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Inhibition of Amino Acid Incorporation *in vitro* by Metabolites of 2-Acetylaminofluorene and by Certain Nitroso Compounds.* (30583)

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Arrhenius and Hultin(1-3) have shown that administration of large amounts of 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), or of certain other carcinogenic amines or amides to rats inhibited the ability of liver preparations to incorporate labeled amino acids into protein when the animals were killed up to 8 hours later. After 8 hours the *in vitro* incorporation of labeled amino acids increased to above normal levels as a result of a corticoid-stimulated mechanism. Inhibition of amino acid incorporation into protein could also be demonstrated when rat liver slices were preincubated with these amines or amides prior to incubation with radioactive amino acids(3,4).

These results suggested that metabolites of these amines and amides were responsible for the inhibition of amino acid incorporation. The present studies were undertaken to determine which metabolites of AAF and AF inhibited amino acid incorporation when added to a rat liver microsome-pH 5 fraction system. 2-Nitrosofluorene, which has been detected as a metabolite of *N*-hydroxy-AAF by Irving(5) and which must therefore also be a metabolite of AAF and AF, inhibited amino acid incorporation strongly. Similar strong inhibition was obtained with nitrosobenzene and 2-nitrosotoluene; these compounds proved not to be carcinogenic when administered subcutaneously under conditions whereby 2-nitrosofluorene is carcinogenic(6).

Materials and methods. Weanling male rats (Holtzman Rat Co., Madison, Wis.) were killed by decapitation, and 10 g of liver was homogenized in 40 ml of medium A of Hawtrey *et al*(7) (0.25 M sucrose, 0.005 M $MgCl_2$, 0.025 M KCl, and 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.6). The nuclei and mitochondria were sedimented at 8,500 × *g* for 15 minutes, and the supernatant fraction was recentrifuged at 105,000 × *g* for 1 hour in a Spinco Model L centrifuge. The microsomal pellet was washed, resuspended, and recentrifuged(7), and finally resuspended in 15 ml of medium A. The pH 5 fraction was precipitated from the microsomal supernatant by the addition of 1 N acetic acid to a pH of 5.2, centrifuged, washed(7), and resuspended in 15 ml of medium A. The protein content of each preparation of microsomes and pH 5 fraction was determined according to Cleland and Slater (8).

For the amino acid incorporation assays each tube contained in a total volume of 1 ml 0.5 μmole guanosine triphosphate, 2.25 μmoles adenosine triphosphate, 18 μmoles phosphoenolpyruvate, 50 μg pyruvate kinase, 35 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.6, 9 μmoles $MgCl_2$, 18 μmoles KCl, 190 μmoles sucrose, 10 μl dimethylsulfoxide (containing test compound), and 1.2-2.5 mg each of microsomal and pH 5 fraction pro-

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tein. 0.25 μc L-leucine-UL-C^{14} (Volk, 180 mc/mmole), L-phenylalanine-UL-C^{14} (Volk, sp. act. 185 mc/mmole), or L-phenylalanine-UL-H^3 (Schwarz BioReseach, sp. act. 1.5 c/mmole) was added per tube; when phenylalanine was used, 25 μg of polyuridylic acid (Pabst Laboratories) was also added per tube. After incubation at 37°C for 15 minutes, 0.1 ml of a saturated aqueous solution of nonradioactive L-leucine or L-phenylalanine was added, and the protein was precipitated by addition of 1.5 ml of 10% trichloroacetic acid. The protein was sedimented by centrifugation, washed twice by resuspension in 3 ml of 5% trichloroacetic acid and centrifugation, extracted at 90°C for 20 minutes with 3 ml 5% trichloroacetic acid, washed twice with 3 ml 5% trichloroacetic acid, and finally dissolved in 2 ml of concentrated formic acid at room temperature for radioactivity determination in a Packard Tri-Carb scintillation counter. All counts were corrected for counting efficiency; the radioactivity of proteins from unincubated reaction mixtures was always less than 1.5% of the radioactivity of protein from incubated control samples.

AF(9), 2-nitrosofluorene(10), *N*-hydroxy-AF(10), *N*-hydroxy-AAF(11), and nitrosobenzene were synthesized in this laboratory. 2-Nitrosotoluene was a product of Aldrich Chemical Co.; each of the nitroso compounds was sublimed *in vacuo,* and these compounds and *N*-hydroxy-AF were stored at —15°C. 1- And 3-hydroxy-AAF were kindly supplied by Dr. T. L. Fletcher, University of Washington, Seattle and 5- and 7-hydroxy-AAF were kindly supplied by Drs. John and Elizabeth Weisburger, National Cancer Institute, Bethesda, Md. These compounds and AAF (Mann Research Lab.) were chromatographed on a 1:1 mixture of Celite (Johns-Manville) and Darco activated charcoal with 60% ethyl acetate—40% benzene prior to use. A solution of each compound in redistilled dimethyl sulfoxide was prepared immediately prior to use.

