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N-Hydroxy-2-fluorenylacetamide, an Active Intermediate of the Mammary Carcinogen N-Hydroxy-2-fluorenylbenzenesulfonamide[1] (38980)

D. MALEJKA-GIGANTI AND H. R. GUTMANN
(Introduced by N. S. Mizuno)

Laboratory for Cancer Research, Veterans Administration Hospital, Minneapolis, Minnesota 55417, and Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

N - Hydroxy - 2 - fluorenylbenzenesulfonamide (*N*-hydroxy-2-FBS)[2] is a potent mammary carcinogen for the female Sprague–Dawley rat by ip administration (1). In work on the metabolism of this compound *in vivo*, we found that the benzenesulfonyl group was detached from the fluorene moiety, since animals that had received *N*-hydroxy-2-FBS excreted *N*-hydroxy-2-FAA, 2-FAA, 3-, 5- and 7-hydroxy-2-FAA, as well as benzenesulfonamide and *p*-hydroxy-benzenesulfonamide in the urine (2). The isolation of *N*-hydroxy-2-FAA suggested that *N*-hydroxy-2-FBS was cleaved to the hydroxylamine, *N*-hydroxy-2-FA, which was then acetylated to *N*-hydroxy-2-FAA. The urinary excretion of metabolites indicative of the desulfonylation of *N*-hydroxy-2-FBS prompted us to investigate whether this reaction occurred in the mammary gland or in some other tissue (2). This was done by injecting *N*-hydroxy-2-FBS labeled with ^{14}C and ^{35}S into the rat and by determining the $^{35}S/^{14}C$ ratio in a number of tissues including the mammary gland. The marked deviation of the $^{35}S/^{14}C$ ratio in the mammary gland from the $^{35}S/^{14}C$ ratio in the labeled compound led us to infer that *N*-hydroxy-2-FBS had been cleaved into ^{35}S- and ^{14}C-containing fragments. However, the mechanism of the cleavage was not clear and it was not known whether *N*-hydroxy-2-FA was one of the products of the cleavage. In the present study, we have obtained evidence that desulfonylation of *N*-hydroxy-2-FBS takes place in the mammary gland and proceeds via the hydroxylamine.

The question, whether mammary carcinogenesis after systemic administration of *N*-hydroxy-2-FBS was due to the administered compound or to its possible metabolites, *N*-hydroxy-2-FA and *N*-hydroxy-2-FAA, also had not been resolved in the previous study (2). It was recognized that both of these carcinogens could mediate the carcinogenicity of *N*-hydroxy-2-FBS. In the current study we have approached this problem by the direct application of *N*-hydroxy-2-FBS, *N*-hydroxy-2-FA and *N*-hydroxy-2-FAA to the mammary gland. In addition, the carcinogenicity of 2-nitrosofluorene for the mammary gland was tested by this technique. The latter compound is a product of the spontaneous decomposition of *N*-hydroxy-2-FBS as shown here. The data obtained in these studies form the basis of this report.

Materials and methods. Labeled and unlabeled compounds. *N*-Hydroxy-2-[9-^{14}C]FBS, mp 130–131°, specific radioactivity = 8.63 mCi/mmole, *N*-hydroxy-2-[9-^{14}C]FA, mp 175°, specific radioactivity = 0.97 mCi/mmole (2) and 2-nitroso-[9-^{14}C]fluorene, mp 79–81°, specific radioactivity = 0.85 mCi/mmole (3), were prepared as described previously. *N*-Hydroxy-2-[9-^{14}C]FAA, mp 147–148°, specific radioactivity = 8.94 mCi/mmole, was obtained from New England Nuclear, Boston, MA and recrystallized once. The uv absorption spectra of all compounds matched those of authentic samples and their radiopurity was shown by thin-layer chromatography (2, 3).

