Conrad, C. M. Branson, *Science* **164**, 426 (1969).
3. J. Kraicer and S. C. Cheng, *Amer. J. Physiol.* **214**, 158 (1968).
4. W. W. Umbreit, T. H. Burris, J. F. Stauffer, in *Manometric Techniques* (Burgess, Minneapolis, 1964), p. 132.
5. G. Ling and R. W. Gerard, *Nature* **165**, 113 (1950).
6. A. L. Hodgkin, *Proc. Roy. Soc. Ser. B Biol. Sci.* **148**, 1 (1958).
7. Adenohypophyses were removed, weighed, and dissolved in $HNO_3$; the $[K^+]$ was determined with a flame photometer after appropriate dilution.
8. W. S. Wilde in *Mineral Metabolism* (Academic Press, New York, 1962), vol. 2A.
9. J. V. Milligan and J. Kraicer, unpublished results.
10. A. Weber, R. Herz, I. Reiss, *J. Gen. Physiol.* **46**, 679 (1963).
11. Since $Ca^{2+}$ is divalent, the Nernst equation has the form $E = 26 \times \log_{10} (C_o/C_i)$.
12. We thank C. Ripley and A. Morris for technical aid. J. L. Gosbee assisted with the removal of adenohypophyses. Supported by McLaughlin Research Fund (Queen's) and M.R.C. (Canada).

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## Reactivity in vivo of the Carcinogen N-Hydroxy-2-acetylaminofluorene: Increase by Sulfate Ion

Abstract. *Injections of sulfate ion in rats given the carcinogen* N-*hydroxy-2-acetylaminofluorene increased (i) the formation of 1- and 3-(methion-*S*-yl)-2-acetylaminofluorene bound to protein in the liver, (ii) the formation of fluorenyl derivatives bound to total protein, ribosomal RNA, and DNA in the liver, and (iii) the toxicity of the carcinogen. These data provide evidence that the highly reactive ester 2-acetylaminofluorene-*N*-sulfate, previously suggested as an ultimate reactive and carcinogenic metabolite of* N-*hydroxy-2-acetylaminofluorene, is formed in the rat liver in vivo.*

The very low nonenzymatic reactivity of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) with tissue nucleophiles in vitro (*1*) suggests that metabolic activation is required to convert this carcinogen into an ultimate reactive and carcinogenic form. The possible nature of this reactive form was suggested by findings that synthetic electrophilic esters of *N*-hydroxy-AAF react readily at neutrality with nucleic acids, proteins, and certain of their nucleophilic components (*1–4*). Furthermore, the products formed by reactions of guanosine and methionine with synthetic esters of *N*-hydroxy-AAF, such as *N*-acetoxy-AAF or AAF-*N*-sulfate (Fig. 1), are identical to products obtained on degradation of liver RNA and protein from rats given AAF or its *N*-hydroxy metabolite (*1, 5, 6*). The ester-like O-glucuronide of *N*-hydroxy-AAF, a major urinary and biliary metabolite (*7*), also reacts in similar ways with the same nucleophiles in vitro, but at a much slower rate (*8*).

The supernatant fraction of homogenates of rat liver contains sulfotransferase activity capable of transferring the sulfonate group from 3′-phosphoadenosine-5′-phosphosulfate to *N*-hydroxy-AAF to form AAF-*N*-sulfate (*6, 9, 10*). Further, on the basis of close correlations between sulfotransferase activity for *N*-hydroxy-AAF in the liver, the amount of hepatic protein-bound 1- and 3-(methion-*S*-yl)-AAF formed from *N*-hydroxy-AAF in vivo, and susceptibility to hepatic carcinogenesis, AAF-*N*-sulfate appears to be an ultimate reactive carcinogenic metabolite of *N*-hydroxy-AAF (*6*). Our results provide evidence for the formation and reactivity of this ester in rat liver in vivo.

