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Role of sulfotransferase from rat liver in the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in *Salmonella typhimurium*

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Studies on the metabolism of 2-acetylaminofluorene (2-AAF) have provided the basis for much of our current understanding of aromatic amine and amide carcinogenesis [1–3]. This compound, 2-AAF, is readily *N*-hydroxylated, both *in vivo* and *in vitro*, by liver enzymes of most rodents to the more potent carcinogen *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-2-AAF) [4–7]. Several findings indicate that the ultimate carcinogen(s) is one or more of the metabolites of *N*-hydroxy-2-AAF, rather than the compound itself [8, 9]. Metabolism of *N*-hydroxy-2-AAF occurs principally in the liver, where it is converted to various reactive electrophiles. The enzyme systems implicated include sulfotransferase(s), deactylase(s), *trans*-acetylase(s) and UDP-glucuronyl transferase(s) [1–3, 9–13]. The relative importance of each of these processes in the conversion of *N*-hydroxy-2-AAF to the ultimate carcinogenic species is not clear.

The sulfate ester of *N*-hydroxy-2-AAF, a strongly electrophilic species, appears to be a metabolite of major importance in the induction of hepatic tumors [9]. Analysis of the hepatic macromolecular bound fluorene derivatives after administration of 2-AAF or *N*-hydroxy-2-AAF *in vivo* indicates, however, that other enzymes in addition to sulfotransferase may contribute to the initiation of liver cancer by these chemicals. For example, after administration of 2-AAF or *N*-hydroxy-2-AAF to rats, 1- and 3-methionyl-2-AAF are the fluorene derivatives covalently bound to liver protein [9]. The major product liberated after hydrolysis of rat liver RNA is *N*-(guanosin-8-yl)-2-acetylaminofluorene and smaller amounts of the 2-aminofluorene analog [4, 14, 15]. In contrast, the major adduct of DNA is *N*-(deoxyguanosin-8-yl)-2-aminofluorene, with smaller amounts of the acetylated form *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene [14–16]. These data suggest that the acetyl group is lost from 2-AAF and *N*-hydroxy-2-AAF before or during arylation of DNA. Moreover, *N*-hydroxy-2-AAF can cause cancer in some tissues which lack sulfation activity toward this *N*-hydroxy derivative [9, 17].

Recently Ames *et al.* [18] developed a simple bacterial assay for detecting mutagenicity of chemicals. In this assay, approximately 90 per cent of the carcinogens tested were mutagens. When examining the mutagenicity of potential metabolites of 2-AAF in this system, Durston and Ames [19] found that 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene were 200–300 times more mutagenic than *N*-hydroxy-2-AAF. When the 9000 *g* supernatant fraction (S-9) from rat liver was included in the assay, the mutagenicity of *N*-hydroxy-2-AAF increased almost 100-fold, whereas mutagenicity of 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene either decreased or remained the same. Felton *et al.* [20] have shown that both microsomes and the postmicrosomal fraction (105,000 *g* supernatant) of the liver can convert *N*-hydroxy-2-AAF into a more mutagenic species. Since the sulfate ester of *N*-hydroxy-2-AAF has been postulated to be the ultimate carcinogen of 2-AAF and *N*-hydroxy-2-AAF, and there is a high correlation between mutagenicity and carcinogenicity, we examined its potential mutagenicity in the Salmonella test system.

The sulfate ester of *N*-hydroxy-2-AAF (a generous gift from Dr. E. Weisburger, National Cancer Institute) was generated *in situ* according to the method used for phenolic compounds by Gregory and Lipmann [21]. *p*-Nitrophenylsulfate (pNPS) was used as the sulfate donor for 3′,5′-adenosine diphosphate (PAP) in the synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the cofactor required for sulfation. This reaction is catalyzed presumably by phenolsulfotransferase [22] in the rat liver postmicrosomal fraction which is prepared by running the 100,000 *g* supernatant over a Sephadex G12 column [23]. *N*-hydroxy-2-AAF is readily sulfated in this system, and this reaction is dependent on the presence of PAP [23]. *p*-Nitrophenylsulfate and PAP were obtained from Sigma Chemical Co. (St. Louis, MO), and other chemicals used were of the best available commercial grade.