N-Hydroxy-2-FBS, mp 138–140° (1), *N*-hydroxy-2-FAA, mp 150–151° (4), *N*-hydroxy-2-FA, mp 170–175°, 2-nitrosofluorene, mp 79–81° (5) and 2-FA, mp 127–129° (6) were prepared by the published pro-

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[2] The abbreviations used are: *N*-hydroxy-2-FBS, *N*-hydroxy-2-fluorenylbenzenesulfonamide; *N*-hydroxy-2-FAA, *N*-hydroxy-2-fluorenylacetamide; 2-FA, 2-fluorenamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

cedures. The uv and infrared spectra of the compounds matched those of authentic samples. The uv and infrared absorption spectra were recorded with a Beckman Acta III and IR-10 spectrophotometer, respectively.

Animal experiments. Female Sprague–Dawley rats were purchased from the Holtzman Company, Madison, WI. The rats used for carcinogenicity tests were 6–8 wk old and those used for enzymatic assays were 12 weeks old. The technique of local application of the compounds to the mammary glands has previously been described (7). The maintenance of the animals, carcinogenicity tests and preparation of tissues for histological examination were carried out as reported previously (7).

Stability of N-hydroxy-2-FBS in the incubation media. *N*-Hydroxy-2-FBS dissolved in methanol, ethanol or DMSO disproportionates readily to 2-nitrosofluorene. The uv spectra of such solutions showed invariably the 360 nm peak characteristic of 2-nitrosofluorene (5). The uv spectra of *N*-hydroxy-2-FBS, free of absorbance at 360 nm, were recorded, however, when the compound was dissolved in diethyl ether. *N*-Hydroxy-2-FBS was stable for several days in an ethereal solution provided the solution was stored at 4° in the dark.

The stability of *N*-hydroxy-2-FBS in aqueous media was examined either at 37 or 4° in a nitrogen atmosphere. An ethereal solution (0.5 ml) of *N*-hydroxy-2-FBS (0.5 μmole) or an aqueous suspension of the compound (0.3 μmole) in 0.9% NaCl containing 7% gum acacia (0.1 ml) was added to 0.01 *M* phosphate buffer, pH 7.4, (10 ml) or to 0.02 *M* HEPES buffer, pH 7.4 (5 ml). After incubation for the times indicated in Fig. 1, the incubation mixtures were cooled in an ice-bath and extracted with ether. The extracts were washed with cold water saturated with nitrogen, dried (Na_2SO_4) and the volume was adjusted with ether to 10 ml. The uv spectra of the solutions were recorded (Fig. 2) and the recovery of *N*-hydroxy-2-FBS was calculated from its absorbance at 278 and 307 nm. 2-Nitrosofluorene was estimated from its absorbance at 360 nm. Incubation of a suspension of *N*-hydroxy-2-FBS in 0.01 *M* phosphate buffer, pH 7.4, at 37°

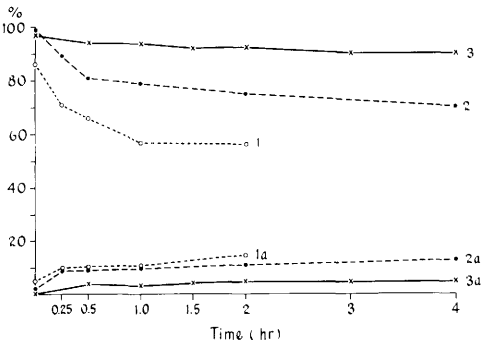

FIG. 1. Recoveries of *N*-hydroxy-2-FBS after incubations of the compound in buffers. 1, *N*-hydroxy-2-FBS in 0.01 *M* phosphate buffer, pH 7.4, at 37°; 2, *N*-hydroxy-2-FBS in 0.02 *M* HEPES buffer, pH 7.4, at 37°; 3, *N*-hydroxy-2-FBS in 0.02 *M* HEPES buffer, pH 7.4, at 4°. 1a, 2a and 3a: 2-nitrosofluorene formed in incubation mixtures 1, 2 and 3, respectively.

resulted in gradual decomposition of the compound. Under these conditions, the recovery of *N*-hydroxy-2-FBS was less than 60% (Fig. 1, Incubation No. 1). However, the compound was stable when it was incubated in 0.02 *M* HEPES buffer, pH 7.4, at 4°. Under these conditions, the recoveries of *N*-hydroxy-2-FBS ranged from 90–95% (Fig. 1, Incubation No. 3) and the formation of 2-nitrosofluorene did not exceed 5% (Fig. 1, Curve 3a). Accordingly, this aqueous system was selected for studies of the metabolism of *N*-hydroxy-2-FBS by the mammary gland.

