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GLUCOCORTICOID BINDING AND RESPONSE IN RAT LIVER AND DIETHYLNITROSAMINE (DENA)-INDUCED HEPATOMAS

R. S. SNART and JOANNA THORNE*
Department of Zoology, The University, Sheffield, S10 2TN, England

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SUMMARY

The binding of [^{3}H] -dexamethasone in normal and diethylnitrosamine treated rat livers has been studied over a 4 month period. It was impossible to distinguish any difference between normal rat livers and diethylnitrosamine (DENA)-induced hepatomas in regard to glucocorticoid binding to receptor proteins, the translocation of receptor–steroid complex into the nucleus or its ability to bind to DNA. Tyrosine amino transferase (TAT) activity in rat livers was refractory to corticosterone and dexamethasone treatments, but following adrenalectomy a highly significant response was observed. It was found that the diethylnitrosamine-induced hepatomas had slightly reduced levels of tyrosine aminotransferase activity, but this enzyme was stimulated significantly by corticosterone and very well by dexamethasone. The plasma corticosterone concentrations measured in diethylnitrosamine treated aminals are significantly lower than normal plasma corticosterone concentrations.

INTRODUCTION

Several steroid unresponsive lymphoma cell lines have recently been described[1, 2] in which either the receptor–glucocorticoid interaction, the subsequent translocation of receptor complex to the nucleus or its interaction with DNA are abnormal. In other cell lines[3, 4] the normal steroid binding and translocation to the nucleus occurs so it is taken that such cells must be abnormal in reactions beyond the nuclear association. A similar approach has been adopted in our investigation of the loss of steroid responsiveness in diethylnitrosamine induced hepatomas[5].

In rat liver the normal gluconeogenic response to glucocorticoids has been described by Weber[6]. This steroid response was found to be limited in slow growing hepatomas and absent in fast growing hepatomas. A similar loss of gluconeogenic activity and hormonal response has been described in diethylnitrosamine induced hepatomas[5,7].

MATERIALS AND METHODS

Wistar rats (180–250 g) were used throughout these studies, the progeny of random matings in the closed colony maintained at Sheffield University. Bilateral adrenalectomies were carried out using the dorsolateral approach, the aminals being allowed free access to Oxoid 86 diet and 0.9% saline after recovery. At the end of each experiment the animals were examined for the absence of adrenal tissue.

For induction of hepatomas, rat litters were taken at 6 weeks of age and maintained on an Oxoid 86 diet with free access to water containing diethylnitrosamine (50 mg/ml).

Characterisation of DENA-induced hepatoma. Six rats which had been treated with DENA for 4 weeks were weighed and killed by a blow to the head. The livers were excised and weighed, and the liver weight expressed as a percentage body weight. This procedure was repeated with other control animals and DENA treated animals at 8, 12 and 16 week intervals. The livers taken from animals at the 16 week stage of treatment with DENA showed considerable tumour development as evidenced by histochemical observation and an observed increase in size.

[^{3}H]-dexamethasone binding to rat liver cytosol and plasma. Rat liver cytosol (50% w/v) was prepared as described previously[8]. An aliquot (2 ml) was applied to the surface of a coarse Sephadex G50 column (2.5 × 45 cm) and eluted with ice cold 18 mM phosphate buffer pH 6.8. The eluate was collected and all the protein containing fractions (10 ml) were pooled. A 1 ml aliquot was incubated for 90 min with [^{3}H]-dexamethasone (10^{-7} M specific activity 29 Ci/mM) at room temperature and this solution was then applied to a Sephadex G50 superfine column (0.9 × 10 cm) and eluted at 4°C with the phosphate buffer. The eluted fractions were collected and the radioactivity in each sample counted. This process was repeated using 50% w/v rat plasma and other cytosol preparations obtained from six normal or six DENA treated animals killed at 4, 8, 12 or 17 week intervals.

The displaceability of the binding was studied using similar separation techniques to determine the protein bound radioactivity following incubation of 1 ml of initial cytosol protein eluate

*Present address: Department of Surgery, University of Liverpool.

with [^{3}H]-dexamethasone (10^{-7} M) for 120 min at room temperature and 1 ml of the same cytosol protein eluate with [^{3}H]-dexamethasone for 60 min and with a 1000 fold excess of unlabelled dexamethasone for a further 60 min at room temperature.

