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INHIBITION OF HEPATIC MACROMOLECULE SYNTHESIS BY SINGLE DOSES OF *N*-HYDROXY-2-FLUORENYLACETAMIDE*

JULIAN B. MARSH and DAVID L. DRABKIN

Department of Biochemistry and Center for Oral Health Research, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

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Abstract—A single intraperitoneal injection of *N*-hydroxy-2-fluorenylacetamide, at a dose of 40 mg/kg, into male rats was shown to inhibit both liver regeneration after partial hepatectomy and proteinuria by rats with experimental nephrosis. These effects were accompanied by inhibition of the incorporation of labeled precursors into hepatic RNA and DNA. No such inhibitory effects were seen in normal rats. No definitive explanation for these results can be given at present, but it is suggested that this compound may be affecting the regulatory mechanisms involved in cell division.

N-OH-FAA† is a potent chemical carcinogen capable of inducing hepatomas after periodic, moderately prolonged intraperitoneal administration to rats.[1] Extensive studies have been carried out on the binding of this compound to DNA and RNA.[2] Barry and Gutmann[3] and Sorof *et al.*[4] have shown that binding to a hepatic basic cytoplasmic protein occurs, and Barry and Gutmann[3] have suggested that this binding may be related to the mechanism of carcinogenesis. In the present work we have found dramatic inhibitory effects of *N*-OH-FAA on protein, RNA and DNA synthesis in regenerating liver and in livers of rats with experimental nephrosis with single doses of the compound as early as 6 hr after it was given. No inhibitory effects were observed in normal animals under the same experimental conditions.

MATERIALS AND METHODS

Male rats of the Holtzman strain, weighing between 130 and 180 g, were used. *N*-OH-FAA of verified purity and *N*-OH-FAA-9-^{14}C were gifts from Dr. Helmut Gutmann. The drug was given intraperitoneally as a suspension in 1% gum acacia in 0·15 M NaCl at a dose of 40 mg/kg except where otherwise indicated. Control animals received injections of 1% gum acacia.

Nephrosis was induced by intravenous injection of sheep antirat kidney serum as previously described.[5] Partial hepatectomy in which two-thirds of the liver was removed was performed under ether anesthesia as previously described.[5] All animals were fed Purina laboratory chow *ad lib.*

Thymidine-methyl-^{3}H (sp. act. 17·8 mc/μmole), orotic acid-6-^{14}C (sp. act. 4·6 μc/μmole) and [^{14}C]amino acids (reconstituted mixture, uniformly labeled, 1·5 mc/mg) were obtained from the New England Nuclear Corp.

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† Abbreviation used: *N*-OH-FAA = *N*-hydroxy-2-fluorenylacetamide.

Protein, RNA and DNA were purified and measured as previously described.[5] Estimation of radioactivity of nucleic acids was carried out by addition of the hot 5% trichloroacetic acid extract to 10 vol. of Aquasol (New England Nuclear Corp.), after which the samples were counted in a liquid scintillation spectrometer. The protein residue was dissolved in 1 N NaOH and dried on filter paper which was then immersed in a toluene-based phosphor for counting. When necessary, quenching corrections were made, and all samples were counted to a 5 per cent probable error. For the measurement of the absorption of labeled *N*-OH-FAA, liver and serum samples were dissolved in NCS Solubilizer (Amersham/Searle Corp.) before adding to the toluene-based phosphor.

RESULTS

When a single dose of *N*-OH-FAA was given to nephrotic rats, a marked decline in proteinuria occurred (Table 1). The effect persisted for at least 2 days. The drug did not modify the small amount of proteinuria seen in normal animals. The decline in proteinuria was related to lower serum protein levels (Table 2). In addition, the rise in liver RNA seen in nephrosis was prevented. Injection of *N*-OH-FAA into normal animals did not decrease serum protein, liver RNA or liver DNA levels, since the values shown in Table 2 are not significantly lower than normal values obtained in our laboratory at the time these experiments were carried out.[5]

When *N*-OH-FAA was given to rats within 1 hr after two-thirds partial hepatectomy (Table 3), at one-third the usual dose, examination of the liver remnant 24 hr later

TABLE 1. EFFECT OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA) ON PROTEINURIA OF NEPHROTIC RATS*

Type of animal	No. of animals	Protein excretion per day Day (mg) 0	Day (mg) 1	Day (mg) 2
Nephrotic	3	611 ± 7·7	613 ± 22	674 ± 49
Nephrotic plus *N*-OH-FAA	4	651 ± 31	331 ± 41†	175 ± 9†
Control plus *N*-OH-FAA	4	37 ± 3·1	41 ± 7·9	32 ± 1·3

* Rats were injected as described in the text at the end of day 0. The numbers after the ± represent the standard error of the mean.

