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The effect of *in vivo* hydrocortisone administration on the labelling index and size of chromaffin tissue in the postnatal and adult mouse

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INTRODUCTION

Corticosteroids are known to increase the size and to delay the disappearance of extra-adrenal chromaffin tissue in the postnatal rat (Lempinen, 1964). In a previous paper (Monkhouse & Coupland, 1985), a similar response has been reported in the prenatal and perinatal mouse where hydrocortisone caused a hyperplastic response in the para-aortic body. There were marked increases in the size and the S-phase labelling indices at both ages. The same work also provided evidence of a less marked, but nevertheless significant, increase in the labelling index of intra-adrenal chromaffin tissue but, possibly due in part to the developmental state of the 'medulla', no effect was observed on the amount of intra-adrenal chromaffin tissue. The present work extends these observations by reporting on the effect of hydrocortisone on the labelling indices and size of intra- and extra-adrenal chromaffin tissue in the postnatal mouse at various ages: 8 days, 14 days (labelling indices only), 21 days and in adults (over 2 months).

MATERIALS AND METHODS

Albino mice (*Mus musculus*, strain CS1) were supplied by Nottingham University Medical School Animal House from closed colonies maintained under standard conditions of temperature and lighting (12 hours light, 12 hours darkness). Animals were allowed free access to water and a normal diet (R and M breeding diet, Heygates Ltd, Northampton).

Administration of hydrocortisone

Hydrocortisone was prepared in an evaporated milk and water medium (resultant concentration 2 mg/ml) as described by Monkhouse & Coupland (1985). Hydrocortisone was administered to mice in the experimental groups three times daily (eight hourly) at a total daily dosage of 40 mg/kg (full dose) or, in some cases, 20 mg/kg (half dose) through a short length of narrow gauge feeding tube (Portex Ltd, Hythe, Kent), the tip of which was placed well back in the oropharynx of the animal. Mice in some of the control groups received an equivalent volume of the evaporated milk and water mixture, but without hydrocortisone; these are referred to as *handled control* groups. Mice in the other control groups, referred to as *unhandled*, received neither hydrocortisone nor the evaporated milk and water mixture.

Age groups

Eight days

Hydrocortisone or milk and water mixture was administered to neonatal mice for 7 days after birth; animals were killed on the eighth postnatal day. Treatment groups at this age were: unhandled control, handled control and experimental (full dose).

14 days

Hydrocortisone was administered to neonatal mice for 13 days after birth; animals were killed on the fourteenth postnatal day. Treatment groups at this age were: unhandled control and experimental (full dose). Only labelling indices were derived from mice in this age group: volumetric analysis was not performed.

21 days

Hydrocortisone was administered for 14 days from the seventh postnatal day; animals were killed on the twenty first postnatal day. Treatment groups at this age were: unhandled control and experimental (full dose).

Adults

Hydrocortisone was administered for 14 days. Animals in the experimental groups were killed on the fifteenth day after drug administration began; those in the unhandled groups were killed at an equivalent age. Treatment groups at this age were: unhandled control, experimental (half dose) and experimental (full dose).

Except in the adult group in which all animals were males, the sex of the mice was not recorded.

Animal and tissue processing

Two hours before being killed, each animal was injected intravenously (adults) or intraperitoneally (other ages) at 9.30 am with 5 μCi/g body weight [6-^{3}H]thymidine, specific activity 5 Ci/mmol (Radiochemical Centre, Amersham, Bucks, or Amersham International plc). Animals were anaesthetised with an intraperitoneal injection of sodium pentobarbitone ('Sagatal', May & Baker Ltd, Dagenham, Essex) and killed by intracardiac perfusion of fixative (2 % glutaraldehyde and 2 % paraformaldehyde in 0·1 M sodium cacodylate buffer, pH 7·2). Tissues were placed in fixative overnight, washed in buffer, dehydrated in graded alcohols and embedded in 'Fibrowax' (Raymond A. Lamb, London NW10) in the usual way. Blocks were sectioned serially at a thickness of 6 μm on a Cambridge rotary rocking microtome and sections were dried on to gelatinised glass slides.