Binding studies using 10^{-11} M [^{3}H-dexamethasone. A 20% w/v cytosol preparation in Krebs ringer was obtained using procedures previously described[8]. The supernatant was dialysed for 2 days using 1/4 in. Visking dialysis tubing against Krebs Ringer which was changed every 12 h. The adsorption spectrum of the diluted protein showed no sign of the characteristic haemoglobin spectrum indicating that blood had effectively been removed. 1 ml aliquots of the dialysate were pipetted into 1/4 in. Visking dialysis tubing and dialysed for 24 h at 4°C againct 5 ml Ringer solution containing 10^{-11} M [^{3}H]-corticosterone (specific activity 41 Ci/mM) samples of both inside and outside solutions (0.5 ml) were counted for radioactivity.

Binding of [^{3}H]-dexamethasone–receptor complex to DNA. Rat liver cytosol (50% w/v) was prepared and the receptor protein separated as described previously through both coarse and superfine Sephadex G50 columns. To a 1 ml sample of this separated protein was added 3×10^{-7} M [^{3}H]-dexamethasone and 50 μl of a stock DNA solution (3.5 mg/ml) (Worthington Calf Thymus DNA). This mixture was incubated for 2 h at 0°C and then for 1 h at room temperature (22°C). The solution was chilled and carefully layered onto a Sepharose 4B column (0.9×25 cm) and eluted with 0.018 M phosphate buffer pH 7.4. 1 ml fractions of the eluate were collected and the radioactivity counted in 0.5 ml of each sample. The OD at 260 and 280 nm was measured in a 0.5 ml sample using a Perkin Elmer U.V. 137 spectrophotometer. This experiment was repeated using control rats and those that had been subjected to DENA treatment for 16–18 weeks.

Nuclear binding of [^{2}H]-dexamethasone–receptor complex. Rat liver nuclei were prepared using the method of Coleman, Mitchell and Hawthorne[9] modified by Gehring and Tomkins[1].

Rats were sacrificed by a blow to the head and the livers rapidly excised, minced and homogenised in ice cold phosphate buffer pH 7.4 (containing 10 mM $MgCl_2$ 2 mM $CaCl_2$) to give a 20% w/v homogenate. This homogenate was diluted to 10% using a 0.5 M sucrose solution and centrifuged at 800 *g* for 10 min using an MSE Mistral 4L. The supernatant was discarded and the pellet resuspended in 1 ml phosphate buffer and spun again at 800 *g* for 10 min. This procedure was repeated three times. A [^{3}H]-dexamethasone receptor complex was obtained by gel filtration of a 50% w/v cytosol preparation and chromatographic separation of the [^{3}H]-dexamethasone (10^{-7} M) equilibrated protein fraction using a superfine G50 column as previously described in the methods section.

A 200 mg sample of the rat liver nuclear pellet was taken for DNA estimation and a further 200 mg sample incubated with 200 μl of the freshly prepared receptor complex in the phosphate buffer solution, for 1 h at 0°C followed by 1 h at 22°C with constant shaking. At the end of this incubation the solution was centrifuged for 5 min at 800 *g*. The supernatant was discarded and the pellet resuspended in 1 ml ice cold phosphate buffer and recentrifuged. This wash procedure was repeated three times. The final pellet was weighed and dissolved in 1 ml NCS (Amersham, Searle) and the radioactivity counted. This experiment was repeated for a group of DENA treated animals at the 16 week stage of treatment.

Tyrosine amino transferase (TAT) activity. This enzyme was estimated by the method of Granner and Tomkins[10] in liver samples taken from six adrenalectomized, normal or diethylnitrosamine treated (16 week stage) animals. The effect of intraperitoneal injection with 1 ml of 1% carboxymethylcellulose carrier with or without 5 mg of corticosterone or 5 mg dexamethasone 5 h before sacrifice was studied using six animals in each experimental group. The effect of a 0.1 ml subcutaneous injection of 10% EtOH with or without 5 μg corticosterone or 5 μg dexamethasone was also investigated.

Protein estimations were carried out using standard techniques and the results expressed in terms of μmol *p*-OH phenylpyruvate liberated/mg protein/h.

Corticosterone assays. In a final experiment the plasma concentrations of corticosterone in normal and DENA treated animals (16 week stage) were compared. The assays were carried out in this department by Miss R. Leonard using the radioimmunoassay procedure described by Schulster, Tait, Tait and Mrotek[11].