For determination of the carcinogenic activities of the nitroso derivatives 2.5 mg of 2-nitrosofluorene or equimolar amounts of nitrosobenzene or 2-nitrosotoluene dissolved in 0.2 ml of tricaprylin without heat was injected subcutaneously twice weekly for 8 weeks into groups of 20 random-bred CD female rats (Charles River Breeding Laboratories, Brookline, Mass.) with initial weights of 90-110 g. The solutions were injected immediately after preparation. The rats were maintained on Wayne Blox and water *ad libitum.* All tumors were fixed in 10% neutral formalin and sectioned at 5-7 μ. The sections were kindly diagnosed by Dr. Henry Pitot of the McArdle Laboratory.

TABLE I. Inhibition of Incorporation of L-Phenylalanine by 2-Acetylaminofluorene and Its Metabolites.

Compound (3×10^{-4}M)	C^{14} or H^3 incorporated into protein (% of control)* Exp 1	Exp 2
2-Nitrosofluorene	8	3
AF	100	100
AAF	93	81
N-Hydroxy-AF	76	97
N-Hydroxy-AAF	96	83
1-Hydroxy-AAF	96	83
3-Hydroxy-AAF	84	84
5-Hydroxy-AAF	80	74
7-Hydroxy-AAF	72	60

* L-Phenylalanine-UL-H^3 was used in Exp 1; incorporation in the control tubes was 25,000 cpm. L-Phenylalanine-UL-C^{14} was used in Exp 2; incorporation in the control tubes was 57,000 cpm.

Results. Amino acid incorporation experiments. Of the fluorene derivatives studied only 2-nitrosofluorene had a marked effect on amino acid incorporation. At a concentration of 3×10^{-4} M it inhibited the incorporation of H^3- or C^{14}-L-phenylalanine into protein to less than 10% of the control level (Table I). Other experiments showed that it similarly inhibited the incorporation of L-leucine. At 3×10^{-4} M levels AF, AAF, *N*-hydroxy-AAF, *N*-hydroxy-AF and 1-hydroxy-AAF caused no significant inhibition, while 3-, 5-, and 7-hydroxy-AAF inhibited the reaction to 60-85% of the control value.

Nitrosobenzene and 2-nitrosotoluene inhibited the incorporation of phenylalanine or leucine to the same extent as 2-nitrosofluorene. No inhibition was obtained at 10^{-5} M concentrations, while each compound inhibited the incorporation to about 25-40% of the control value at 10^{-4} M levels (Table II).

The inhibition could not be attributed to

TABLE II. Inhibition of Amino Acid Incorporation by Various Nitroso Derivatives.

Inhibitor	Molarity	C^{14} or H^3 incorporated in protein (% of control)* Exp 1	Exp 2
2-Nitrosofluorene	5×10^{-4}	4	
	1×10^{-4}	24	38
	5×10^{-5}		62
	1×10^{-5}	100	
Nitrosobenzene	1×10^{-4}	27	41
	5×10^{-5}		64
	1×10^{-5}	100	
2-Nitrosotoluene	1×10^{-4}	19	25
	5×10^{-5}		59
	1×10^{-5}	100	

* L-Phenylalanine-UL-H^3 was used in Exp 1; 32,000 cpm were incorporated into protein in the control tubes. L-Leucine-UL-C^{14} was used for Exp 2; 16,000 cpm were incorporated into protein by the control samples.

reaction of the nitroso compounds with the amino acids. Only one radioactive spot, which moved with the same R_F (0.47-0.64) as authentic C^{14}-L-phenylalanine and which accounted for over 95% of the radioactivity added, was obtained on chromatography of the medium before or after incubation. For this purpose the supernatant solution after addition of trichloroacetic acid was extracted with ethyl ether, concentrated, and chromatographed on Whatman No. 1 filter paper strips in 2-butanol-3% ammonia (5:2)(12). The radioactivity was scanned in a Nuclear Chicago strip counter.

