Effects of Adrenocorticotropic Hormone and Growth Hormone on the Metabolism of N-Hydroxy-N-2-fluorenylacetamide and on Physiologic Parameters

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Summary

The effects of large doses of adrenocorticotropic hormone (ACTH) and of growth hormone on physiologic parameters and on the metabolism of N-hydroxy-N-2-fluorenylacetamide (N-OH-FAA) were investigated in Fischer male rats. ACTH, 96 or 80 units, and growth hormone, 12 or 10 mg, were injected s.c. daily for 10 consecutive days into animals prefed either control diet or the N-OH-FAA-containing diet (160 ppm) for 4 weeks prior to the hormonal treatment. ACTH, but not growth hormone treatment, led to increased rectal temperature and body weight loss. Water intake and urine output were elevated by either hormone. ACTH injections enlarged the adrenals 6- to 7-fold and practically involuted the thymus. ACTH caused a significant percentile weight increase in the liver, kidney, heart, and pituitary, but growth hormone did not.

14C-labeled N-OH-FAA was given on the last day of the hormonal treatment. Urinary excretion of total metabolites, of glucosiduronic acids, and particularly of the glucuronide of N-OH-FAA was increased by both hormones and was especially notable in the carcinogen-prefed animals. These changes suggest that ACTH and growth hormone decreased dehydroxylation and deacetylation of N-OH-FAA. The radioactivity bound to liver proteins was increased, remarkably so in the animals treated with growth hormone.

The results indicate that ACTH plays a paramount role in inducing marked physiologic and metabolic changes, eventually leading to the enhancement of hepatoma formation, as seen in animals fed carcinogen and implanted with MtT, a functional pituitary tumor. The contributing role of growth hormone, however, should not be neglected.

Introduction

The endogenous pituitary hormones from a transplantable pituitary tumor, Furth MtT/F4, which produces large amounts of ACTH,2 growth hormone, and prolactin (2, 11, 40), enhanced hepatocarcinogenesis by N-OH-FAA (43), a more proximate and active carcinogen derived by metabolism from FAA (9, 27, 28, 44). The underlying mechanisms of the rapid hepatoma formation caused by MtT appeared to rest in part on an increased metabolic rate, indicated by a higher body temperature, and a lower conversion of N-OH-FAA to detoxified products, due to decreased dehydroxylation and deacetylation (39).

Bates et al. (3) analyzed the physiologic changes caused by MtT by employing large doses of exogenous ACTH, growth hormone, and prolactin in female Fischer rats and succeeded in reproducing the splanchomegalic syndrome seen in MtT-bearing animals. The present experiments aimed to assess the roles of ACTH and growth hormone, major hormones produced by MtT, on the physiologic parameters and on the metabolism of N-OH-FAA in male Fischer rats. In order to simulate the conditions of our previous study (39) and also to enhance the metabolic effect, ACTH and growth hormone were given to the animals prefed the carcinogen prior to the hormonal treatment, in addition to the rats fed a control diet.

Materials and Methods

Seven-week-old male rats of the Fischer strain obtained from the NIH Animal Production Section were used. The animals were divided into 6 groups of 4 animals each. The rats of 3 groups were fed a semisynthetic control diet, A (39, 43), and the animals of the other 3 groups were given a carcinogen diet, K, which contains 160 ppm N-OH-FAA in Diet A. The diets were administered for 4 weeks prior to the treatment with hormones. The 1st of each diet group served as a control without treatment (Groups A and K). The animals of the 2nd group were given ACTH (A + ACTH and K + ACTH), and those of the 3rd group were treated with the bovine growth hormone (A + GH and K + GH).

The doses of the hormones and schedule of the treatment were based on the experiment of Bates et al. (3) in female Fischer rats. The hormones were injected s.c. twice a day, in the morning and in the late afternoon, for 10 consecutive days. The doses of the hormones were based on the body weight at the start of the hormonal regimen. ACTH (Achar gel, 80 units/ml, Armour Pharmaceutical Company, Kankakee, Ill.) was given in daily doses of 96 and 80 units for rats fed A and K diet, respectively. The bovine growth hormone was obtained from Dr. Wilhelmi, Emory University, through the courtesy of the NIH Endocrine Study Section; daily doses of 12 and 10 mg were administered to the rats fed A and K diets, respectively.