† Significantly different from the corresponding mean of uninjected nephrotic rats, as judged by $P < 0.05$.

TABLE 2. SERUM PROTEIN AND LIVER RNA LEVELS IN NEPHROTIC RATS GIVEN *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA)*

Type of animal	Serum protein (mg/ml)	Liver RNA (mg/g)
Control plus *N*-OH-FAA	71·6 ± 1·88 (5)	8·4 ± 0·44 (4)
Nephrotic	54·9 ± 2·31 (7)	12·5 ± 0·99 (6)
Nephrotic plus *N*-OH-FAA	36·2 ± 1·77 (9)†	9·6 ± 0·49 (4)†

* Animals were sacrificed 2 days after the drug was given. The numbers after ± represent the standard error of the mean and the numbers in parentheses represent the number of animals.

† Significantly different from the means of untreated nephrotic rats, as judged by $P < 0.05$.

revealed a significant decrease in RNA, but not DNA. Normally, DNA levels do not rise at this early time, and these results suggest that the drug was not causing pathological changes of cells but was probably interfering with RNA synthesis. Since catabolism of RNA is appreciable, the net effect would be a drop in the RNA level. When the livers were examined 4 days later, it can be seen (Table 4) that regeneration lagged considerably behind in the animals receiving the drug.

TABLE 3. LIVER RNA and DNA AFTER INJECTION OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA) IN PARTIALLY HEPATECTOMIZED RATS*

Conditions	Liver RNA (mg/g)	Liver DNA (mg/g)
Partial hepatectomy	9·3 ± 0·34 (5)	3·5 ± 0·18 (5)
Partial hepatectomy, plus *N*-OH-FAA	6·6 ± 0·09 (3)†	3·4 ± 0·15 (3)

* Animals given 13 mg/kg of *N*-OH-FAA intraperitoneally 1·5 hr after removal of the liver, and RNA and DNA measured 24 hr later.

† Significantly different from the mean of the uninjected animals as judged by $P < 0·05$. The numbers in parentheses represent the number of animals, while those after the ± represent the standard error of the mean.

TABLE 4. LIVER REGENERATION AND SERUM PROTEIN LEVELS AFTER INJECTION OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA)*

Conditions	Per cent regeneration	Serum protein (mg/ml)
Partial hepatectomy	92 ± 3·8 (3)	51·6 ± 0·6 (3)
Partial hepatectomy, plus *N*-OH-FAA	54 ± 1·9 (3)†	44·2 ± 1·7 (3)†

* Animals given 13 mg/kg of *N*-OH-FAA intraperitoneally 1·5 hr after removal of the liver. Regeneration and serum protein levels were measured 4 days later. The per cent regeneration was calculated, using the equation

$$\frac{100\ (\text{g final total liver}) - ((\text{g liver excised}/0{\cdot}68) - \text{g liver excised})}{\text{g of liver excised}}$$

in which 0·68 = (g left lateral lobe + median lobe)/(g original total liver).[6]

† Significantly different from the mean of the uninjected animals, as judged by $P < 0·05$. The numbers in parentheses represent the number of animals, while those after the ± represent the standard error of the mean.

The incorporation of labeled precursors into protein, RNA and DNA was not inhibited by the injection of *N*-OH-FAA into normal rats (Table 5). At 24 hr, a slight increase in RNA labeling occurred. In rats with established (24 hr) nephrosis, a significant inhibition of labeling of protein, RNA and DNA was seen (Table 6). The effect was greatest on DNA labeling. When the drug was given at the same time as the antikidney serum injection, the greatest effect was seen again on DNA. These findings are in general agreement with the fact that the greatest drop in proteinuria in nephrotic rats occurred between day 1 and day 2 (Table 1).

