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Effect of Hypophysectomy or *p*-Hydroxypropiophenone on Hepatic Precancerous Changes in Rats Given Thioacetamide or 3'Methyl-4-Dimethylaminoazobenzene. (31914)

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Prior to the onset of demonstrable malignant neoplasia in rats fed 3'methyl-4-dimethylaminoazobenzene (3'Me-DAB) hepatic adenylic acid (5'AMP) deaminase (E. C. 3.5.4.6.) activity of the liver was increased (1) and the deaminase activity of primary liver tumors which subsequently developed was higher than normal liver(2). Hypophysectomy, which inhibits liver tumor induction in rats fed 3'Me-DAB(3), inhibited increases in 5'AMP deaminase activity of precancerous liver(1).

Single intraabdominal injections of 3'Me-DAB also caused increases in hepatic deaminase activity and these increases were accompanied by decreased incorporation of labeled orotic acid into hepatic nuclear ribonucleic acid (RNA)(4). When hypophysectomized animals were given single injections of 3'Me-DAB, enzyme activity was not appreciably increased and incorporation of labeled orotic acid was not decreased(5). Thus, both tumor induction(3) and metabolic lesions in precancerous liver(1,2,4,5) caused by 3'Me-DAB were prevented or delayed by altering the endocrine status of the rat.

Thioacetamide is hepatocarcinogenic in rats (6-8). Like 3'Me-DAB, it too caused increases in hepatic 5'AMP deaminase activity (9) and changes in hepatic nuclear RNA metabolism(8-11). The purpose of the present work was to ascertain whether these effects of thioacetamide upon hepatic tissue were influenced by altering the endocrine status of the rat.

Materials and methods. Rats used in this study were Holtzman females weighing 150-180 g or hypophysectomized females weighing 110-140 g (purchased from Hormone Assay Laboratories, Chicago). They were fed Rockland mouse and rat diet or a semi-synthetic diet(12). Hepatocarcinogens were either fed or injected intraabdominally. When fed, 3'Me-DAB was added to the semi-synthetic diet(12) at 0.06%; thioacetamide was added at 0.066%. When injected, 3'Me-DAB was dissolved in corn oil and injected once at 250 mg/kg body weight; thioacetamide was dissolved in 0.9% NaCl and was injected daily at 50 mg/kg body weight. Orotic acid-

TABLE I. Effect of Hypophysectomy on Increase of Adenylic Acid (5'AMP) Deaminase Activity Following Injections of 3'Methyl-4-dimethylaminoazobenzene (3'Me-DAB) or Thioacetamide.

	5'AMP Deaminase activity, μmoles NH_3/mg protein			
	Oil control	3'Me-DAB	Saline control	Thioacetamide
Normal rats*	(12) 0.87 ± .06†	(16) 1.80 ± .11 207%‡	(11) 1.08 ± .02	(10) 1.68 ± .07 156%
Hyped " §	(7) 0.81 ± .03	(12) 0.94 ± .02 116%	(10) 1.20 ± .09	(11) 1.85 ± .07 154%

Animals injected with oil or 3'Me-DAB were killed 96 hr after a single injection; saline or thioacetamide was injected 4 times once daily and the animals were killed 24 hr after the last injection.

* Values given for oil control and 3'Me-DAB appeared previously(4).

† Numbers in parentheses represent numbers of animals. Values are averages ± S.E.

‡ Calculated by: $\frac{\text{Carcinogen injected}}{\text{Control}} \times 100 = \%$.

§ Values given for control and 3'Me-DAB appeared previously(5).

$6^{14}C$ was dissolved in 0.9% NaCl and injected intraabdominally at 3.3 μc/rat 30 minutes prior to killing.

Livers were homogenized in 0.25 M sucrose. Homogenates were centrifuged either at 105,000 × *g* for enzyme studies(13) or at 1250 × g for isolation of nuclei(14). Nuclei were further purified by centrifugation through 2.2 M sucrose(15).