The inhibitory effect of 2-nitrosofluorene was not reversed by sedimentation and resuspension of the liver fractions. In this experiment the liver microsomes and pH 5 fraction were incubated separately in medium A (approx. 2 mg of protein in 0.3 ml) with 10 μl of dimethyl sulfoxide or a dimethyl sulfoxide solution of 2-nitrosofluorene (3×10^{-4} M) for 10 minutes at 0°C. The fractions were then reisolated (by centrifugation at 105,000 $\times$ g or precipitation at pH 5.2, respectively). When the microsomes and pH 5 fraction which had been incubated with dimethylsulfoxide were assayed together, one-half of the original activity was recovered. On the other hand, assay of the 2-nitrosofluorene-treated fractions in the absence of additional inhibitor yielded only 3% of the original activity.

Carcinogenicity study. While mammary carcinomas and sarcomas at the injection site were each found by 8 months in approximately one-half of the rats injected with 2-nitrosofluorene (total dose—40 mg), no tumors were found in the rats injected with an equimolar amount of nitrosobenzene and only one benign mammary tumor occurred in a rat injected with this level of 2-nitrosotoluene (Table III).

Discussion. These data suggest that the nitroso derivatives may be important intermediates in the inhibition of amino acid incorporation by liver preparations from rats treated *in vivo* with carcinogenic amines or in liver slices incubated with these compounds. The formation of 2-nitrosofluorene from *N*-hydroxy-AAF by liver microsomes has been described by Irving(5), but the reactivity of the nitroso derivative has negated our attempts to estimate the amounts formed *in vivo*. The possible role of other metabolites as inhibitors of amino acid incorporation has not been excluded; thus the weak inhibition with 7-hydroxy-AAF could be due to its deacetylation to the more reactive aminofluorenol. Arrhenius and Hultin (4) suggested that the 7-hydroxy derivative might be involved on the basis of the smaller inhibition observed *in vivo* or with slices with 7-fluoro-AAF or 7-fluoro-AF as compared to the non-fluorinated derivatives. However, the

TABLE III. Carcinogenic Activities of Subcutaneously Injected 2-Nitrosofluorene, Nitrosobenzene, and 2-Nitrosotoluene.*

Compound injected	No. of rats with tumors by 8 months: Mammary carcinomas	Sarcomas at injection site
2-Nitrosofluorene	9†	10
Nitrosobenzene	0	0
2-Nitrosotoluene	0‡	0
None (solvent only)	0	0

* Groups of 20 female rats were injected subcutaneously twice weekly for 8 wk with 2.5 mg of 2-nitrosofluorene or equimolar amounts of the other compounds.

† One rat had 4 mammary carcinomas, one rat had 5, and the other rats each bore single mammary carcinomas.

‡ One rat had a benign mammary tumor.

rate of oxidation of the amine to the nitroso derivative or the stability of the latter compound could also be important factors.

The oxidation of aniline and its *N*-alkyl derivatives to nitrosobenzene has been demonstrated with liver microsomes by Kiese and Uehleke(13). Thus, the lack of inhibition of amino acid incorporation following administration of aniline(4) as compared to the carcinogenic amines may reflect only quantitative differences in the formation or stability of the nitroso derivatives. Although Arrhenius and Hultin(4) observed some correlation between the inhibition of amino acid incorporation and the carcinogenicity of the amine administered, the *in vitro* inhibition by the nitroso derivatives cannot be correlated with their carcinogenicities. Thus, while repeated subcutaneous injections of 2-nitrosofluorene into female rats resulted in moderate yields of mammary carcinomas and of sarcomas at the injection site, neither nitrosobenzene nor 2-nitrosotoluene produced tumors under the same conditions.

The rapidity of reaction of 2-nitrosofluorene with reduced glutathione at neutral pH (10) and the requirement of sulfhydryl groups for activation of amino acids and incorporation of the activated amino acids into protein(14,15) suggest that the inhibition results from the reaction of 2-nitrosofluorene with sulfhydryl groups on these enzymes.

Summary. 2-Nitrosofluorene, 2-nitrosotoluene, and nitrosobenzene, at concentrations of 5×10^{-5} M or greater inhibited the incorporation of amino acids into protein by a rat liver microsome-pH 5 fraction system. 2-Acetylaminofluorene, 2-aminofluorene, the *N*-hydroxy derivatives of these compounds, and the 1-, 3-, 5-, and 7-hydroxy derivatives of 2-acetylaminofluorene had little effect on amino acid incorporation at these levels. Repeated subcutaneous injections of 2-nitrosofluorene into female rats induced moderate incidences of mammary carcinomas and of sarcomas at the injection site by 8 months. Similar injections of nitrosobenzene or of 2-nitrosotoluene were not carcinogenic.

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