Since the specific activity of the RNA precursor pool was not measured, it is not possible unequivocally to equate isotope incorporation into RNA with the rate of synthesis. In the regenerating liver (Table 3) the decline in the amount of RNA at

TABLE 5. EFFECT OF INJECTION OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA) ON THE INCORPORATION OF LABELED PRECURSORS INTO PROTEIN, LIVER RNA AND LIVER DNA IN NORMAL RATS*

Time of exposure to drug (hr)	Per cent change in labeling compared with uninjected controls			
	Serum protein	Liver protein	Liver RNA	Liver DNA
1			− 2·0 ± 4·5 (12)	− 1·0 ± 8·6 (13)
6	0·0 ± 7·6 (11)	−10·0 ± 5·6 (11)	+11·0 ± 13 (5)	−12·0 ± 11 (5)
24	+6·0 ± 10 (6)	+12 ± 7·8 (6)	+26 ± 10·6 (7)†	+ 7·0 ± 7·6 (7)

* Rats received 1 μc of labeled amino acids, 10 μc of labeled thymidine, or 0·5 μc of labeled orotic acid in 0·5 ml 0·15 M NaCl intraperitoneally 1 hr before sacrifice. The figures in parentheses represent the number of animals. The figures after ± represent the standard error of the mean. Generally, three controls and three injected animals were run as a group at the same time. The per cent change in labeling was calculated by averaging the total amount of radioactivity in the purified compound per g wet weight of liver for the control animals and calculating the per cent difference of each of the carcinogen-injected animals from the mean of the controls. In this way, differences from week to week and differences between groups of rats were minimized. In the control animals, the average specific activity of serum protein was 38, liver protein 41, liver RNA 5700 and liver DNA 960 counts/min/mg respectively.

† Significantly different from zero, as judged by $P \leqq 0{\cdot}05$.

TABLE 6. EFFECT OF INJECTION OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA) ON THE INCORPORATION OF LABELED PRECURSORS INTO PROTEIN, RNA AND DNA IN LIVERS OF NEPHROTIC RATS*

Time after anti-kidney serum injection (hr)	Time of exposure to *N*-OH-FAA (hr)	Per cent change in labeling compared with untreated controls			
		Serum protein	Liver protein	Liver RNA	Liver DNA
24	6	−27 ± 1·6 (5)†	−21 ± 8·2 (5)†	−28 ± 10 (9)†	−40 ± 7·7 (9)†
0	24	− 6 ± 5·3 (3)	+ 5 ± 6·3 (9)	−18 ± 6·1 (3)	−75 ± 9·8 (3)†

* Animals were given labeled precursors as described in footnote to Table 4. The time after anti-kidney serum injection represents the time at which the *N*-OH-FAA was given.

† Significantly different from zero, as judged by $P \leqq 0{\cdot}05$.

26 hr, combined with the decreased incorporation of label after *N*-OH-FAA administration makes it probable that RNA synthesis was depressed. In the case of the nephrotic rats, the amount of hepatic RNA after 48 hr was less in the presence of the carcinogen (Table 2) but we did not find a significant change in RNA content at 24 hr, when the labeling studies were carried out. If the precursor nucleotide pool remained constant, then there was an inhibition of RNA synthesis when the drug was present for 6 hr in a 1-day nephrotic rat, but no significant inhibition when the drug was given over a 24-hr period. It is possible that a stimulation of RNA synthesis by the drug, seen in normal animals at 24 hr (Table 5), is partially masking the inhibition in nephrotic rats at this time.

The most dramatic effects were seen on DNA and RNA labeling in livers from partially hepatectomized rats (Table 7). Even at one-third of the usual dose, almost

complete inhibition of the rise in DNA synthesis seen 24 hr after partial hepatectomy occurred. The parent compound, 2-amino fluorene, and its acetylation product were less inhibitory than *N*-OH-FAA (Table 8).

