of $CaCl_2$ extract of UT, a low level of $1e^-$ oxidation of N-OH-2-FAA was shown (6). The addition of 4% Cetab or 4% Cetac + 0.1 M KBr to the UT extract (Expt. #5) or to LPO (Expt. #2) caused 75-86% of the N-OH-2-FAA to be converted to 2-NOF with formation of only trace amounts of N-AcO-2-FAA. Likewise, the major product was 2-NOF when Cetab extracts of UT were incubated with N-OH-2-FAA in the presence of 0.4% Cetab and H_2O_2 (Expt. #6). In the incubation mixtures metabolizing N-OH-2-FAA, small amounts of 2-FAA were also formed. The presence of the amides has been reported previously among the products of chemical (18,19) or enzymic oxidations of N-arylhydroxamic acids (3-5). N-OH-2-FAA incubated with heat-inactivated enzymes and H_2O_2/Br^- (Expts. #3 and 7) was recovered unchanged. To determine if 2-NOF could have arisen from N-AcO-2-FAA,

---

Fig. 1. Formation of 2-NOF from N-OH-2-FAA by LPO (A) and $CaCl_2$ extract of rat UT (B) in the presence of H_2O_2 and Br^-. LPO (5 µl, 0.9 µg protein, 0.008 G.U.) was added to 0.1 M Na^+ phosphate buffer, pH 7.4. $CaCl_2$ extract of rat UT (15 µl, 20 µg protein, 0.008 G.U.), prepared as described in Materials and Methods, was added to 0.5 M NaCl/0.01 M Tris-HCl buffer, pH 7.2. Buffers contained 4% Cetab (●——●), 4% Cetac + 0.1 M KBr (○——○), 0.1 M KBr (□——□), or 4% (w/v) Tween-20 + 0.1 M KBr (▲——▲). N-OH-2-FAA (72 nmoles in 9 µl methanol) and H_2O_2 (90 nmoles in 9 µl) were added to give a final volume of 0.9 ml. Controls contained Cetac with no KBr (△——△) or Cetab with no H_2O_2 or LPO or UT extract immersed in a 100°C bath for 15 min (■——■). Changes in absorbance at λ_{max} (360-370 nm) were monitored continuously for 20 min with a Hitachi 110A Spectrophotometer. Nanomoles 2-NOF were calculated based on ε from a standard 2-NOF solution in buffer with detergents (ε, 18,400) or without (ε, 14,000).

Table 1. Products of Oxidation of N-OH-2-FAA by LPO and UT Extracts

Expt. #	Catalytic System	Reaction medium	Products (nmoles) 2-FAA	N-AcO-2-FAA	2-NOF	Unreacted Substrate (nmoles)	Total Recovery (%)
1	LPO	A	0.50	0.93	1.00	66.5	81.1
2	LPO	A + 4% Cetab or A + 4% Cetac + 0.1 M KBr	1.20	Trace	73.0	1.23	88.7
3	Boiled LPO	A + 4% Cetab	0	0	0	80.3	94.4
4	UT-$CaCl_2$	B	Trace	0	0	87.5	103
5	UT-$CaCl_2$	B + 4% Cetac + 0.1 M KBr	1.50	Trace	64.0	10.2	89.0
6	UT-Cetab	A + 0.4% Cetab	1.64	0.42	47.0	3.12	82.8
7	Boiled UT-Cetab	A + 0.4% Cetab	0	0	0	60.0	95.2
8	LPO	A + 1 or 4% Cetab	1.84	*	0	69.0	87.7**
9	UT-Cetab	A + 0.4% Cetab	0.30	*	0	18.5	101**

Buffers were: A, 0.1 M Na phosphate buffer, pH 7.4, and B, 0.01 M Tris-HCl buffer, pH 7.2, containing 0.5 M NaCl. The incubation mixtures of Expts. 1-5 are described in the legend to Fig. 1. They contained N-OH-2-FAA, 85 nmoles in 8 µl methanol. The incubation mixtures of Expts. 6-7 consisted of extract of UT peroxidative activity (15 µl in 0.08 M sodium phosphate buffer, pH 6.3, containing 20% glycerol and 0.02% Cetab, 0.007 G.U.) and N-OH-2-FAA, 63 nmoles in 8 µl methanol. Total volume was 1.0 ml. N-AcO-2-FAA, 83 and 20 nmoles in 8 µl methanol, was the substrate in Expts. 8 and 9, respectively. The reaction was started by addition of H_2O_2 at 0.1 mM, except for Expt. 1, in which it was at 0.005 mM. When formation of 2-NOF reached its maximum, as determined spectrally (Fig. 1), the incubation mixtures were extracted and extracts analyzed by HPLC as described in Materials and Methods. Values are the means of 2 or 3 determinations with average S.D. of 10%.

*Added as substrate. **Includes small amounts of N-OH-2-FAA.

the acetate ester was incubated with LPO (Expt. #8) or UT-Cetab extract (Expt. #9) and H_2O_2 in media containing Cetab. About 90% of the N-AcO-2-FAA was recovered unchanged which indicated that, if formed, it was stable in these systems.