Autoradiography and staining

Sections were dewaxed in xylene, hydrated in graded alcohols and dipped in Ilford K2 photographic emulsion at 43 °C in a darkroom under Ilford safelight F904. The emulsion was allowed to gel on a cold plate and the slides were exposed in dry conditions for 15 days at 4 °C in light-tight boxes. Control slides for background radiation and latent image fading were included. The autoradiographs were developed for 8 minutes in Kodak D19 developer at 19 °C, washed in distilled water for 1 minute and fixed for about 2 minutes in ammonium thiosulphate ('Hypam': distilled water; 1:10). Sections were stained in Gurr's Giemsa stain, washed in water, air dried and mounted in picolyte resin.

Labelling indices

Para-aortic body

At least 3000 chromaffin cells were counted in the para-aortic body of 8 days old experimental mice. In the control groups at this age, and in all groups at other ages, the para-aortic body was no longer present as a discrete mass (see Results).

Adrenal chromaffin cells

At least 4000 adrenal chromaffin cells were counted in each adrenal gland. Right and left sides were not recorded separately although only one gland from each animal was taken. The numers of adrenaline-storing and noradrenaline-storing cells were noted, together with the numbers of each that were labelled. The labelling index was expressed as a percentage of the total number of cells.

A nucleus was counted as labelled when it was associated with 6 or more grains. Independent and random counting and sampling systems were applied such that no particular area of either adrenal or para-aortic body was unduly favoured.

Cell number per unit area in para-aortic body of 8 days old mice

Chromaffin cell nuclear dimensions in 8 days old para-aortic bodies were measured under the $\times 160$ objective using a calibrated scale. One hundred nuclei from each animal were measured in two dimensions at right angles and these values were used to calculate the mean nuclear profile dimension per animal (with the normal statistical parameters). These values were multiplied by $4/\pi$ (Abercrombie, 1946) to take account of the distribution of nuclear chord lengths as seen in tissue sections and thus to derive a closer approximation to the true values.

Nuclear packing was assessed by counting under the $\times 100$ objective the number of nuclei per unit area, randomly selected. Ten such areas were inspected for each para-aortic body. Values were corrected to exclude nuclear fragments, using the dimensions derived above, with the formula of Abercrombie (1946).

Volumetric analysis

The areas of the para-aortic body and the whole adrenal gland, with that of the medulla recorded separately, were measured on every tenth consecutive serial section using a Kontron MOP-AMO3 optical picture analyser in conjunction with a Wild microscope and drawing tube attachment. The volume of the structure is given by the formula

$$V = (\Sigma A)\, d/m^2$$

where V = volume; ΣA = sum of areas of slices; d = distance between slices (in this case 60 μm); and m = linear magnification factor (in this case $\times 149{\cdot}88$).

Statistics

All numerical results were treated as part of a normally distributed population and are expressed in the Tables as mean and standard error of the mean. Statistical significance was determined by Student's unpaired t-tests. P values are given to one or two significant figures; the value taken as being significant was $P < 0{\cdot}02$.

RESULTS

Hydrocortisone led to a deterioration in the condition of the experimental animals throughout the period of administration similar to that reported at earlier ages (Monkhouse & Coupland, 1985). Oral administration of hydrocortisone necessitated the inclusion of at least one group of handled control animals. It was apparent from the results that there were no differences between the two control groups (handled and unhandled); this accorded with the results from handled control animals reported earlier in fetal mice and this matter was not given further consideration.

Light microscopy

In the 8 days old experimental animals, the para-aortic body was situated ventral to, and on either side of the aorta near the level of the renal vessels. Ganglion cells were closely associated, often intermingled, with it. It was well vascularised with a characteristic arrangement of thin walled blood vessels; the chromaffin cells were clearly identifiable.