The instability of AAF-*N*-sulfate (half-life in water of less than 1 minute) (*4*) precludes its isolation from liver, and the loss of sulfate ion on reaction with nucleophiles makes it impossible to infer its presence from the structures of reaction products. However, the enzymatic formation of AAF-*N*-sulfate in vitro is dependent on 3′-phosphoadenosine-5′-phosphosulfate (*6, 9, 10*), and the data of Büch *et al.* (*11*) indicate that the amount of sulfate ion in vivo is a limiting factor in the formation of *p*-acetylaminophenyl sulfate from *p*-hydroxyacetanilide in the rat. Thus our attention was directed to the effect of sulfate ion on the reactivity of *N*-hydroxy-AAF in the liver of the rat, especially in rats given prior treatment with *p*-hydroxyacetanilide to deplete the pool of sulfate ion in vivo.

Male rats (250 to 300 g, CD-randombred, Charles River Breeding Laboratory, Wilmington, Mass.) were injected according to the schedules in Table 1 and Fig. 2. The amount of 1- and 3-(methion-*S*-yl)-AAF bound to protein in the liver was estimated from the

Table 1. Stimulation by sulfate ions of binding in vivo of fluorenyl derivatives of *N*-hydroxy-AAF to protein-bound methionine or to the total protein, ribosomal RNA, and DNA in rat liver. In experiment 1, *p*-hydroxyacetanilide (19 mg/100 g of body weight; in 1*M* NaCl or 0.5*M* $Na_2SO_4$) was injected at 0 and 3 hours; *N*-hydroxy-AAF was injected at 4 hours. In experiment 2, *p*-hydroxyacetanilide (19 mg/100 g of body weight; in 2*M* NaCl, 1*M* $Na_2SO_4$, 1*M* sodium phosphate, or 2*M* sodium acetate, all at *p*H 7) was injected at 0 hour; *N*-hydroxy-AAF was injected at 4 hours. In experiment 3, water, 1.5*M* NaCl plus 0.25*M* $MgCl_2$, or 0.75*M* $Na_2SO_4$ plus 0.25*M* $MgSO_4$ were injected at 0 hour; *N*-hydroxy-AAF was injected at 3 hours. In experiment 4, the injections were as in experiment 3, except *N*-hydroxy-AAF was injected at 2 hours. In experiment 5, the injections were as in experiment 2, except $C^{14}$-labeled *N*-hydroxy-AAF was injected at 4 hours. All injections were intraperitoneal (0.5 ml/100 g of body weight). The *N*-hydroxy-AAF was suspended in 1.75 percent gum acacia, which in experiments 1, 3, and 4 contained the same concentrations of salts as the solutions injected at 0 time. The differences between the amounts of bound fluorenyl derivatives for the livers of rats injected with sulfate or chloride ions had *P* values of $< 0.05$ for DNA and ribosomal RNA and of $< 0.01$ for protein (Student's *t*-test). The results are expressed as the mean ± 1 standard deviation; numbers of rats are in parentheses.

| Expt. | *N*-hydroxy-AAF (mg/100 g) | Time rats killed after *N*-hydroxy-AAF injection (hr) | Amount of fluorenyl derivatives with injection of | | |
|---|---|---|---|---|---|
| | | | Sulfate | Chloride | No anion |
| | | | *Bound to methionine in protein*† | | |
| 1* | 5 | 16 | 29 ± 6 (6) | 8 ± 4 (6) | |
| 2* | 5 | 16 | 38 ± 8 (5) | 14 ± 3 (6)‡ | |
| 3 | 12 | 2 | 78 ± 19 (13) | 55 ± 13 (7) | 51 ± 23 (6) |
| 4 | 20 | 2 | 98 ± 14 (4) | 63 ± 19 (4) | 68 ± 12 (4) |
| | | | *Bound to protein*§ | | |
| 5* | 5 | 16 | 1470 ± 220 (5) | 780 ± 140 (5) | |
| | | | *Bound to ribosomal RNA*§ | | |
| 5* | 5 | 16 | 325 ± 90 (5) | 155 ± 70 (5) | |
| | | | *Bound to DNA*§ | | |
| 5* | 5 | 16 | 280 ± 95 (5) | 200 ± 70 (5) | |