Preparation of the mammary gland and liver; conditions of incubation. All steps were carried out in a cold room at 4°. Mammary tissue from the thoracic-abdomino-inguinal region of 12 rats were removed bilaterally, dissected free of lymph nodes, weighed and minced with scissors. Fat-free parenchymal cells were prepared by the method of Moon *et al.* (8). Cell viability was tested by mixing the cell suspension with an equal volume of 1% (w/v) trypan blue and examining the cells microscopically. Approximately 80% of the cells in the final pellet excluded trypan blue and were, by this criterion, viable. The livers of the rats from which the mammary tissue had been obtained were perfused with cold 0.9% NaCl and excised. The liver (1 g) or the pellet of mammary parenchymal cells (1 g) was suspended in 9 g of 0.02 *M* HEPES, pH 7.4, and subjected to sonication for 0.5 min at a frequency of 20,000 Hz and an

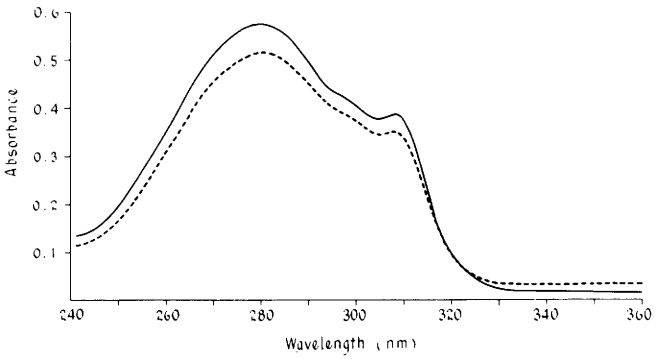

FIG. 2. Ultraviolet absorption spectra of *N*-hydroxy-2-FBS. (——) Compound extracted from 0.02 *M* HEPES buffer, pH 7.4, 4°, at 0 time; (-----) compound extracted from 0.02 *M* HEPES buffer, pH 7.4, after incubation for 4 hr at 4°. Solvent, diethyl ether.

average output power of 60–70 W (Sonifier Model S-110, Branson Instruments, Inc., Danbury, CO). The protein content of the sonicate was determined by the method of Lowry *et al.* (9). The sonicate was saturated with nitrogen before use in the incubations. Each incubation system consisted of 0.2 μmole of the [9-^{14}C]labeled compound dissolved in 0.2 ml diethyl ether and of an aliquot of the sonicate containing 8 mg protein. The volume of the mixture was adjusted to 2 ml with 0.02 M HEPES buffer, pH 7.4, saturated with nitrogen. Incubation mixtures from which the sonicates were omitted served as controls. The incubations were carried out for 4 hr at 4° in flasks placed in a nitrogen atmosphere. The incubation was terminated by the addition of methanol (0.5 ml) containing unlabeled 2-FA (1.0 μmole) and cold acetone (6 ml).

Determination of free and bound 2-[9-^{14}*C*]-*FA.* The precipitated proteins were separated by centrifugation at 600*g* and the sediments were washed twice with acetone. The acetone wash was added to the supernatant liquid and the acetone was removed with a stream of nitrogen. Distilled water (2 ml) was then added and the supernatant liquid was made alkaline (pH 9) with dilute NaOH. The 2-[9-^{14}C]FA was extracted and purified to constant specific radioactivity by thin-layer chromatography on silica gel as previously described (3). The sedimented proteins were partially hydrolyzed with 3 *M* NaOH (2 ml) at 37° for 2 hr (10). The hydrolysis was carried out in the presence of unlabeled 2-FA (1.0 μmole). After incubation, each hydrolysate was diluted with distilled water (8 ml) and duplicate aliquots (0.5 ml) were counted. The 2-[9-^{14}C]FA was extracted from the hydrolysate and purified to constant specific radioactivity by thin-layer chromatography (3). The amounts of 2-[9-^{14}C]FA extracted from the hydrolysate represent 2-FA released from macromolecular adducts (10).