Further information on methods. All the methods used in the present study have been successfully used in similar systems, though our experience in studying nuclear uptake of the receptor steroid complex was that this could not be satisfactorily measured if the nuclear separation is carried out in the presence of excess amounts of labelled steroid. We have therefore incubated the nuclei in the presence of our previously separated receptor–steroid complex. Although the room temperature incubation that occurs will result in an increased rate of dissociation our results have shown that in the presence of these very low concentrations of labelled steroid nuclear binding does occur. We have also chosen throughout this work to express the steroid binding in terms of the height of protein bound peak rather than the area as only qualitative comparisons are possible.

The measurement of displaceable binding was carried out by added excess of unlabelled steroid

after initial equilibration with labelled steroid, as studies that simply lower the specific activity of the radioactively labelled steroid may not distinguish between covalently bound and tightly associated steroid.

In studying the binding of 10^{-11} M corticosterone and dexamethasone, care has been taken with this single concentration to demonstrate the very tight binding of corticosterone. This degree of binding cannot be accounted for by the main steroid receptor present in liver cytosol preparations. Our previous detailed studies[8] have analysed the K_A and site concentration of "receptors" that could account for this binding. The failure of dexamethasone to bind to these highly specific receptors may be understood in terms of a greater structural specificity of these higher affinity sites.

RESULTS

Using a superfine Sephadex G50 column, [^{3}H]-dexamethasone bound to rat liver cytosol protein separates (Fig. 1) as a clearly defined peak of radioactivity from the free steroid which elutes outside the void volume of the column. As the concentration of [^{3}H]-dexamethasone (3×10^{-7} M) in the incubate was chosen as sufficient to saturate the receptor, the height of this binding peak has been taken as a measure of the receptor binding capacity. There was no tight binding of dexamethasone to plasma trancortin. In six experiments the mean ±S.E.M peak height was determined as 1894 ± 74 counts per min per 500 μl eluant whereas in the presence of a 1000 fold excess of unlabelled dexamethasone the mean peak height was 446 ± 56 counts per min per 500 μl eluant, indicating a 76% displacement of the binding.

The ratio of liver weight to total body weight was measured throughout the progress of tumour development (Fig. 2). Over the initial 4 to 8 week period there appears to be a slight decrease in liver weight relative to that observed in controls, however this difference was not significant. After 12 weeks of treatment the ratio in treated rats was 28% higher than in control rats ($P < 0.01$) and after 16 weeks of treatment this ratio was 89% higher ($P < 0.001$). In this diagram it can be seen that normally the ratio of liver weight to body weight decreases as the rats reach maturity. The increase in liver weight relative to body weight observed in DENA treated animals is accompanied by a gradual deterioration in the condition of the liver and lack of growth in the rats. The livers showed considerable hepatoma development with some necrosis.

The result of binding studies carried out over the period of treatment with DENA are shown in Fig. 3. It can be seen that the binding capacity for [^{3}H]-dexamethasone increases slightly with age in both DENA treated and control groups.

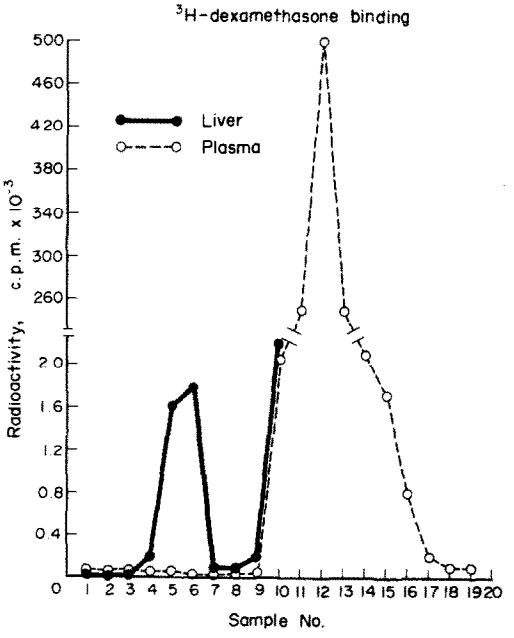

Fig. 1. Shows the separation of [^{3}H]-dexamethasone bound to rat liver cytosol protein, on a superfine Sephadex G50 column (●——●). The failure of [^{3}H]-dexamethasone to bind to plasma protein, under similar conditions, is shown (○---○). Not all fractions containing free steroid have been counted.