* Given prior treatment with *p*-hydroxyacetanilide. † Expressed as micrograms of *o*-methylmercapto-AAF liberated per 5 g of liver. ‡ When phosphate or acetate ions were injected in place of chloride ions, the values were 12 ± 1 (3) and 11 ± 3 (3), respectively. § Expressed in picomoles per milligram.

amount of 1- and 3-methylmercapto-AAF released by alkaline treatment of the liver proteins (*6*). The DNA and ribosomal RNA were isolated from the livers of rats given *N*-hydroxy-AAF-9-$^{14}$C (Tracerlab, Waltham, Mass.; diluted to a specific activity of 2.8 $\mu$c/mg) according to the procedure of Irving and Veazey (*12*), except that the DNA was purified by digestion in 0.3*N* KOH at 37°C for 12 hours, and then precipitated by addition of HCl to a *p*H of 3 and of trichloroacetic acid (final concentration, 2.5 percent). The DNA contained less than 1 percent of RNA as judged by the orcinol test; the ratios of absorbances at 259 to 280 nm were 1.91 ± 0.05 and 2.06 ± 0.01 for the DNA and ribosomal RNA, respectively. The protein was precipitated from 10 ml of the combined phenolic extracts with 20 ml of acetone, and the precipitate was extracted with 20 ml each of acetone, ether, and 95 percent ethanol. Before the radioactivity was determined (Packard Tricarb scintillation counter) the DNA was digested by deoxyribonuclease, the RNA was digested with 0.3*N* KOH, and the protein was dissolved in Hyamine hydroxide (Packard).

In the first studies the release of 1- and 3-methylmercapto-AAF by alkaline treatment of the liver proteins of rats given *N*-hydroxy-AAF was used as an index of the amount of 1- and 3-(methion-*S*-yl)-AAF bound to protein in the liver (*6*) (Fig. 1). When rats were given *p*-hydroxyacetanilide and then sulfate ion, two to three times more 1- and 3-methylmercapto-AAF (*o*-methylmercapto-AAF) was released from the liver proteins 16 hours after a subsequent dose of *N*-hydroxy-AAF than when the rats were given chloride, phosphate, or acetate ion instead of sulfate ion (Table 1, experiments 1 and 2). A 50 percent increase in methionyl-AAF derivatives bound to protein in the liver was obtained on administration of sulfate ion as compared to chloride ion or no ion without prior treatment with *p*-hydroxyacetanilide when larger doses of *N*-hydroxy-AAF were given and when the rats were killed only 2 hours after administration of the carcinogen (experiments 3 and 4). These effects appear to result from changes in the concentration of 3′-phosphoadenosine-5′-phosphosulfate in vivo, because the sulfotransferase activity for *N*-hydroxy-AAF in the liver (*6*) was not altered by the injections of *p*-hydroxyacetanilide or of the salt solutions.

Larger amounts of radioactivity from labeled *N*-hydroxy-AAF were bound to DNA, ribosomal RNA, and protein in the livers of rats previously given *p*-hydroxyacetanilide with sulfate ion rather than with chloride ion (Table 1, experiment 5). The greater stimulation by sulfate ion for ribosomal RNA binding (109 percent increase) and protein binding (largely cytoplasmic, 89 percent increase) as compared to that for DNA binding (40 percent increase) is consistent with the localization of the sulfotransferase activity for *N*-hydroxy-AAF of rat liver in the soluble fraction. Other reactive metabolites of *N*-hydroxy-AAF, such as its *O*-glucuronide, may also be involved in the formation of fluorenyl derivatives bound to macromolecules in vivo (*5, 8, 13*).