Reaction mixtures for the assay of 5'AMP deaminase activity contained: 5'AMP, 13.3 mM; sufficient 105,000 × *g* supernatant to give a protein content of 1.5-2.5 mg/ml; and 0.05 M citrate buffer, pH 6.0(13). Incubation was for 30 minutes at 37° in air. Extent of deamination was based on the amount of ammonia or inosinic acid (IMP) produced. Ammonia was determined colorimetrically(16) while IMP was determined spectrophotometrically after elution from Dowex-1 formate cycle columns(13).

RNA of liver nuclei was extracted by the neutral-salt extraction procedure described by Allfrey and Mirsky(17). Two fractions of nuclear RNA were obtained: a slowly labeling "non-nucleolar" nuclear (NNN) RNA fraction extracted with 0.1 M phosphate buffer pH 7.1; and a more rapidly labeling "nucleolar" RNA fraction insoluble in 0.1 M phosphate buffer and 1 M NaCl. These two fractions are not identical with those recognized by Koulish and Kleinfeld (18), but their relationship to cytological entities has been discussed(4,5,9). Nucleoproteins of the two sub-fractions were extracted by the method of Steele *et al*(19); RNA was determined colorimetrically by the method of Volkin and Cohn(20); DNA by the method of Burton (21); and protein by the method of Lowry *et al*(22). Isotope concentrations were determined in a gas flow counter with appropriate corrections for background and self-absorption.

Results and discussion. Thioacetamide injections given once daily caused increases in hepatic 5'AMP deaminase activity through 6 injections(9). Since hypophysectomy prevented increases caused by 3'Me-DAB injections(4,5) it was important to know whether thioacetamide injections increased the hepatic deaminase activity of hypophysectomized rats. The responses of hypophysectomized rats to 3'Me-DAB and thioacetamide are compared in Table I. There was essentially no increase in 5'AMP deaminase activity following a single injection of 3'Me-DAB. On the other hand, hypophysectomized rats responded as readily as normal rats to thioacetamide injections.

When hypophysectomized rats were given multiple injections of thioacetamide followed by a pulse of labeled orotic acid, the concentration of RNA in the "nucleolar" fraction was significantly increased. Increased incorporation of labeled orotic acid into the NNN fraction also was observed. These data are shown in Table II. Nearly identical results were previously observed in normal rats injected with thioacetamide(9). From these data, and those of Table I, we concluded that

TABLE II. Effect of Thioacetamide on Metabolism of Nuclear Ribonucleic Acid (RNA) in Liver of Hypophysectomized Rats.

	RNA concentration, μg RNA/mg protein	
	"Non-nucleolar" Nuclear RNA	Nucleolar RNA
Saline	(8) 38 ± 2*	(7) 60 ± 3
Thioacetamide	(9) 45 ± 3	(9) 74 ± 3†

	Orotic acid-6^{14}C incorporation into RNA			
	"Non-nucleolar" Nuclear RNA		Nucleolar RNA	
	cpm/mg protein	cpm/mg DNA	cpm/mg protein	cpm/mg DNA
Saline	(8) 109 ± 10	(8) 298 ± 41	(8) 930 ±115	(8) 2846 ± 364
Thioacetamide	(7) 174 ± 13†	(7) 618 ± 62†	(7) 604 ± 54	(7) 3734 ± 598

Dosage schedules for thioacetamide and saline were as described in Table I. Orotic acid-6^{14}C, 3.3 μc/rat, was injected 30 min prior to killing. Nuclei were separated from homogenates (14) and then purified by centrifugation through 2.2M sucrose(15). Sub-fractionation of RNA was by the Allfrey-Mirsky procedure(17).

* Values are averages ± S.E. Numbers in parentheses represent number of animals.

† Values greater than corresponding saline values at 0.1 P level or greater.

TABLE III. Effect of *p*-Hydroxypropiophenone (PHP) on Increases in Rat Liver 5'AMP Deaminase Activity Caused by Feeding Thioacetamide (TA).