TABLE 7. EFFECT OF INJECTION OF *N*-HYDROXY-2-FLUORENYLACETAMIDE (*N*-OH-FAA) ON THE INCORPORATION OF LABELED PRECURSORS INTO RNA AND DNA BY LIVERS OF PARTIALLY HEPATECTOMIZED RATS*

Dosage of drug (mg/kg)	Per cent change in labeling compared with uninjected controls	
	RNA	DNA
40	−86 ± 6·1 (3)†	−96 ± 1·7 (3)†
13	−59 ± 8·1 (3)†	−93 ± 1·8 (3)†

* *N*-OH-FAA was injected 1 hr after removal of two-thirds of the liver and the labeled precursors injected 24 hr later. Incorporation was measured 1 hr later. The average specific activity of RNA and DNA in control animals was 10,700 and 11,800 counts/min/mg respectively.

† Significantly different from zero, as judged by $P \leq 0{\cdot}05$.

TABLE 8. EFFECT OF AMINO FLUORENE COMPOUNDS ON RNA AND DNA LABELING IN LIVERS OF PARTIALLY HEPATECTOMIZED RATS*

Compound	Per cent change in labeling compared with controls	
	RNA	DNA
2-Amino fluorene	−21 ± 8·7 (3)	−39 ± 17 (3)
N-acetyl-2-amino fluorene	−39 ± 3·2 (3)†	−66 ± 4·1 (3)†

* Compounds (40 mg/kg), injected intraperitoneally 1 hr after partial hepatectomy. Labeled precursors were injected 24 hr later and animals sacrificed 1 hr later.

† Significantly different from zero, as judged by $P \leq 0{\cdot}05$.

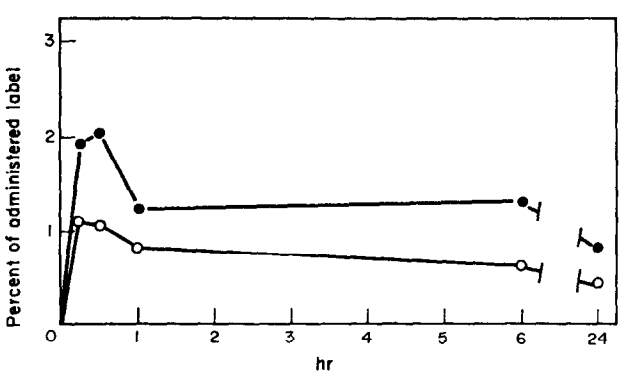

FIG. 1. Absorption of *N*-hydroxy-2-fluorenylacetamide-9-^{14}C after intraperitoneal injection of 40 mg/kg (sp. act. 0·24 mc/m-mole) suspended in 1% gum acacia. The total amount of label appearing in the liver (●——●) and serum (○——○) was measured, assuming approximately 4 per cent of the body weight for each.

After injection of *N*-OH-FAA-9-^{14}C, an estimate of the amount of radioactivity appearing in serum and liver over a 24-hr period can be obtained from the data in Fig. 1. About 1–2 per cent of the administered label appears in the liver in 6 hr. When the compound is given intraperitoneally dissolved in propylene glycol, about twice the amount is present in serum and liver.

DISCUSSION

The results of the present investigation show clearly that *N*-OH-FAA at a dose of 40 mg/kg given intraperitoneally to normal Holtzman rats of about 150 g had no inhibitory effect on the incorporation of labeled precursors into liver or serum protein, and liver RNA and DNA. When these processes are accelerated, either by partial hepatectomy or by induction of nephrosis, a single injection of the carcinogen prevents the increase in nucleic acids, especially DNA, seen under these circumstances. Although the macromolecule precursor pool sizes were not measured after injection of the drug, in every case the incorporation of isotope paralleled the directional changes in total amounts of DNA, RNA and protein. The inhibitory effects of the carcinogen in nephrotic or regenerating liver were more pronounced on DNA labeling than on RNA labeling and least, though significant, on the incorporation of labeled amino acids into protein. If the effects of the carcinogen on labeling are on the precursor pool size and not on synthesis, it would be difficult to explain the changes in amounts of DNA and RNA shown in Table 2 and Table 4.