Intraperitoneal injection of 5 mg of

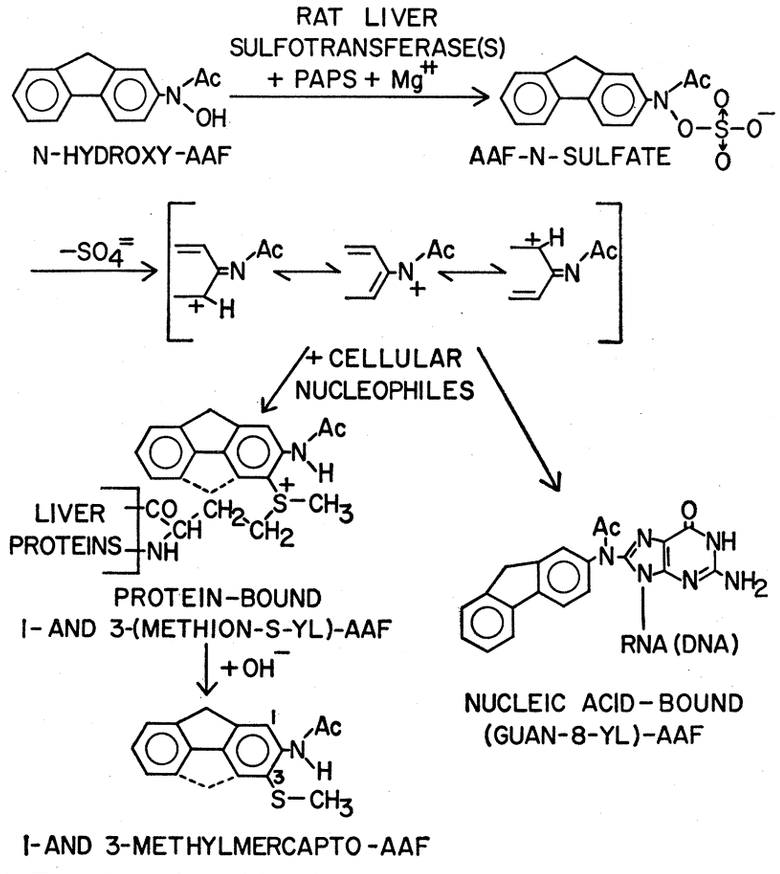


Fig. 1. Formation and reactivity of 2-acetylaminofluorene-*N*-sulfate (AAF-*N*-sulfate) in the rat liver (*1, 6, 9, 10*). *PAPS*, 3′-phosphoadenosine-5′-phosphosulfate.

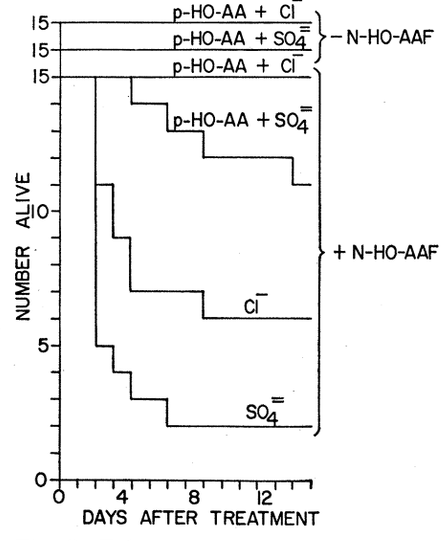


Fig. 2. Enhancement of the toxicity of *N*-hydroxy-AAF (*N*-HO-AAF) for the rat by concurrent administration of sulfate, rather than chloride, ions. Male rats (350 to 440 g) were injected intraperitoneally with 0.5 ml per 100 g of body weight of the following solutions or suspensions: at 0 and 2 hours with 0.25*M* *p*-hydroxyacetanilide (*p*-HO-AA) in 1*M* NaCl or 0.5*M* $Na_2SO_4$ and at 4 hours with 1.75 percent gum acacia in 2*M* NaCl or 1*M* $Na_2SO_4$ containing 10 mg of *N*-hydroxy-AAF per milliliter. Rats that did not receive *p*-hydroxyacetanilide or *N*-hydroxy-AAF were injected on the same schedule with comparable solutions lacking only these components.