Radioactivity measurements. The radioactivity of all samples was determined in Scintisol-Complete (10 ml) (Isolab, Inc., Elkhart, IN) by liquid scintillation spectrometry. All samples were counted in duplicate with an error not exceeding 5%, and the counts were corrected for quenching. The counting efficiencies were 70–80%.

Results and Discussion. The desulfonylation of *N*-hydroxy-2-FBS was examined in sonicates of mammary parenchymal cells and liver. The primary product that arises when the benzenesulfonyl group of *N*-hydroxy-2-FBS is removed by hydrolysis is *N*-hydroxy-2-FA. Because of the instability of this hydroxylamine, there is no adequate method available for its direct assay in micro amounts. However, *N*-hydroxy-2-FA is reduced to 2-FA by preparations of rat liver and mamary gland (Table I), and this reaction was utilized to demonstrate the formation of *N*-hydroxy-2-FA from the *N*-hydroxy-2-FBS by mammary gland. About 25 nmoles of 2-FA were extracted from the incubation mixtures in the free form and an additional 18 nmoles were obtained from the

TABLE I. CONVERSION OF *N*-HYDROXY-2-FA, *N*-HYDROXY-2-FBS AND *N*-HYDROXY-2-FAA TO 2-FLUORENAMINE BY SONICATES OF MAMMARY GLAND AND LIVER.

Substrate[a]	Nanomoles of 2-FA isolated[b]		Nanomoles of 2-FA isolated from macromolecular adducts[c]	
	From mammary gland	From liver	Of mammary gland	Of liver
N-Hydroxy-2-[9-^{14}C]FA	20.3 ± 1.74	8.57 ± 1.94	13.4 ± 1.69	15.4 ± 1.69
N-Hydroxy-2-[9-^{14}C]FBS	23.6 ± 1.85	12.6 ± 1.38	18.2 ± 0.77	24.6 ± 2.25
N-Hydroxy-2-[9-^{14}C]FAA	0.75 ± 0.34	1.50 ± 0.23	1.40 ± 0.18	3.71 ± 0.16

[a] Each incubation system contained 8.0 mg of protein and 200 nmoles of labeled substrate. Conditions of incubation and the specific radioactivities of the substrates are described in the text.

[b] Assayed by inverse isotope dilution. The values are the means ± SD from three experiments and are corrected for the radioactivity of the controls that contained no protein.

[c] The macromolecular adducts were partially hydrolyzed as described in the text and 2-FA was assayed by inverse isotope dilution. The values are the means ± SD from three experiments.

alkaline hydrolysis of macromolecular adducts. A minimum of 43 nmoles of *N*-hydroxy-2-FA, equivalent to 21.5% of *N*-hydroxy-2-FBS, was formed, therefore, by the enzymatic cleavage of the N-S bond of *N*-hydroxy-2-FBS. In contrast, only minor amounts of 2-FA were isolated when the arylhydroxamic acid, *N*-hydroxy-2-FAA, was incubated with mammary gland. This indicated that the bond that links the acetyl group to the nitrogen is more resistant to enzymatic hydrolysis than is the N–S bond of *N*-hydroxy-2-FBS.

Theoretically, 2-FA can also arise from the enzymatic reduction of 2-nitrosofluorene (3, 5). As mentioned above, *N*-hydroxy-2-FBS decomposes spontaneously in polar solvents and phosphate buffer to 2-nitrosofluorene and sulfinic acid. In the incubation system used in the present experiments, the decomposition of *N*-hydroxy-2-FBS was limited to 3–5% (Fig. 1). Since 2-nitrosofluorene incubated with mammary gland under identical conditions as *N*-hydroxy-2-FBS yielded 12% 2-FA, it was estimated that at most 0.6% of the 2-FA isolated upon incubation of *N*-hydroxy-2-FBS with mammary gland could have been derived from 2-nitrosofluorene. Moreover, 2-nitrosofluorene was not seen on chromatograms of ether extracts of mammary gland incubated with *N*-hydroxy-2-FBS. It was concluded that the 2-FA isolated in the above experiments came from *N*-hydroxy-2-FA and that the enzymatic hydrolysis of *N*-hydroxy-2-FBS by mammary gland yields *N*-HO-2-FA.