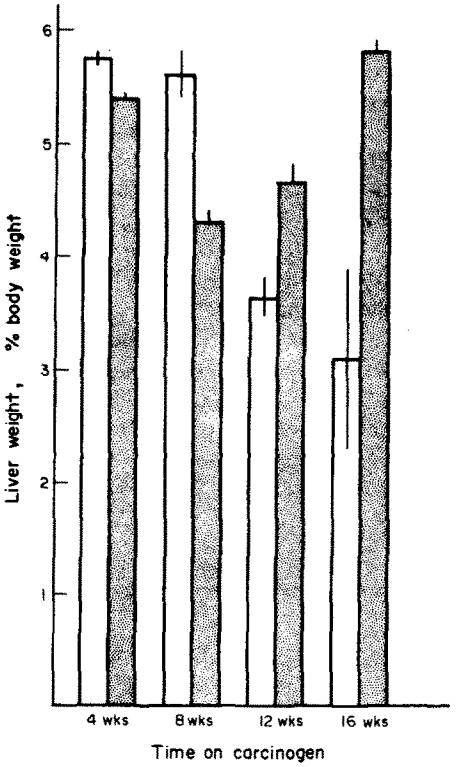

Fig. 2. Shows the normal variation of liver weight as a percentage of body weight over a 16 week period in six control rats (open histograms), compared with that observed in diethylnitrosamine treated rats studied over the same period (shaded histograms). Results show the mean ±S.E.M.

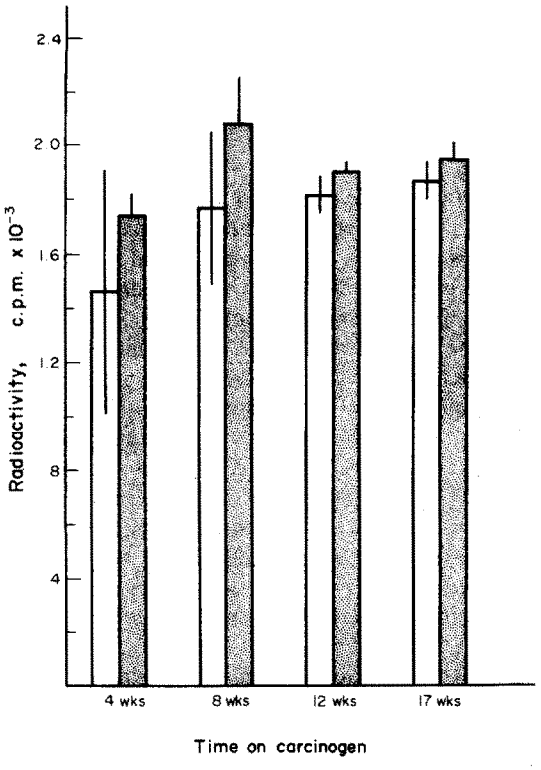

Fig. 3. Comparison of the [^{3}H]-dexamethasone binding in liver cytosol protein obtained from six control rats (open histograms) or six diethylnitrosamine treated rats (shaded histograms) at various stages during hepatoma development. The histograms represent the mean ± S.E.M. counts per min at the peak of the superfine Sephadex G 50 separation.

At no period during the treatment was there any significant difference in the levels of binding in control and treated rat livers, indicating that there is no deletion of the major glucocorticoid receptor as had previously been suspected by Dalton and Snart[5]. It was therefore decided to study the binding of 10^{-11}M [^{3}H]-corticosterone and 10^{-11} M [^{3}H]-dexamethasone to rat liver cytosol. It can be seen (Table 1) that the ratio of bound/free steroid observed for [^{3}H]-corticosterone was high for both control and DENA treated rats. This is taken to confirm that no deletion of specific receptors for [^{3}H]-corticosterone has occurred following DENA treatment. However, it is of interest to note that the binding observed with low concentrations of [^{3}H]-dexamethasone indicates that dexamethasone cannot bind to the tighter binding receptor sites[8]. These experiments should not be complicated by the presence of transcortin in the liver cytosol preparation, as transcortin is present in relatively small amounts even when the liver has not been exsanguinated[12].

The binding of the [^{3}H]-dexamethasone receptor complex to DNA (Fig. 4) is shown in terms of the amount of radioactivity eluting in the void volume on a Sepharose 4B column. In this void volume DNA separates from protein and free steroid. It is shown that the receptor–steroid complex does not separate within the void volume except in the presence of DNA. In the absence of receptor only a limited amount of [^{3}H]-dexamethasone elutes with the DNA and it was found that in both control and DENA treated animals there was no significant difference in the DNA binding of the receptor complex. Spectrophotometric analysis of the eluted DNA containing samples indicated that the protein: DNA ratio was 2:1, whereas in the applied sample this ratio was 100:1.