Table 1 shows that, in the presence of cofactors for sulfation and postmicrosomal fraction from rat liver, covalent binding of *N*-hydroxy-2-AAF to proteins was much greater than in the absence of the cofactor *p*-nitrophenylsulfate. When the postmicrosomal fraction was replaced by bovine serum albumin, little covalent binding occurred. When

Table 1. Effects of sulfation and *p*-nitrophenol on covalent binding of *N*-hydroxy-2-acetylaminofluorene to protein*

Incubation	*p*-Nitrophenol added	*N*-hydroxy-2-AAF (pmoles bound mg^{-1} min^{-1}) ^{14}C-label	^{3}H-label
Complete system	No	408	448
	Yes	78	153
Minus cofactors for sulfation (pNPS + PAP)	No	18	98
	Yes	20	93
Bovine serum albumin in place of supernatant fraction	No	18	38

* The complete system for incubation contained 0.5 mM *N*-hydroxy-2-AAF (^{14}C-acetyl or generally labeled with ^{3}H; sp. act. 10 mCi/m-mole and 6 Ci/m-mole, respectively), 4 mg/ml of postmicrosomal supernatant protein, 10 mM *p*-nitrophenylsulfate and 0.02 mM PAP. The concentration of added *p*-nitrophenol was 0.2 mM and of added bovine serum albumin was 4 mg/ml. The time of incubation was 5 min at 37°.

Table 2. Mutagenic effect of *N*-hydroxy-2-AAF in the presence of postmicrosomal fraction from rat liver*

Additions on plate	Revertant/ plate
Postmicrosomal fraction (PF)	1270
PF + *p*-nitrophensylsulfate + PAP	518
PF + *p*-nitrophenylsulfate + PAP + *p*-nitrophenol	902
PF + *p*-nitrophenol	1428
Phosphate buffer 0.90% NaCl in place of PF	90

* The mutagenicity test was carried out according to Ames *et al.* [18] with strain TA 1538, using 3 mg of postmicrosomal fraction/plate, instead of the supernatant fraction after centrifugation of liver homogenate at 9000 *g* for 10 min. In addition, each plate contained 1 μg *N*-hydroxy-2-AAF. The concentrations of other agents, when added, were: 10 mM *p*-nitrophenylsulfate, 20 μM PAP and 0.2 mM *p*-nitrophenol as indicated.

p-nitrophenol, presumably a competitive inhibitor for the sulfation of *N*-hydroxy-2-AAF, was included in the reaction mixture, the covalent binding of *N*-hydroxy-2-AAF was decreased. These results are in agreement with those of DeBaun *et al.* [9], who also found that *p*-nitrophenol inhibits sulfation of *N*-hydroxy-2-AAF.

The effect of sulfation of *N*-hydroxy-2-AAF by the postmicrosomal fraction on its mutagenicity in the Salmonella test system is shown in Table 2. The postmicrosomal fraction, the cofactors and *p*-nitrophenol were not mutagenic by themselves (data not shown). *N*-hydroxy-2-AAF gave rise to relatively few revertants in the absence of the postmicrosomal fraction. In the presence of the postmicrosomal fraction, however, there was a marked increase in the mutagenicity of *N*-hydroxy-2-AAF. This increase may be due to deacetylation of *N*-hydroxy-2-AAF, since *N*-hydroxy-2-aminogluorene is highly mutagenic in the test system [20, 24].

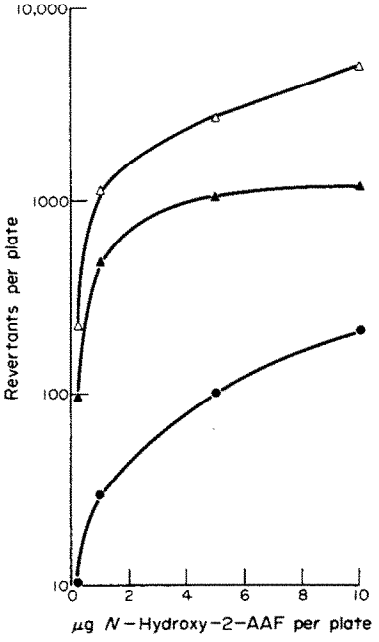

Fig. 1. Effect of sulfotransferase activity in rat liver postmicrosomal fraction on *N*-hydroxy-2-AAF mutagenicity. Key: (△——△) postmicrosomal fraction without cofactors for sulfotransferase; (▲——▲) postmicrosomal fraction with cofactors for sulfotransferase; and (●——●) *N*-hydroxy-2-AAF without the postmicrosomal fraction. Three mg of postmicrosomal protein/plate was used.