The results of HPLC analyses confirmed the high levels of conversion of N-OH-2-FAA to 2-NOF determined spectrally (Fig 1). These high levels and the low levels of N-AcO-2-FAA formed indicated that in the presence of Br^-, $1e^-$ oxidation of N-OH-2-FAA did not occur to an appreciable extent. This was confirmed by ESR spectroscopy. Whereas N-O-2-FAA could be easily detected in incubations containing 0.06 G.U. of LPO and 0.03 mM H_2O_2 without Br^-, it was not detected in incubations containing 1% Cetab or 0.1 M KBr under conditions which led to rapid generation of 2-NOF.

2-NOF could conceivably result from oxidation of N-OH-2-FA formed by enzymic deacetylation of N-OH-2-FAA. To determine if deacetylase activity was present in the UT extracts, we used as substrates acetanilide as well as 2-FAA and N-OH-2-FAA (20). We found no deacetylase activity with or without

Table 2. Effect of Chemical Oxidants on Formation of 2-NOF from N-OH-2-FAA

Oxidant	pH	2-NOF (nmoles)
OBr^-	3	183
$OBr^- + H_2O_2$	3	19.0
$Br^- + H_2O_2$	3	0
H_2O_2	3	0
OBr^-	7	134
$OBr^- + H_2O_2$	7	10.7
OBr^-	7	167
OBr^- + TME	7	0
OBr^-	10	185
$OBr^- + H_2O_2$	10	0
BrO_3^-	7	0
$BrO_3^- + H_2O_2$	7	0
O_2 (50% methanol)	7	0
O_2 (0.1 M Na phosphate)	7	0

OBr^- (210 nmoles in 25 µl), generated immediately before use by addition of saturated Br_2 solution to N_2-bubbled 0.01 N NaOH, was added to 2.5 ml N_2-bubbled 0.1 M Na phosphate buffer (pH 3, 7 or 10) in anaerobic reference (R) and sample (S) cuvettes. H_2O_2 (210 nmoles in 22 µl) or TME (2.5 µmoles in 40 µl methanol) was immediately added to both R and S followed by N-OH-2-FAA (190 nmoles in 21 µl methanol) to S. 2-NOF was determined from the increase in absorbance at λ_{max} (360-370 nm, ϵ, 14,000). Values are the means of 2 or 3 determinations with average S.D. of 15%. OBr^- was estimated by addition of KI to buffers and titration with Na thiosulphate. Absorbance was likewise monitored with $KBrO_3$ (0.1 M) with or without H_2O_2 (0.1 mM). Solutions containing KBr (0.1 M) or bubbled with O_2 (1 bubble/sec) were incubated 3.5 h at room temperature and absorbance monitored.

added Br^-. Further, the deacetylase inhibitor NaF did not inhibit the H_2O_2/Br^--dependent oxidation of N-OH-2-FAA to 2-NOF. These results indicated that a deacetylase was not involved in the conversion of N-OH-2-FAA to 2-NOF by UT extract/H_2O_2/Br^-.

We propose that LPO or a peroxidative enzyme(s) in UT extract in the presence of H_2O_2 oxidizes Br^- to OBr^- (HOBr) which then oxidizes N-OH-2-FAA to 2-NOF. LPO is known to oxidize Br^- and I^- (21). Br_2 in dilute NaOH rapidly oxidized N-OH-2-FAA to 2-NOF in buffers, pH 3-10 (Table 2). Addition of TME prevented oxidation by the enzymic and chemical oxidizing systems, apparently by trapping OBr^- (22). Addition of H_2O_2, which reportedly converts OBr^- to singlet oxygen (23), resulted in losses of absorbance of OBr^- and ability to oxidize N-OH-2-FAA (Table 2). Hence, OBr^- (HOBr), but not singlet oxygen, is involved in oxidation of N-OH-2-FAA. No oxidation to 2-NOF occurred when N-OH-2-FAA in buffer or 50% methanol was bubbled with O_2

or when incubated with Br^- and/or H_2O_2 at pH 3.0 for several hours. Likewise, BrO_3^- ± H_2O_2 did not oxidize N-OH-2-FAA.

In oxidations catalyzed by peroxidases, it is unknown if the oxidizing species acts as an enzyme-hypohalous acid complex or as a free hypohalous acid according to Equation 2:

$$E + H_2O_2 \longrightarrow EO + H_2O \xrightarrow{Br^-} [EOBr] \longrightarrow E + OBr^- \qquad (2)$$

where E denotes enzyme and EO its peroxide derivative (21). Incubation of LPO (0.011 G.U.) and 0.13 mM H_2O_2 in 1.0 ml total volume with 0.1 M Na phosphate buffer, pH 7.4, containing 1% Cetab and 91 nmoles N-OH-2[1'-^{14}C]FAA resulted in complete conversion to 2-NOF in 20 min. After extraction of 2-NOF with methyl-tert butyl ether (4x), the aqueous phase contained 100% of the radioactivity indicating the presence of labeled acetyl group. This suggests the following reaction:

$$\text{N-OH-2-[1'-}^{14}\text{C]FAA} + OBr^- \longrightarrow \text{2-NOF} + CH_3{}^{14}COO^- + Br^- \qquad (3)$$

Based on the mechanism of oxidation of N-arylhydroxamic acids with H_2O_2 (24), we propose that N-OH-2-FAA undergoes oxidative cleavage to acetic acid and a hypothetical intermediate, N-bromo-N-2-fluorenylhydroxylamine, which would decompose to 2-NOF and Br^-. There is no evidence that N-OH-2-FA is an intermediate in this oxidation, as proposed for chloroperoxidase-catalyzed oxidation of N-arylamines to the nitroso compounds (25,26).