Although the mammary gland desulfonylated *N*-hydroxy-2-FBS to *N*-hydroxy-2-FA, this reaction is not directly implicated in mammary carcinogenesis by systemically administered *N*-hydroxy-2-FBS since *N*-hydroxy-2-FA was inactive upon direct application to the gland (Table II). A second possibility underlying the carcinogenicity of *N*-hydroxy-2-FBS might be the nonenzymatic disproportionation of the compound to yield 2-nitrosofluorene. However, 2-nitrosofluorene exhibited only weak carcinogenicity at the site of application. The high potency of systemically administered *N*-hydroxy-2-FBS toward the mammary gland is, therefore, not attributable to the formation of 2-nitrosofluorene by decomposition of *N*-hydroxy-2-FBS. The possibility that systemically administered *N*-hydroxy-2-FBS is active *per se* upon reaching the mammary gland was ruled out because *N*-hydroxy-2-FBS applied directly to the gland was inactive (Table II). The only metabolite of *N*-hydroxy-2-FBS that showed high potency after local application to the mammary gland was *N*-hydroxy-2-FAA. This compound produced, in two separate series of rats, predominantly malignant tumors (64%) of the mammary gland. Most of the tumors were located at the site of application (Table II). It seems very likely, therefore, that this carcinogenic fluorenylhydroxamic acid which is a urinary metabolite of *N*-hydroxy-2-FBS (2) accounts for the carcinogenicity of systemically administered *N*-hydroxy-2-FBS. The most plausible pathway by which *N*-

TABLE II. CARCINOGENICITIES OF *N*-HYDROXY-2-FA, 2-NITROSOFLUORENE, *N*-HYDROXY-2-FBS, AND *N*-HYDROXY-2-FAA AFTER A SINGLE APPLICATION TO THE RAT MAMMARY GLAND.

Compound applied[a]	Rats with tumors/rats used	Latent period (months)	Tumor incidence	
			At site of application	At distant site
N-Hydroxy-2-FA	0/6	—	0	0
2-Nitrosofluorene	2/6	7–8	1/6 (1[b])[c]	1/6 (1[d])
N-Hydroxy-2-FBS	0/7	—	0	0
N-Hydroxy-2-FAA[e]	5/7	3–6	5/7 (5[f])	0
N-Hydroxy-2-FAA[g]	4/6	5–9	4/6 (4[h])	1/6 (2[i])

[a] A single dose of 0.02 mmole of each compound was applied to the left thoracic mammary glands.
[b] One mammary adenocarcinoma.
[c] The numbers in parentheses are the number of tumors.
[d] One mammary adenocarcinoma in the left inguinal region.
[e] This series was run concurrently with the other compounds.
[f] Three mammary adenocarcinomas, one fibroadenoma, one pleomorphic sarcoma.
[g] This series was run independently at a different time.
[h] Three mammary adenocarcinomas, one fibroadenoma.
[i] One fibroadenoma in the right thoracic region, one fibroadenoma in the right inguinal region.