Addition of the cofactors for sulfation in the presence of the postmicrosomal fraction and *N*-hydroxy-2-AAF caused a sharp decrease in mutagenicity (from 1270 to 518 revertants). This decrease is mutagenicity was largely reversed by *p*-nitrophenol, which did not decrease the number of revertants when the cofactors for sulfation had been omitted (Table 2). Figure 1 shows the effect of the sulfotransferase activity on the mutagenicity of *N*-hydroxy-2-AAF. We observed no toxicity (i.e. killing of bacteria) in the presence or absence of the active sulfotransferase at the concentrations of *N*-hydroxy-2-AAF tested.

These results show that, under conditions where the sulfate ester of *N*-hydroxy-2-AAF is formed, the mutagenicity of *N*-hydroxy-2-AAF is greatly decreased in the bacterial test system. A simple explanation of the data would be that the sulfate ester of *N*-hydroxy-2-AAF is not as mutagenic as other metabolites of *N*-hydroxy-2-AAF produced by the postmicrosomal fraction. Thus, when the PAPS-generating system is absent, other metabolites, possibly *N*-hydroxy-aminofluorene, which is very mutagenic [24], would be produced at a maximal rate. However, when a PAPS-generating system is included, the formation of this mutagenic metabolite(s) would be decreased with simultaneous formation of the sulfate ester of *N*-hydroxy-2-AAF. This finding that the presence of a PAPS-generating system in the postmicrosomal fraction decreases the mutagenicity of *N*-hydroxy-2-AAF in the Salmonella test system is unexpected since Maher *et al.* [25] showed that the sulfate ester was very mutagenic when DNA, isolated from *Bacillus subtilis* strain SB 19, was reacted with the sulfate ester of *N*-hydroxy-2-AAF and incorporated into strain T3 which was blocked in tryptophan synthesis. In the case of the Salmonella test system, however, the sulfate ester of *N*-hydroxy-2-AAF may have reacted with protein or other macromolecules before arylation of bacterial DNA could occur. Supporting this interpretation is the observation that the sulfate ester of *N*-hydroxy-2-AAF is not carcinogenic when injected subcutaneously [2, 9]. Another explanation for the lack of mutagenic activity of the sulfate ester of *N*-hydroxy-2-AAF could be that ionized compounds do not pass through the cell wall as easily as non-ionized. The sulfate ester of *N*-hydroxy-2-AAF, in addition to being a highly unstable compound, is ionized at pH 7.4, and this may contribute to its lack of mutagenic activity in the Salmonella test system.

The results presented in Table 1 indicate that without the sulfation system being present in the postmicrosomal fraction there is more covalent binding of generally labeled [^{3}H]*N*-hydroxy-2-AAF than of acetyl-^{14}C-labeled compound. This result could be explained by the loss of the ^{14}C-label during deacetylation or *trans*-acetylation reactions. Thus, in the absence of sulfation, [acetyl-^{14}C]labeled *N*-hydroxy-2-AAF does not bind covalently to postmicrosomal protein to a greater extent than it does to bovine serum albumin (Table 1). Covalent binding of the ^{3}H-labeled compound to protein of the postmicrosomal fraction in the absence of sulfation cofactors is much higher than that to bovine serum albumin. This finding suggest that arylating metabolites are formed without activation of the sulfotransferase system.

In summary, our results indicate that metabolic activation of *N*-hydroxy-2-AAF by a process(es) other than the one catalyzed by sulfotransferase is more important in mutagenesis in *Salmonella typhimurium* than activation by sulfotransferase. It may be that the Salmonella test system is unable to detect a short-lived highly reactive potential mutagen such as the sulfate ester of *N*-hydroxy-2-AAF because this species arylates other more readily available macromolecules before reaching bacterial DNA.

In view of the strong correlation between chemically induced cancer and mutagenicity by the corresponding compounds in the Salmonella test system, metabolites of *N*-hydroxy-2-AAF other than the sulfate ester may play a significant role in the carcinogenesis of *N*-hydroxy-2-AAF. At present, however, no conclusion about the relative carcinogenicity of these metabolites can be drawn.