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CHART 1. Body weight curves of male Fischer rats on various experimental regimens. The 1st points of the curve give weights after 3 weeks on Diets A and K. Hormone injections began after the 4th week, Day 0, and continued for 10 days, the daily dose being given divided in 2 injections in the morning and the afternoon. On Day 7 all rats were placed on Diet A, and labeled N-OH-FAA was injected on Day 10 with the last dose of hormone. Hollow symbols, Diet A; filled symbols, Diet K; O----O, A; □----□, A + GH; △----△, A + ACTH; ●----●, K; ■----■, K + GH; ▲----▲, K + ACTH. N-OH-FAA, N-hydroxy-N-2-fluorenylacetamide; GH, growth hormone; ACTH, adrenocorticotropic hormone.

CHART 2. Rectal temperatures of male Fischer rats on various experimental regimens, during, and for 4 days prior to, injection of hormones. Symbols are given on Chart 1. Note that groups on carcinogen had a generally lower temperature than controls, but their temperature rose after Day 7 when all rats were on control diet. Adrenocorticotropic hormone (ACTH) was pyrogenic. The temperature changes in the groups of rats treated with ACTH coincided with the loss in body weight (Chart 1).
The rectal temperature was measured twice a day, in the morning (9:00–10:00 A.M.) and in the late afternoon (5:00–6:00 P.M.), using an electronic thermocouple.

On the last day of the hormonal treatment, 10 mg/kg of N-OH-FAA-9-14C (specific activity 5.2 \times 10^6 \text{cpm/mg}; 0.89 \text{me/mmole}) suspended in steroid suspending vehicle (a gift of the Cancer Chemotherapy National Service Center, National Cancer Institute) were injected i.p. immediately after the morning administration of the hormones. The animals were placed in metabolism cages, and urine was collected in ice-cold receivers. For 3 days prior to injection of labeled N-OH-FAA, the rats which had been fed Diet K were placed on control Diet A to eliminate free carcinogen.

The animals were killed, by removal of blood from the abdominal aorta followed immediately by perfusion through the portal vein with isotonic saline solution, 24 hr after administration of the labeled carcinogen. The procedures for identification of the urinary metabolites and for the measurement of radioactivity bound to the liver protein were as previously reported (39, 42), with minor modification. Briefly, the free compounds were removed from the urine by ether extraction at pH 6. The glucosiduronic acids were hydrolyzed by incubation with bacterial \(\beta\)-glucuronidase followed by ether extraction. The sulfuric acid conjugates were extracted with ether at pH 6, after acid hydrolysis. After evaluation of the ether, ethanol solutions of the residues from these extracts were chromatographed on paper in a cyclohexane solvent system (cyclohexane, \(i\)-butanol, acetic acid, water, 16:4:2:1). Radioautographic technics were employed to locate and quantitate the individual metabolites.

A liver homogenate was prepared with 4 volumes of ice-cold distilled water per gm of liver, and the proteins were precipitated with 3.5 volumes of ethanol. After extensive washing in centrifuge tubes with acetone (4 times), ethanol (once), ether-ethanol (once), and ether (once), the proteins were dried in vacuo. The radioactivity of all samples was determined with a liquid scintillation counter. The liver proteins were counted after complete digestion with Hyamine. Samples from each tissue or fraction were processed in duplicate or in triplicate. Thus, the averages shown in Tables 2 and 3 are based on 8 or 12 individual analyses. The values were self-consistent and also in accord, where appropriate, with those previously published (cf. Ref. 39, 42).

Tissues were fixed in 10% formalin or Bouin's fixative and stained with hematoxylin and eosin.