In studies of the translocation of the [^{3}H]-dexamethasone–receptor complex into rat liver/hepatoma nuclei expressed as the percentage of receptor complex bound to nuclei relative to the total counts, it was discovered (Table 2) that there was no significant difference in binding between control rats and those treated with DENA. However, while the standard error for the mean control group was relatively small, that for the DENA treated animals was large. This variability is believed to some extent to be due to the inhomogeneity of the hepatoma tissue. The reassociation of steroid with receptor after room temperature equilibration is believed to take place as the concentration of hormone is very low.

Hormone-stimulated tyrosine amino transferase (TAT) activity in normal and DENA treated livers (Fig. 5) shows that the level of TAT activity expressed in μmol p-OH phenyl pyruvate/mg protein/mg protein/h in adrenalectomized rats was greatly increased following intraperitoneal injection with 5 mg doses of corticosterone or dexamethasone ($P < 0.001$); these steroid-induced

Table 1. Binding of 10^{-11} M corticosterone and dexamethasone in cytosol preparations

		Outside (d.p.m./0.5 ml)	Inside (d.p.m./0.5 ml)	Distribution coefficient
[^{3}H]-Dexamethasone (10^{-11} M)	Control	463 ± 46 (6)	1122 ± 162 (6)	2.46
	DENA treated	423 ± 48 (6)	859 ± 66 (6)	2.32
[^{3}H]-Corticosterone (10^{-11} M)	Control	306 ± 39 (5)	2033 ± 143 (6)	6.61
	DENA treated	256 ± 64 (5)	1516 ± 594 (5)	6.14

Rat liver cytosol was prepared as a pooled sample from six animals. The cytosol was dialysed for 2 days against Krebs Ringer pH 7.4 with 12 hourly changes of Ringer. 1 ml fractions were then dialysed for 24 h against 5 ml Krebs Ringer containing 10^{-11} M [^{3}H]-corticosterone or 10^{-11} M [^{3}H]-dexamethasone. Outside and inside samples (0.05 ml) were taken for assay of radioactivity. The experiment was repeated using six diethylnitrosamine (16 weeks) treated animals.