Laboratory of Chemical Pharmacology
National Heart and Lung Institute, National Institutes of Health, Bethesda, MD 20014
GERARD J. MULDER*
JACK A. HINSON

Section on Molecular Toxicology
Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20014, *U.S.A.*
WENDEL L. NELSON†
SNORRI S. THORGEIRSSON

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REFERENCES

1. C. Heidelberger, *Ann. Biochem.* **44,** 79 (1975).
2. J. A. Miller, *Cancer Res.* **30,** 559 (1970).
3. J. H. Weisburger and E. K. Weisburger, *Pharmac. Rev.* **25,** 1 (1973).
4. J. H. Miller and E. C. Miller, *Prog. exp. Tumor Res.* **11,** 270 (1969).
5. S. S. Thorgeirsson, D. J. Jollow, H. A. Sasame, I. Green and J. R. Mitchell, *Molec Pharmac.* **9,** 398 (1973).
6. P. D. Lotlikar, K. Wertonan and L. Luha, *Biochem. J.* **136,** 1140 (1973).
7. K. P. Baetcke, B. J. Gough and T. E. Shellenberger, *Biochem. Pharmac.* **23,** 1745 (1974).
8. J. R. DeBaun, J. Y. Rowley, E. C. Miller and J. A. Miller, *Proc. Soc. exp. Biol. Med.* **129,** 268 (1968).
9. J. R. DeBaun, E. C. Miller and J. A. Miller, *Cancer Res.* **30,** 577 (1970).
10. H. Bartsch, M. Dworkin, J. A. Miller and E. C. Miller, *Biochim. biophys. Acta* **286,** 272 (1972).
11. C. M. King, *Cancer Res.* **34,** 1503 (1974).
12. C. C. Irving, R. A. Veazey and J. T. Hill, *Biochim. biophys. Acta* **179,** 189 (1969).
13. C. C. Irving and L. T. Russell, *Biochemistry* **9,** 2471 (1970).
14. E. Kriek, *Chem. Biol. Interact.* **1,** 3 (1969/70).
15. C. C. Irving and R. A. Veazey, *Cancer Res.* **29,** 1799 (1969).
16. E. Kriek, *Cancer Res.* **32,** 2042 (1972).
17. C. C. Irving, D. H. Janss and L. T. Russell, *Cancer Res.* **31,** 387 (1971).
18. B. N. Ames, L. D. Lee and W. E. Durston, *Proc. natn. Acad. Sci. U.S.A.* **70,** 782 (1973).
19. W. F. Durston and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **71,** 737 (1974).
20. J. S. Felton, D. W. Nebert and S. S. Thorgeirsson, *Molec. Pharmac.* **12,** 217 (1976).
21. J. D. Gregory and F. Lipmann, *J. biol. Chem.* **229,** 1081 (1957).
22. J. D. Gregory, in *Methods of Enzymology*, Vol. 5, p. 977 (1962).
23. G. J. Mulder, J. A. Hinson and J. R. Gillette, *Biochem. Pharmac.*, in press.
24. J. McCann, E. Choi, E. Yamasaki and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **72,** 5135 (1975).
25. V. M. Maher, E. C. Miller, J. A. Miller and W. Szybalski, *Molec. Pharmac.* **4,** 411 (1968).

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Effect of Levamisole on phytohemagglutinin-stimulated human lymphocytes*

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Levamisole† (1-tetramizole), a potent anthelminthic, has been shown to have immuno-stimulatory activity in animals and man [1]. It is currently being investigated clinically in the treatment of neoplastic disease [2], rheumatoid arthritis [3], systemic lupus [4], and other disorders presumed to be associated with impaired cellular immunity. Previous studies on the effect of Levamisole on lymphocyte transformation *in vitro* have produced conflicting results. Copeland *et al.* [5] found no stimulation of resting human lymphocytes *in vitro* by the drug, nor could they demonstrate either enhancement or inhibition of lymphocyte transformation by specific or non-specific mitogens; Pabst and Crawford [6] showed significantly greater responses in human lymphocytes to *Candida albicans*, measles virus and purified protein derivative of *Tubercle bacilli* in the presence of Levamisole. Wachi *et al.* [7] found suppression of RNA synthesis of human lymphocytes cultured with phytohemagglutinin (PHA) and Levamisole in 3-day cultures, but little or no effect on DNA synthesis. When these cultures were continued for 4 days, however, they reported a stimulatory effect on DNA synthesis. In order to further clarify the effects of Levamisole on human lymphocytes, studies were undertaken with both resting and phytohemagglutinin-stimulated normal and anergic lymphocytes, and a normal cell line which has been maintained in continuous culture.

Lymphocytes were obtained from normal healthy volunteers and a selected group of patients with diseases associated with anergy, after consent was obtained. One of these patients was a 53-year-old male with severe rheumatoid arthritis, who had not received cytotoxic drugs or corticosteroids, and from whom aspirin was withheld for 1 week prior to obtaining the cells [8]. There were two patients with Hodgkin's disease, one was a 42-year-old male with

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