### Results

**Physiologic Changes.** At the start of hormonal treatments, the animals which had been on the carcinogen diet for 4 weeks weighed about 30 gm less than the controls (Chart 1). ACTH caused a decrease in the body weight of both carcinogen- and control-diet-fed animals. Growth hormone failed to alter body weight appreciably. The food intake of the animals treated with ACTH or growth hormone decreased slightly within 4 days after the beginning of the injections but thereafter returned to near the control levels of 11–14 gm of A diet, 7–10 gm/rat/day in K diet

Steroid suspending vehicle is an injectable sterile solution of sodium chloride, 9 gm; sodium carboxymethyl cellulose 7 LP, 5 gm; polysorbate 80, 5 ml; benzyl alcohol, 9 ml; and distilled water to 1 liter.
group, respectively. The food intake of the rats treated with ACTH in either A or K diet group showed no change, whereas the other groups revealed a decrease in food intake during the last 24-hr period, after injection of labeled N-OH-FAA. The average food intake (in gm) per rat (for each group) during this period was as follows: Group A, 5.2; K, 6.9; A + ACTH, 12.8; K + ACTH, 10.0; A + GH, 7.3; and K + GH, 6.0. The water intake was increased in both ACTH- and growth-hormone-treated groups regardless of the diets. The mean values (in ml) per day for 3 consecutive days prior to the injection of the labeled carcinogen were as follows: Group A, 11.3; A + ACTH, 14.5; A + GH, 14.1; K, 9.3; K + ACTH, 14.4; and K + GH, 12.6. Corresponding to the water intake, urinary volumes were also larger with the 2 hormones.

ACTH treatment gave rise to a pronounced elevation in the rectal temperature of the animals in both diet groups, but especially so in the K diet group. The means of morning and afternoon temperatures are plotted in Chart 2. On the 8th day, the differences between the temperatures of the ACTH group and those of the control group were 0.7°C in A-diet-fed and 0.8°C in K-diet-fed animals. Growth hormone, however, caused no significant rise in the body temperature.

ORGAN WEIGHTS. The most marked changes caused by ACTH were enlarged adrenals and involuted thymuses (Table 1). Also, ACTH induced a sizable percentile weight increase in the liver, heart, pituitary, and especially in the kidneys. However, the spleen weighed less. There were no real changes in the organ weights attributable to the 2 diets in the 4-week feeding period.

Growth hormone slightly increased liver and kidney weights, per 100 gm body weight, and moderately raised the weight of the adrenals and spleens. The weight of the pituitary in Group A + GH and that of the thymus in Group K + GH were slightly decreased. Neither ACTH nor growth hormone altered the weight of testes.

HISTOPATHOLOGIC OBSERVATIONS. The livers of all the groups fed the carcinogen showed fibrosis in the portal and interlobular areas. The degenerative changes, namely, vacuolated cytoplasm and increased numbers of karyolytic nuclei, in the parenchymal liver cells were more pronounced in Group K + ACTH than in Group K. The livers of Group A + ACTH revealed more vacuolative change in the cytoplasm of the liver cells in the centrolobular area, compared with Group A. Although a few small foci of hyperplasia were found in the livers of some rats fed the carcinogen, there was no indication of enhancement of these changes at this stage by ACTH or growth hormone. Growth hormone led to no remarkable effect on the liver morphology in the rats prefed either A or K diet.

Either prefeeding the carcinogen or hormonal treatment caused degeneration of tubular epithelium of the kidney. Vacuolar degeneration in the epithelium of the convoluted tubules was noted in the animals treated with ACTH. However, the pronounced degenerative changes in the kidneys observed in MtT-bearing animals (11, 39, 43) could not be reproduced by this short experimental regimen.

The adrenals of the rats treated with ACTH revealed hyperplasia of the zona fasciculata and many vacuoles in the hypertrophic cells in this zone.

URINARY METABOLITES OF N-OH-FAA. The total radioactivity from injected labeled carcinogen excreted in the urine was higher in Group K than in Group A (Table 2). Both ACTH and growth hormone increased the output of the urinary radioactivity more so in the A + ACTH group compared with the A + GH group. In K-diet-fed groups, a greatly enhanced excretion of the urinary radioactivity was also induced by the 2 hormones.

Radioactivity in feces paralleled that seen previously (39), but comparative quantitation is unreliable in view of the pronounced differences in fecal output in the short experimental period. The larger food intake in the ACTH-groups led to elimination of more radioactivity with the bulkier stools. The animals injected with growth hormone had small stools.