Weeks diets were fed	5'AMP Deaminase activity, μmoles NH_3/mg protein			
	Control	TA	PHP	TA + PHP
0	(6) 1.32 ± .07*			
2	(6) 1.11 ± .04	(6) 1.71 ± .04	(6) .97 ± .05	(6) 1.68 ± .12
4	(6) 1.03 ± .08	(6) 1.83 ± .05	(6) .84 ± .06	(6) 2.08 ± .14
6	(6) .96 ± .10	(6) 1.90 ± .22	(6) .88 ± .05	(6) 2.02 ± .15
8	(6) .79 ± .07	(6) 1.79 ± .14	(6) .77 ± .02	(6) 2.00 ± .21
12	(6) .67 ± .08	(6) 1.76 ± .09	(4) .81 ± .06	(6) 1.68 ± .07

Control animals were fed a semi-synthetic diet(12). TA was added to this diet at 0.066% while PHP was added at 1.67%.

* Values are averages ± S.E. Numbers in parentheses represent numbers of animals.

acute toxic effects of thioacetamide on rat liver 5'AMP deaminase or RNA metabolism did not require the presence of a functioning pituitary. This conclusion contrasts sharply with the one reached when considering acute toxic effects of 3'Me-DAB on the same facets of liver metabolism in the presence or absence of a functioning pituitary(4,5).

The estrogenic compound *p*-hydroxypropiophenone (PHP) inhibited hepatocarcinogenesis with both 3'Me-DAB(23) and DAB(24). We reported(1) that PHP delayed or inhibited increases in 5'AMP deaminase caused by feeding 3'Me-DAB. As shown in Table III, when thioacetamide and PHP were fed simultaneously throughout 12 weeks, there was no indication of interference with the increase in deaminase activity.

Since PHP did not inhibit increases in 5'AMP deaminase activity caused by thioacetamide feeding (Table III) but did inhibit increases caused by 3'Me-DAB(1), it was important to determine whether these response patterns would be sustained when the carcinogens were administered by injection. Accordingly, rats were fed a diet(12) containing 1.67% PHP for 1, 2 or 3 weeks and then given single injections of 3'Me-DAB or 4 once daily injections of thioacetamide. The data are shown in Table IV. One week of PHP feeding caused no interference and both carcinogens caused increases in hepatic 5'AMP deaminase activity. But, after 2 or 3 weeks on PHP containing diets, 5'AMP deaminase activity among 3'Me-DAB injected animals was considerably less than that among animals fed the control diet. On the other hand, animals of the PHP fed group responded to thioacetamide as well or better than those receiving control diet. Since PHP possesses

TABLE IV. Effect of PHP on Increases in Rat Liver 5'AMP Deaminase Activity Caused by Injections of 3'Me-DAB or Thioacetamide.

	5'AMP Deaminase activity, μmoles IMP/mg protein			
	Basal diet		Basal diet + PHP	
Time diets were fed	3'Me-DAB injected	Thioacetamide injected	3'Me-DAB injected	Thioacetamide injected
1 wk	(3) 3.55 ± .33*	(4) 3.12 ± .13	(3) 3.09 ± .31	(4) 3.42 ± .36
2 "	(4) 3.13 ± .12	(4) 2.26 ± .10	(2) 1.66 ± .66	(4) 2.25 ± .13
3 "	(4) 3.69 ± .77	(4) 2.81 ± .23	(2) 1.90 ± .03	(4) 3.36 ± .40

Control diet was a semi-synthetic diet(12) and PHP was added to this diet at 1.67%.
3'Me-DAB and thioacetamide were injected as described in Table I.
* Values are averages ± S.E. Numbers in parentheses represent numbers of animals.

potent estrogenic activity(25), it seems reasonable to conclude: that PHP administration altered the endocrine status of the rat; and, that this alteration mediated the effects of 3'Me-DAB on 5'AMP deaminase activity, but not the effects of thioacetamide.