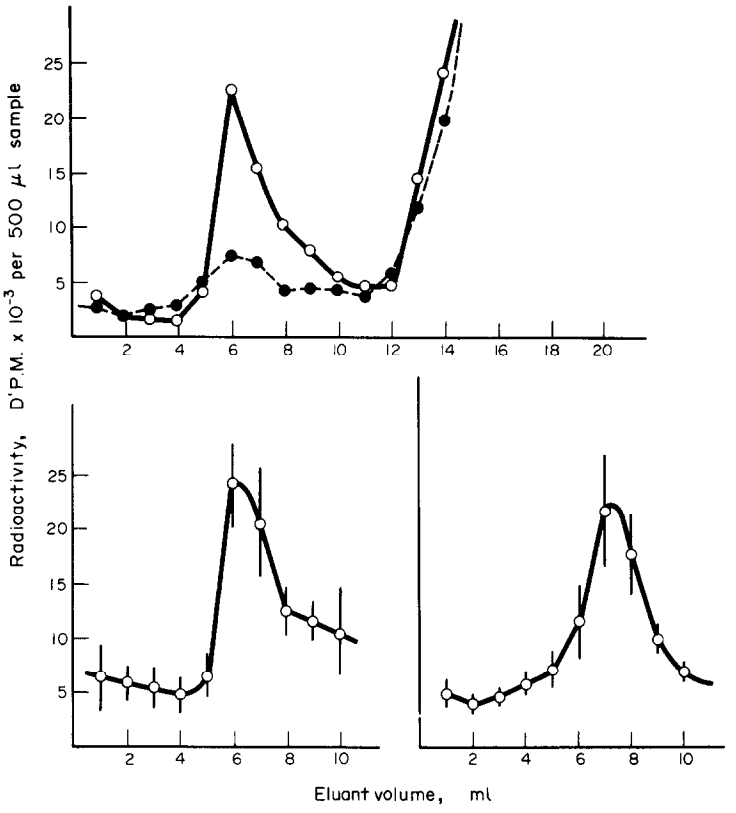

Fig. 4. The binding of [^{3}H]-dexamethasone–receptor complex to calf thymus DNA. (A) 1 ml sample of the [^{3}H]-dexamethasone–receptor complex prepared using a superfine Sephadex G50 column was incubated in the presence or absence of 175 μg DNA for 2 h at 0° followed by 1 h at 22°C. The solution was then chilled and applied to a Sepharose 4B column (0.9 × 25 cm). 1 ml fractions were collected and the radioactivity counted. (●---●) receptor complex alone. (○——○) Receptor complex plus DNA. (B) Shows the separation profile for [^{3}H]-dexamethasone receptor complexes bound to DNA as a mean ± S.E.M. of six 16 week control (L.H.S.) and six 16 week DENA treated rats (R.H.S.).

Table 2. Binding of [^{3}H]-dexamethasone receptor to rat liver nuclei

Cytosol	RS (d.p.m./ml)	NRS (d.p.m./ml)	% Total	m	S.E.
Control	6669	2362	35.4	30.27	± 3.20
	4758	1161	24.4		
	3748	1163	31.1		
DENA	6669	3064	45.9	30.73	± 8.55
treated	4758	1430	30.0		
	3748	609.7	16.3		

[^{3}H]-dexamethasone–receptor complex prepared using a superfine Sepadex G50 column was indubated with 200 mg nuclear pellet for 1 h at 0°C followed by 1 h at 22°C with constant shaking. The nuclei were centrifuged and washed three times and then dissolved in 1 ml NCS and the radioactivity measured as d.p.m./ml. RS represents the initial radioactivity of the complex and NRS the nuclear bound receptor–steroid complex.

increases are not significantly different from each other. On the other hand 5 μg doses of corticosterone or dexamethasone injected subcutaneously (Fig. 6), stimulated the enzyme differentially. Corticosterone stimulated only an 88% increase ($P < 0.001$). This difference in TAT stimulation by corticosterone and dexamethasone is highly significant.

Adrenalectomy of DENA treated rats (16 weeks) was impractical because these amimals could not survive the operation for longer than a day. Therefore the hormonal stimulation of TAT activity within the hepatomas has been compared (Figs 5 and 6) with corresponding values obtained in control animals. It can be seen that although TAT activities in DENA treated rat livers is lower than in normal livers this difference is only significant at the $P = 0.05$ level. However, this

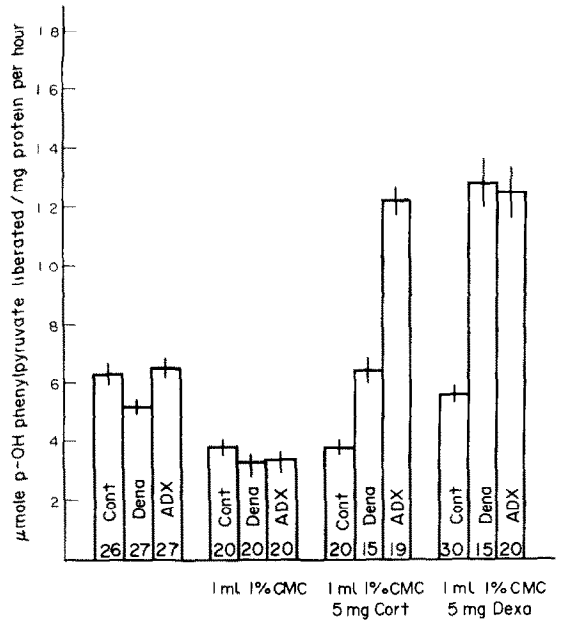

Fig. 5. Shows in histogram form the mean ± S.E.M. (n) liver tyrosine amino transferase activities measured in control, diethylnitrosamine treated or adrenalectimized rats at the 16 week stage of treatment. The effects of intraperitoneal injections with 1 ml carboxymethylcellulose (1% suspension) in the absence or presence of 5 mg corticosterone or 5 mg dexamethasone are shown by the respective histograms.