Both ACTH and growth hormone increased the urinary glucosiduronic acid fraction, particularly in the K-diet-fed groups. The most noticeable change among individual glucuronides due to the hormonal treatments was seen in the glucosiduronic acid of N-OH-FAA (Table 3). ACTH and growth hormone increased this metabolite in A- and in K-diet-fed groups. K diet alone also raised this compound. The other conjugates, with 7-OH-, 5-OH-, and 3-OH-FAA, also contributed to the elevation of the total glucuronide fraction, although to a lesser degree than the N-OH-FAA derivative. An unknown material, with a mobility of 0.24-0.37, which appeared between 5- and 3-OH-FAA on the chromatograms, showed an increase after the treatment with the 2 hormones, more so in the carcinogen-fed groups.

Sulfuric acid conjugates showed only minor changes due to the hormonal treatments. The major metabolites of this type were conjugates of 7-hydroxy derivatives of 2-FAA and 2-FA. There were also several weaker spots in the autoradiographs at the region of 5-OH-FAA (Rf 0.15-0.24), an unknown (Rf 0.24-0.37) between 5-OH- and 3-OH-FAA, 3-OH-FAA (Rf 0.37-0.52),...
**ACTH, Growth Hormone, and N-OH-FAA Metabolism**

**TABLE 3**

| Urinary Metabolites of N-OH-FAA as Glucosiduronic Acids in Rats as Function of Carcinogen Pretreatment and Hormone Injection |
|---|---|---|---|---|
| Group | 7-OH-FAA (%) | 5-OH-FAA (%) | Unknown (%) | N-OH-FAA (%) |
| A | 1.3 | 2.4 | 0.30 | 0.43 | 1.4 |
| K | 2.1 | 4.0 | 0.51 | 1.1 | 4.8 |
| A + ACTH | 1.7 | 4.3 | 0.39 | 0.97 | 3.0 |
| K + ACTH | 3.7 | 6.9 | 1.7 | 1.5 | 15.3 |
| A + GH | 2.2 | 3.6 | 0.74 | 0.99 | 3.9 |
| K + GH | 4.8 | 5.3 | 2.5 | 1.5 | 20.3 |

* For abbreviations, see Table 2, footnote a.

1-OH-FAA, etc., represents N-(7-hydroxy-2-fluoren)acetamide. Addition of the amounts of individual metabolites shown in the table yields a lower value than that shown for the glucosiduronic acid fraction in Table 2 for 2 reasons. Only the main metabolites are listed. Also, there is radioactivity attributable to other metabolites (42) or to isotope distributed along the chromatographic paper strip as well as at the origin and near the solvent front.

An unknown metabolite with Rf 0.24–0.37, between 5-OH- and 3-OH-FAA on chromatograms.

N-OH-FAA does not separate from small amounts of 1-OH-FAA.

1-OH-FAA (Rf 0.52–0.61), and a material (Rf 0.77–0.90) faster than N-OH-FAA. These had been observed earlier (42).

The total radioactivity in the liver of all the carcinogen-fed groups was lower than that of the corresponding A-diet-fed groups (Table 2). ACTH as well as growth hormone increased the total radioactivity in the liver of animals on both diets.

The radioactivity bound to liver proteins was also less in groups on K compared with the A-diet-fed groups. Growth hormone increased the protein-bound radioactivity in both groups; but ACTH, only slightly in the carcinogen-prefed group.

**Discussion**

In this study physiologic changes and modification of the metabolism of the carcinogen N-OH-FAA caused by a functional pituitary tumor, MtT, could be mimicked by employing individual exogenous pituitary hormones. The effect was not as pronounced as observed in the MtT-bearing animals, probably because of the short term of the hormonal treatment. One of the most striking alterations due to the endogenous pituitary hormones supplied by MtT was a marked increase in the body temperature, accompanied by loss of body weight despite increased food intake (39) (see Addendum 1). This condition could be reproduced by ACTH alone, although there was no significant increase of food intake, again owing to the brief period of treatment. Thus, in 1 way ACTH from MtT seems to promote hepatoma formation by increasing the metabolic rate in the rats fed carcinogen and implanted with this pituitary tumor. From this point of view, the report that high environmental temperature is an important factor favoring a high incidence of Lucké renal tumor of the leopard frog is of interest (33). In contrast, Larson et al. (24) found that lower body temperature delayed measurement of the hepatotoxic effect of carbon tetrachloride. Although bacteriologic examination was not performed on the animals treated with ACTH, an increased body temperature caused by infection due to hypercorticism can be ruled out. The ACTH-treated animals appeared healthy and showed no loss of appetite during the hormonal treatment. Furthermore, gross and microscopic examinations of the lungs and other organs revealed no significant degree of infection.