The levels of Br^- and H_2O_2 and of halide-oxidizing peroxidases in mammals suggest that the above oxidation of N-OH-2-FAA to 2-NOF, as well as oxidation of similar compounds, could occur in vivo. The mechanism by which 2-NOF is carcinogenic and mutagenic is unknown, although it could provide a source of 2-FA-DNA adducts which are the major adducts formed in vivo after administration of N-OH-2-FAA (27).

ACKNOWLEDGEMENTS

We thank Dr. Jed F. Fisher, Dept. of Chemistry, University of Minnesota, for the helpful discussions concerning mechanism of the reaction. We also thank Ms. Jean Suilman and Mr. Richard Decker for technical assistance. Supported by PHS Grant CA-28000 awarded by the National Cancer Institute, DHHS, and U.S. Veterans Administration.

REFERENCES

1. Malejka-Giganti, D., Rydell, R.E. and Gutmann, H.R. (1977) Cancer Res. 37, 111-117.
2. Allaben, W.T., Weeks, C.E., Weis, C.C., Burger, G.T. and King, C.M. (1982) Carcinogenesis 3, 233-240.
3. Bartsch, H. and Hecker, E. (1971) Biochim Biophys. Acta 237, 567-578.
4. Floyd, R.A., Soong, L.M. and Culver, P.L. (1976) Cancer Res. 36 1510-1519.

5. Ritter, C.L., Malejka-Giganti, D. and Polnaszek, C.F. (1983) Chem.-Biol. Interactions. 46, 317-334.
6. Malejka-Giganti, D. and Ritter, C.L. (1985) In: Proceedings of the International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules, Gorrod, J.W., and Damani, L.A., eds., Ellis Horwood Ltd., Chichester, (in press).
7. Ritter, C.L. and Malejka-Giganti, D. (1985) Proc. Am. Assoc. Cancer Res. 26, 105.
8. Ames, B.M., Gurney, E.G., Miller, J.A. and Bartsch, H. (1972) Proc. Nat. Acad. Sci. USA 69, 3128-3132.
9. Ames, B.N., Lee, F.D. and Durston, W.E. (1973) Proc. Nat. Acad. Sci. USA 70, 782-786.
10. Miller, E.C., McKechnie, D., Poirier, M.M. and Miller, J.A. (1965) Proc. Soc. Exp. Biol. Med. 120, 538-541.
11. Miller, J.A. and Miller, E.C. (1969) Progr. Exp. Tumor Res. 11, 273-301.
12. Lyttle, C.R. and DeSombre, E.R. (1977) Proc. Nat. Acad. Sci. USA 74, 3162-3166.
13. Wagai, N. and Hosoya, T. (1982) J. Biochem. 91, 1931-1942.
14. Miller, E.C., Miller, J.A. and Hartmann, H.A. (1961) Cancer Res. 21, 815-824.
15. Barry, E.J., Malejka-Giganti, D and Gutmann, H.R. (1969/70) Chem.-Biol. Interactions 1, 139-155.
16. Zieve, F.J. and Gutmann, H.R. (1971) Cancer Res. 31, 471-476.
17. Ryzewski, C.N. and Malejka-Giganti, D. (1982) J. Chromatogr. 237, 447-456.
18. Forrester, A.R., Ogilvy, M.M. and Thomson, R.H. (1970) J. Chem. Soc. C, 1081-1083.
19. Bartsch, H., Traut, M. and Hecker, E. (1971) Biochim. Biophys. Acta 237, 556-566.
20. Santti, R.S. and Hopsu-Havu, V.K. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 753-766.
21. Morrison, M. and Schonbaum, G.R. (1976) Ann. Rev. Biochem. 46, 861-888.
22. Foote, C.F., Wexler, S., Ando, W. and Higgins, R. (1968) J. Am. Chem. Soc. 90, 975-981.
23. Piatt, J. and O'Brien, P.J. (1979) Eur. J. Biochem. 93, 323-332.
24. Challis, B.C. and Challis, J. (1970) The Chemistry of Amides, Zabicky, J., ed., pp. 786-794., Interscience Publishers, London.
25. Corbett, M.D., Chipko, B.R. and Baden, D.G. (1978) Biochem. J. 175, 353-360.
26. Corbett, M.D., Chipko, B.R. and Batchelor, A.O. (1980) Biochem. J. 187, 893-903.
27. Kriek, E. (1974) Biochim. Biophys. Acta 355, 177-203.