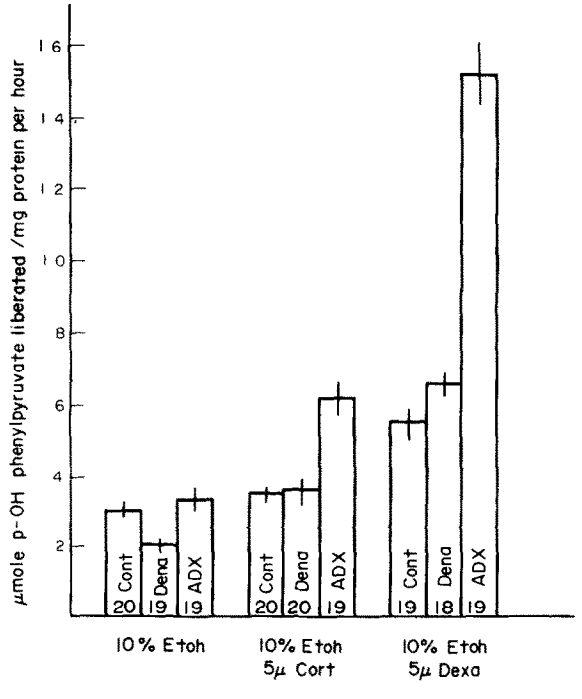

Fig. 6. Shows in histogram form the mean ± S.E.M. (n) liver tyrosine amino transferase activities measured in control, diethylnitrosamine treated or adrenalectomized rats at the 16 week stage of treatment. The effects of subcutaneous injections with 0.1 ml 10% EtOH in the absence or presence of 5 μg corticosterone or 5 μg dexamethasone are shown by the respective histograms.

observation also holds true for the CMC ($P < 0.05$) and EtOH ($P < 0.001$) injected groups. In the DENA treated rats 5 μg corticosterone caused at 85% increase in untreated rats. Corticosterone (5 mg) caused a 97% increase in hepatoma TAT activity compared with an insignificant change in normal livers. Dexamethasone (5 mg) caused a 235% increase in hepatoma TAT activity. This response in percentage terms is similar to that obtained using a lower dose of dexamethasone (5 μg) which caused a 261% increase in TAT activity in DENA treated rat livers as opposed to a 46% increase in livers from untreated rats. These responses are compared to those obtained using adrenalectomized rats in Figs 5 and 6.

Finally the serum corticosterone concentrations were measured in both treated and untreated animals at the 16 week stage. These values were found to be 62.2 ± 4.5 (4) μg% and 13.9 ± 0.7 (5) μg% respectively ($P < 0.001$) indicating a considerable reduction of serum corticosterone levels following DENA treatment.

DISCUSSION

Adrenalectomy does not lead to a large decrease in liver TAT activity but only in adrenalectomized animals is it possible to demonstrate a large corticosteroid response following an injection. The refractory nature of liver activity in normal animals represents an unresolved problem[13, 14].

Our results indicate that liver TAT measured in DENA treated rats is less refractory to glucocorticoid treatment. This effect of DENA does not appear to be due to any change in steroid–receptor characteristics and we are tempted to suggest that it may reflect a decreased steroid metabolism within the liver.

Conjugated steroid metabolites appear in the plasma[15] two to four hours after an increase in plasma corticosteroid concentration. This lag period may be taken to suggest that limiting metabolic enzymes in liver are activated in response to corticosteroid action. Such an effect would alter the tissue sensivity to hormone and provide a basis for understanding the refractory nature of normal liver quite apart from any change in steroid–receptor characteristics. We might further suggest that if this metabolic response is depressed by DENA treatment then the resulting feedback inhibition due to unmetabolised glucocorticoid would decrease ACTH release and in the long term seriously affect adrenal function. Hadjiolova and Hadjiolova[16] have reported effects of DENA treatment on the adrenal and the lower plasma corticosterone concentrations measured in the present work are consistent with their findings.

We would further argue that a decreased steroid metabolism in DENA treated rat livers could account for the increased sensitivity to the hormone.

It would also allow a possible explanation of the apparent loss of *in vitro* hormonal response demonstrated in our previous studies[5]. In order to obtain maximum steroid binding and an *in vitro* response in normal livers, an initial preincubation was required. It was believed that during this period any endogenous hormone is "metabolically" removed and the tissue reprimed .With the hepatoma these *in vitro* conditions may not have been suitable for obtaining a response. However, the two experiments cannot be compared too closely particularly as our present DENA treatment was restricted to 4 mth, at which stage a limited response to corticosterone might still be expected. Nonetheless, it is quite clear that dexamethasone can maximally activate TAT, so we are inclined to believe that the altered steroid sensitivity may be due to changes in steroid metabolism rather than any fundamental change in steroid-receptor characteristics/function.

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