Polydipsia and polyuria accompanied by a diabetes mellitus-like condition, observed in MtT-bearing animals (39), may be attributable to both ACTH and growth hormone, which cause disturbances in water metabolism by inhibiting water reabsorption through the degenerative tubular epithelium of the kidney, and also affect carbohydrate metabolism (10, 21). ACTH may be more responsible than growth hormone for the marked alterations in organ weight of MtT-bearing animals (43). Nevertheless, the changes in the rats treated with exogenous pituitary hormones were not as severe as those observed in the animals implanted with MtT, except for the enlarged adrenals and the completely involuted thymus.

There were some sex differences in the response to the exogenous hormones, chiefly in relation to body weight. In this study growth hormone induced no outstanding change in weight of male Fischer rats, whereas it was responsible for notable growth in female Fischer rats in the similar experiments of Bates et al. (3). However, a comparison of the response of body and organ weights in the male and female rats indicates that male animals are more sensitive to ACTH than female animals. ACTH increased the pituitary weight, per 100 gm, in male rats but decreased it in female animals. Similar changes in the pituitary weight were observed in the MtT-bearing animals (43).

ACTH and growth hormone exerted like effects on the urinary metabolites of N-OH-FAA, increasing urinary excretion of total metabolites, of glucosiduronic acids, and especially the glucuronide of N-OH-FAA. These changes were enhanced in the carcinogen-prefed groups. Ring-hydroxylated metabolites were rather less affected. Thus, both pituitary hormones seem to decrease dehydroxylation and deacetylation of N-OH-FAA, yielding higher levels of N-OH-FAA and lower amounts of detoxified products (see Addendum 2). The in vitro and in vivo transformations of N-OH-FAA have been subjects of recent reports (15, 19, 26, 39, 42).

Lotlikar et al. (25) observed that administration of ACTH largely restored the excretion of N-OH-FAA after injection of a single dose of FAA in hypophysectomized rats. Cortisone and deoxycortisone acted likewise in adrenalectomized-hypophysectomized rats, whereas growth hormone had little effect. Thus, ACTH seems to exert its effect on the metabolism of either N-OH-FAA or FAA in the direction of maintaining higher levels of N-OH-FAA. The present observation is 1 of the few sizable demonstrated effects of ACTH or adrenal hormones on drug metabolism (6, 8, 12, 20, 30, 37). The basic underlying mechanism warrants further attention, especially inasmuch as stress-induced alteration in these hormones may, thus, indirectly affect drug action.

Growth hormone yielded higher levels of protein-bound activity in liver than ACTH or controls, reflecting not only the higher urinary levels of N-OH-FAA but possibly also a greater availability of molecular targets. Despite the larger extent of ex-
cretion of radioactivity in the urine in the hormone-supplemented animals, the activity bound to liver was higher than in controls. It could be, therefore, that an important part of the hormone effect is to act in unison with carcinogen and multiply the number of cellular entities which are in a carcinogen-susceptible state. One is always impressed with the uniform distribution of carcinogen throughout the target organ, yet cancer development, in contrast to toxic action, is a rather localized phenomenon. It seems as though only certain cells bathed with carcinogen were actually in a responsive condition. Hormones may aid in uncovering such entities. The mechanism is presently unclear, yet both ACTH, and indirectly adrenal hormones, as well as growth hormone potentially can act on many molecular targets (17, 21, 22, 29, 32, 38, 45). Additional effort hopefully will discover the crucial selected area where hormone and carcinogen action meet and overlap.