It is difficult at present to provide a definitive explanation for these results. One possibility is that the carcinogen is more toxic to both nephrotic and regenerating liver than to normal liver. In the normal rats, there was no mortality seen up to 4 days after the drug was given. De Baun *et al.*[7] reported a 60 per cent mortality due to hepatic necrosis when 50 mg/kg of *N*-OH-FAA was given intraperitoneally to CD Charles River rats weighing about 400 g. Our rats were given 40 mg/kg and weighed about 150 g. Reduction of the dose by two-thirds in the regenerating liver did not greatly alter the inhibitory effect, and the animals were able to begin liver regeneration over a 4-day period (Table 4). Nevertheless, we cannot rule out differential toxicity as an explanation for these results.

Direct inhibition of DNA synthesis is also a possibility. It is difficult to understand why the drug did not inhibit this process in the normal animals, unless the greatly increased rate of DNA synthesis in nephrotic or regenerating liver makes it easier to observe inhibition. A further possible explanation of the results is that the carcinogen may interfere with the process by which accelerated cell division is initiated or regulated in the liver. In spite of much work[6,8] the mechanism of stimulation of cell growth after partial hepatectomy remains unexplained, and the same can be said for the hepatocellular effects of nephrosis.[5] Interference with the action of a hormone is a plausible explanation of the present findings and further work along these lines is contemplated.

It is questionable whether the present observations are related to the mechanism of carcinogenesis. Covalent linkage of the fluorene nucleus to protein and to nucleic acids has been established.[2] Basic cytoplasmic proteins in the liver are the main carriers of bound carcinogen[3] and there is evidence that the extent of formation of hepatic protein-bound fluorene derivatives is related to the susceptibility to carcinogenesis by various species. In the present work, *N*-OH-FAA was a more potent inhibitor of liver

regeneration than the parent compound 2-amino fluorene or its acetyl derivative, in agreement with the relative carcinogenicity of these compounds.[1,7]

Direct inhibitory and stimulatory effects of carcinogenic amines on hepatic protein synthesis have been observed.[9] In the case of 2-amino fluorene, the stimulatory effects may be related to the requirement for induction of hydroxylating enzymes.[10] It is noteworthy that Arrhenius and Hultin[9] found that the stimulatory effect of 2-amino fluorene on protein synthesis was absent in adrenalectomized rats. No inhibitory or stimulatory effects of *N*-OH-FAA on protein synthesis in normal rats were observed in the present experiments. In a recent communication Zieve and Gutmann[11] have reported that *N*-OH-FAA specifically inhibits rat liver RNA polymerase in normal rats injected with the carcinogen dissolved in propylene glycol. We believe this apparent difference in our results may be due to the fact that twice the amount of the labeled drug is present in serum and liver when given at the same dosage dissolved in propylene glycol.

Whatever the correct explanation for our observations proves to be, we believe these experiments may provide a useful technique for further exploration of the problem of induction of hepatic cell division and its alteration in chemical carcinogenesis.

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REFERENCES

1. E. C. Miller and J. A. Miller, *Pharmac. Rev.* **18,** 805 (1966).
2. C. M. King and B. Phillips, *J. biol. Chem.* **244,** 6209 (1969).
3. E. J. Barry and H. R. Gutmann, *J. biol. Chem.* **241,** 4600 (1966).
4. S. Sorof, E. M. Young and P. L. Fetterman, *Expl Cell. Res.* **20,** 253 (1960).
5. J. B. Marsh, D. L. Drabkin and L. Poppe, *Metabolism* **19,** 392 (1970).
6. D. L. Drabkin, *J. biol. Chem.* **171,** 395 (1947).
7. J. R. de Baun, J. Y. R. Smith, E. C. Miller and J. A. Miller, *Science, N.Y.* **167,** 184 (1970).
8. N. L. R. Bucher, *New Engl. J. Med.* **277,** 686 (1967).
9. E. Arrhenius and T. Hultin, *Cancer Res.* **22,** 823 (1962).
10. H. R. Gutmann and R. R. Erickson, *J. biol. Chem.* **244,** 1729 (1969).
11. F. J. Zieve and H. R. Gutmann, *Fedn Proc.* **29,** 340 (1970).