We also sought to determine whether adrenalectomy inhibited increases in 5'AMP deaminase activity caused by thioacetamide or 3'Me-DAB. Here, when inhibitions were seen, they occurred principally in animals injected with 3'Me-DAB. The magnitude of the inhibitions, however, was small and inhibition was sustained for only a short interval after operation. Thus, under our conditions, we were unable to determine whether adrenal function mediated effects of either carcinogen on 5'AMP deaminase activity.

A proximate carcinogen has been defined as the molecular structure responsible for malignant transformation, or one which is more closely related to the ultimate structure than the administered compound(26). It has been clearly demonstrated that DAB and its carcinogenic derivatives undergo metabolism to proximate carcinogens(26). Changes in the endocrine status of the rat profoundly influenced tumor induction(3) as well as the appearance of certain biochemical lesions in the precancerous liver(1). In view of this, it seems appropriate to postulate that both events are caused by the proximate carcinogen and the conversion of azo dyes to proximate carcinogens occurs by pathways which are influenced by the endocrine status of the liver. The failure of altered endocrine status to prevent these same biochemical lesions after thioacetamide administration suggests that: (a) thioacetamide is the proximate carcinogen; or (b) pathways of metabolic conversion to the proximate carcinogen are not influenced by altered endocrine status. It would be interesting to know whether hypophysectomized rats fed thioacetamide would develop cancer of the liver.

Summary. When either thioacetamide or 3'methyl-4-dimethylaminoazobenzene (3'Me-DAB) was injected intraabdominally into rats, liver adenylic acid (5'AMP) deaminase activity was significantly increased. When these two carcinogens were administered to hypophysectomized rats, the 5'AMP deaminase activity of thioacetamide injected rats was increased, but not that of 3'Me-DAB injected animals. Incorporation patterns of orotic acid-6^{14}C into two sub-fractions of rat liver nuclear RNA among thioacetamide injected rats were not affected by hypophysectomy. Feeding *p*-hydroxypropiophenone (PHP) to rats failed to diminish the increase in liver 5'AMP deaminase activity caused by thioacetamide, whether fed or injected. But, increases caused by injections of 3'Me-DAB were inhibited. The relationship between the endocrine status of the rat and the conversion of thioacetamide and 3'Me-DAB to proximate carcinogens was discussed.

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Quantitative Studies on Spheroplast Formation by the Complement System and Lysozyme on Gram Negative Bacteria.* (31915)

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Gram negative bacteria of the genera *Vibrio, Salmonella, Shigella, Escherichia, Brucella* and *Hemophilus* may be killed by the complement (C′) system(1). Some of the members of these groups, particularly rough forms, are extremely sensitive to small amounts of fresh normal serum, which serves as a source of both natural antibody and C′. Other organisms are not killed unless antiserum is present in addition to C′, and a third group, under the usual experimental conditions, is refractory to C′ despite sensitization by antiserum(2). Among the Enterobacteriaceae, resistance to normal serum has been associated with the capsular (K) antigenic content of the organism(3) and, more significantly, from a biomedical point of view, with the virulence of the organism (4).

Although the bactericidal and bacteriolytic reactions mediated by serum substances have not generally been distinguished, recent observations have indicated that the bactericidal reaction requires antibody and C′, whereas the lysis of the killed cells also requires the enzyme lysozyme(5,6). C′ activity is not unique in rendering cells of gram negative bacteria susceptible to lysozyme attack since starved cells at an abnormal pH(7) or treatment with polymyxin B sulfate(8) will accomplish this result. Obviously different mechanisms may lead to cell lysis by lysozyme.

Bacteria rendered non-viable by the C′ system are not grossly distorted, but are lysed only upon subsequent addition of lysozyme, or in a stabilizing milieu, converted to spheroplasts(5). The quantitative relationships between the susceptibility of an organism to C′ and to lysozyme have not been determined. It was not known whether cells killed by C′ derived from relatively sensitive organisms were equally or differently sensitive to lysozyme. Consequently, cells of organisms

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