There have been several reports concerning the involvement of the pituitary hormones in hepatoma induction. Griffin and his associates (16, 35, 36) found that ACTH and growth hormone restored hepatocarcinogenesis by 3'-methyl-4-dimethylaminoazobenzene in hypophysectomized rats. However, combined administration of ACTH and insulin did not reinstate the carcinogenic effect in hypophysectomized animals treated with N-2-fluorenyldiacetamide (31). According to Chany and Boy (7), Goodall (13), and Hoch-Ligeti (18) [see also Lacassagne (23) and Wahl et al. (41)], cortisone promoted hepatocarcinogenesis, but Reuber (34) has recently reported that ACTH inhibited tumor induction with N-2-fluorenyldiacetamide. Cortisone likewise reduced the carcinogenic potency of methylcholanthrene (1). Possibly a partial explanation for these varied effects rests on the level of active carcinogen, related in turn to the relative importance of activation versus detoxification reactions (44). Bielschowsky et al. (4, 5) observed that growth hormone favored tumor development in normally refractory thyroidecromized rats treated with N-OH-FAA and to deacetylate the acetyl derivatives. Higher levels of N-OH-FAA would be available, therefore, for conjugation with UDP-glucuronic acid which seems as though only certain cells bathed with carcinogen were actually in a carcinogen-susceptible state. One point cited is the rate of conjugation of N-OH-FAA with UDP-glucuronic acid which might be increased. Hence the compound would be more readily excreted as a conjugate. Although in vitro dehydroxylation and deacetylation have not been studied here, it is believed that the data obtained with livers from rats bearing MtT, as reported in prior experiments (39), can be extrapolated to the present series. Our earlier results showed that the results from rats with MtT and/or N-OH-FAA had lower ability both to dehydroxylate N-OH-FAA and to deacetylate the acetyl derivatives. Higher levels of N-OH-FAA would be available, therefore, for conjugation with glucuronic acid, and excretion in the urine, as has been found (Table 3, this paper, and Table 4, Ref. 39).

In conclusion, this study suggests that ACTH plays a major role in MtT-bearing animals, inducing considerable physiologic changes as well as marked modification of the metabolism of N-OH-FAA, which is involved in faster hepatoma formation. However, in view of the pronounced changes in the urinary metabolites of N-OH-FAA, the higher radioactivity bound to liver proteins after growth hormone, and the synergism between pituitary hormones (3), the contributing role of growth hormone should not be neglected in the interpretation of the MtT effects. In any case, both ACTH and growth hormone appeared to exert appreciable additive action with the carcinogen in regard to its metabolism and also to their joint effect on the liver.

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References


Addenda

ADDENDUM 1. Additional data on this point were obtained following a suggestion of Drs. D. Rall and N. Berlin, National Cancer Institute. The respiratory carbon dioxide production, measured as barium carbonate, was determined as evidence of increased metabolic rate in rats bearing MtT. Control rats with an average body weight of 166 gm, 4 months old, rectal temperature 37.6°C, produced 2.20 gm of BaCO₃/hr; other controls with equal body weight to MtT group, 205 gm, 15 months old, rectal temperature 37.7°C, yielded 2.00 gm of BaCO₃. MtT-bearing rats, 8 weeks after implantation, body weight 207 gm, 4 months old, rectal temperature 36.5°C, gave 2.95 gm of BaCO₃. It was also noted that the temperature of the MtT-bearing rats tended to drop if the rats were deprived of food even for a few hr.

ADDENDUM 2. It has been pointed out that “the pituitary hormones could affect urinary levels of N-hydroxy-FAA glucuronide by a number of other mechanisms.” One point cited is the rate of conjugation of N-OH-FAA with UDP-glucuronic acid which might be increased. Hence the compound would be more readily excreted as a conjugate. Although in vitro dehydroxylation and deacetylation have not been studied here, it is believed that the data obtained with livers from rats bearing MtT, as reported in prior experiments (39), can be extrapolated to the present series. Our earlier results showed that the results from rats with MtT and/or N-OH-FAA had lower ability both to dehydroxylate N-OH-FAA and to deacetylate the acetyl derivatives. Higher levels of N-OH-FAA would be available, therefore, for conjugation with glucuronic acid, and excretion in the urine, as has been found (Table 3, this paper, and Table 4, Ref. 39).
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