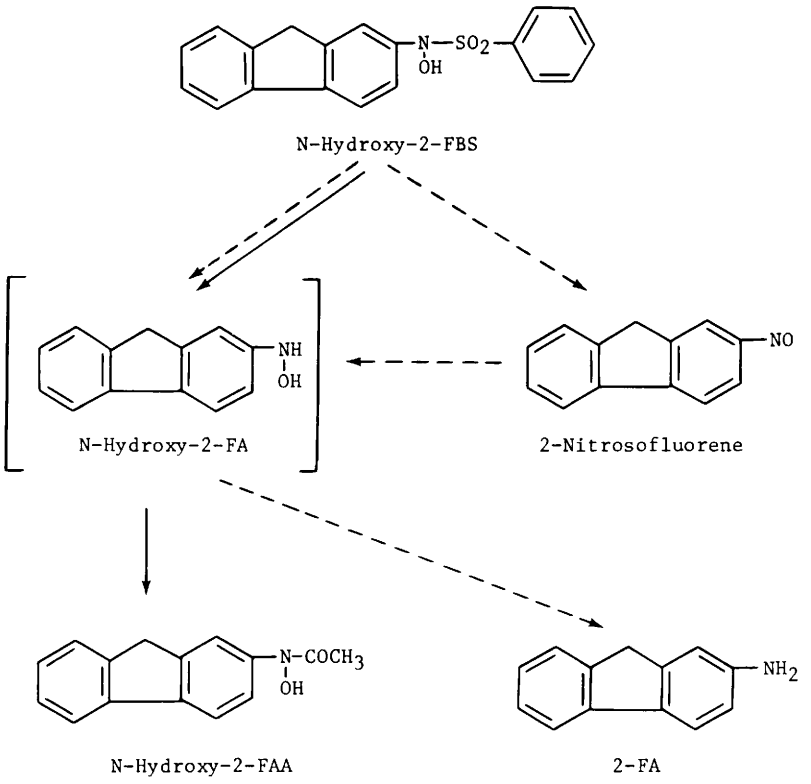

FIG. 3. Reactions of the carcinogen *N*-hydroxy-2-FBS *in vitro* and *in vivo*. - - - - → Reactions demonstrated *in vitro*; ——→ reactions demonstrated *in vivo*.

hydroxy-2-FAA arises from *N*-hydroxy-2-FBS proceeds through desulfonylation and acetylation of the intermediate, *N*-hydroxy-2-FA, as indicated in Fig. 3. Desulfonylation takes place in liver as well as in the mammary gland (Table I). The location where acetylation of the hydroxylamine takes place is not known at present. The mammary gland may

be excluded as the site of acetylation because *N*-hydroxy-2-FA applied to the gland directly was not carcinogenic. It is probable that *N*-hydroxy-2-FA is acetylated elsewhere (11) and that the resulting *N*-hydroxy-2-FAA is transported to the mammary gland where it acts directly as a carcinogen. This view is supported by data showing that conversion of the fluorenylhydroxamic acid to an "ultimate" carcinogen(s) occurs in liver but does not take place in mammary gland (7, 12, 13).

Summary. The mechanism of mammary carcinogenesis by *N*-hydroxy-2-FBS, a highly potent mammary carcinogen for the female rat by ip administration, has been investigated. Previous work *in vivo* indicating hydrolytic cleavage of the nitrogen–sulfur bond has been confirmed with the use of sonicates of mammary gland. One of the products of the hydrolysis was *N*-hydroxy-2-FA identified by its conversion to 2-FA. Since carcinogenicity tests by local application showed that *N*-hydroxy-2-FA was not carcinogenic for the mammary gland, desulfonylation of *N*-hydroxy-2-FBS by mammary gland does not account for mammary carcinogenesis. *N*-Hydroxy-2-FBS applied directly to the mammary gland was not carcinogenic and 2-nitrosofluorene, the product of the spontaneous decomposition of *N*-hydroxy-2-FBS, exhibited only weak carcinogenicity upon local application. In contrast, *N*-hydroxy-2-FAA, a urinary metabolite of *N*-hydroxy-2-FBS, was highly carcinogenic by local application and very likely mediates the action of *N*-hydroxy-2-FBS. A metabolic pathway for the conversion of *N*-hydroxy-2-FBS to *N*-hydroxy-2-FAA is presented. This pathway involves the intermediate formation, by mammary gland or liver, of *N*-hydroxy-2-FA. The site of the subsequent acetylation of the hydroxylamine is unknown at present although the mammary gland appears to be excluded.

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