*N*-hydroxy-AAF per 100 g of body weight resulted in the death of approximately 60 percent of the rats as a consequence of massive periportal necrosis in the liver (Fig. 2) (*14*). The toxicity of *N*-hydroxy-AAF was decreased by prior treatment of the rats with *p*-hydroxyacetanilide, but concurrent administration of sulfate ion partially overcame this protective effect (Fig. 2). An apparent increase in the toxicity of *N*-hydroxy-AAF on administration of sulfate ion as compared to chloride ion was also noted in the absence of prior treatment with *p*-hydroxyacetanilide. The protection afforded by *p*-hydroxyacetanilide is consistent with the decreased toxicity and carcinogenicity of AAF observed by Yamamoto *et al.* (*15*) in rats fed large amounts of acetanilide. *p*-Hydroxyacetanilide is a major metabolite of acetanilide in several mammals (*16*).

The increased binding of fluorenyl derivatives from *N*-hydroxy-AAF in vivo to hepatic protein, ribosomal RNA, and DNA, and the apparent increase in the toxicity of *N*-hydroxy-AAF when rats are supplemented with sulfate ion thus suggest that the highly reactive ester AAF-*N*-sulfate is formed from this carcinogen in vivo (Fig. 1). These data and the correlations between factors that influence the hepatocarcinogenicity of 2-aminofluorene derivatives and the sulfotransferase activity for *N*-hydroxy-AAF in the rat (*6*) support the view that AAF-*N*-sulfate is an ultimate reactive carcinogenic metabolite of *N*-hydroxy-AAF in the rat liver. It is not known which of the interactions between the ultimate carcinogenic form, or forms, of *N*-hydroxy-AAF and tissue constituent, or constituents, are critical in the conversion of normal hepatic cells to neoplastic cells. The amounts of the fluorene derivatives bound to protein and nucleic acids in the livers of animals administered AAF or *N*-hydroxy-AAF have generally paralleled susceptibility to hepatic carcinogenesis (*17*). However, the roles of specific proteins and nucleic acids in this carcinogenic process remain to be determined.

The subcutaneous tissue of rats has been employed in relatively direct tests of the carcinogenicity of possible metabolites of *N*-hydroxy-AAF, and the synthetic lipid-soluble esters *N*-acetoxy-AAF and *N*-benzoyloxy-AAF are more carcinogenic than *N*-hydroxy-AAF at this site (*1*). However, subcutaneous injections of AAF-*N*-sulfate have produced only a few tumors (*1*); the short half-life in water and the ionic nature of this reactive ester probably prevent adequate dosage of the cells. The potential biological activity of AAF-*N*-sulfate is evident from its high activity as a mutagen in vitro for a bacterial transforming DNA (*4*).

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### References and Notes

1. J. A. Miller and E. C. Miller, *Progr. Exp. Tumor Res.* **11**, 273 (1969).
2. P. D. Lotlikar, J. D. Scribner, J. A. Miller, E. C. Miller, *Life Sci.* **5**, 1263 (1966).
3. E. C. Miller, U. Juhl, J. A. Miller, *Science* **153**, 1125 (19[illegible] E. Kriek, J. A. Miller, U. Juhl, E. C. Miller, *Biochemistry* **6**, 177 (1967).
4. V. M. Maher, E. C. Miller, J. A. Miller, W. Szybalski, *Mol. Pharmacol.* **4**, 411 (1968).
5. E. Kriek, *Biochim. Biophys. Acta* **161**, 273 (1968).
6. J. R. DeBaun, E. C. Miller, J. A. Miller, *Cancer Res.*, in press; *Proc. Amer. Ass. Cancer Res.* **10**, 18 (1969).
7. J. W. Cramer, J. A. Miller, E. C. Miller, *J. Biol. Chem.* **235**, 250 (1960); C. C. Irving, *Cancer Res.* **22**, 867 (1962); *J. Biol. Chem.* **240**, 1011 (1965); ——, R. Wiseman, Jr., J. T. Hill, *Cancer Res.* **27**, 2309 (1967).
8. E. C. Miller, P. D. Lotlikar, J. A. Miller, B. W. Butler, C. C. Irving, J. T. Hill, *Mol. Pharmacol.* **4**, 147 (1968).
9. C. M. King and B. Phillips, *Science* **159**, 1351 (1968).
10. J. R. DeBaun, J. Y. Rowley, E. C. Miller, J. A. Miller, *Proc. Soc. Exp. Biol. Med.* **128**, 307 (1968).
11. H. Büch, W. Rummel, K. Pfleger, C. Eschrich, N. Texter, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **259**, 276 (1968).
12. C. C. Irving and R. A. Veazey, *Biochim. Biophys. Acta* **166**, 246 (1968).
13. ——, J. T. Hill, *ibid.* **179**, 189 (1969).
14. We thank Dr. S. Goldfarb for examination of the histological sections.
15. R. S. Yamamoto, R. M. Glass, H. H. Frankel, E. K. Weisburger, J. H. Weisburger, *Toxicol. Appl. Pharmacol.* **13**, 108 (1968).
16. R. T. Williams, *Detoxication Mechanisms* (Wiley, New York, 1959), p. 433.
17. F. Marroquin and E. Farber, *Cancer Res.* **25**, 1262 (1965); C. C. Irving, R. A. Veazey, R. F. Williard, *ibid.* **27**, 720 (1967); J. Y. R. Smith, M.S. thesis, University of Wisconsin (1969).
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## Immunoglobulin Production: Method for Quantitatively Detecting Variant Myeloma Cells

Abstract. *Mouse myeloma cells in continuous culture were cloned in soft agar. The clones were assayed for their ability to synthesize heavy and light chains of gamma globulin by immunoprecipitation directly in the agar. A minor population secreting only light chains was identified. The two cell types were recloned and a low incidence of conversion of 7S to light-chain production was demonstrated. This technique can be used to isolate rare variants of cells secreting specific macromolecules.*

Plasma cell tumors that produce immunoglobulin can be induced in Balb/c mice and maintained by animal passage (*1*). Some of these tumors are defective in immunoglobulin production. Variants have been described which synthesize partially assembled molecules, unassembled heavy and light chains, or light chains only; other variants do not synthesize detectable immunoglobulin polypeptide chains (*1, 2*). These defects in synthesis and assembly are usually present when the tumor is first examined, although some have arisen during animal passage (*3*) or in tissue culture lines which had originally synthesized complete molecules (*2*). We now present a method for quantitatively detecting variants in cultured cell lines and describe the frequency and characteristics of those variants that produce light chains in an MPC 11 cell line.

MPC 11 is a tumor originally obtained from Dr. J. Fahey, which produces $\gamma G_{2b}$ immunoglobulin. The types of $\gamma$-globulin molecules produced and their synthesis, assembly, and secretion by this tumor have been described (*4*). The tumor has been adapted to grow as a suspension culture in spinner bottles. These cells have grown continuously for almost 2 years in Dulbecco's modified Eagle medium (Grand Island Biological Co.) supplemented with nonessential amino acids and horse serum (20 percent). The line produces predominantly 7*S* $\gamma$-globulin but also secretes small amounts of half molecules, light chain dimers, and free light chains (*5*).

Cells which no longer secrete molecules containing heavy chains were detected by first cloning the cultured cells in soft agar by a slight modification of a known technique (*6*). Feeder layers