In the 8 days control groups the para-aortic body was present as only a small aggregation of chromaffin tissue, often an attenuated strand of cells in the wall of one of the abdominal great vessels. This was large enough in all animals to obtain values for volumetric analysis, but large enough only in 3 out of 10 control animals to obtain statistically meaningful cell counts for the computation of labelling indices. In older animals, the para-aortic bodies of both experimental and control animals were fragmented so much that they were no more than isolated collections of chromaffin cells, often barely distinguishable from the paraganglionic groups of cells scattered throughout the peri-aortic tissues, sometimes intimately associated with the walls of the great vessels. In 14 and 21 days old mice these scattered chromaffin cells were noticeably more frequent in the inter-renal regions of experimental mice than in the control groups, but no attempt at quantitation was made. The inter-renal areas of adult experimental animals were no different from those of adult control animals.

The adrenal medullas (Figs. 1, 2) were in all cases well formed and easily identifiable with readily discernible adrenaline-storing and noradrenaline-storing cells, even (though less intensely staining) in 8 days old mice.

Numerical results

8 days old para-aortic body

Hydrocortisone caused an obvious rise in the labelling index of the 8 days old para-aortic body (Table 1) from a mean value of 4·1% in 3 (out of 10) control animals to a mean value of 8·4% in the experimental group ($P = 1{\cdot}3 \times 10^{-4}$). This was mirrored by volumetric analysis (Table 2); the volume increased almost tenfold from a control value of just over 1 $mm^3 \times 10^{-3}$ to an experimental mean value of 11·0 $mm^3 \times 10^{-3}$ ($P = 4{\cdot}9 \times 10^{-9}$).

Adrenal medulla

Labelling indices (Table 3). Hydrocortisone had no effect on the labelling indices of either adrenaline- or noradrenaline-storing cells, and neither were there significant differences between these two cell types within any one age group (in all cases $P > 0{\cdot}02$). The present results, together with those of Monkhouse & Coupland (1985), display a fall in labelling indices from the perinatal values (indices for adrenaline- and noradrenaline-storing cells not recorded separately) of 6·0% in the

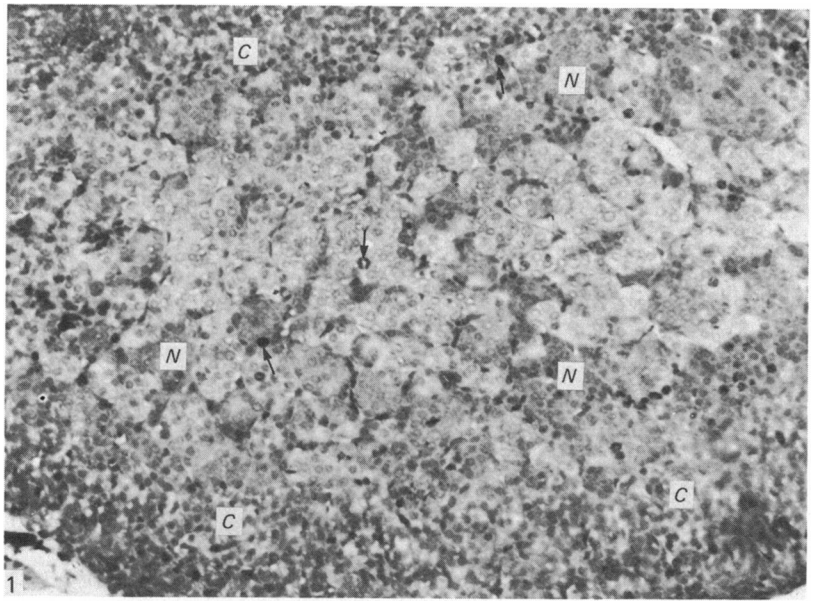

Fig. 1. Autoradiograph of 8 days old adrenal gland showing cortex (*C*) and medulla with darkly staining groups of noradrenaline-storing cells (*N*); some labelled cells are arrowed. Giemsa. ×75.

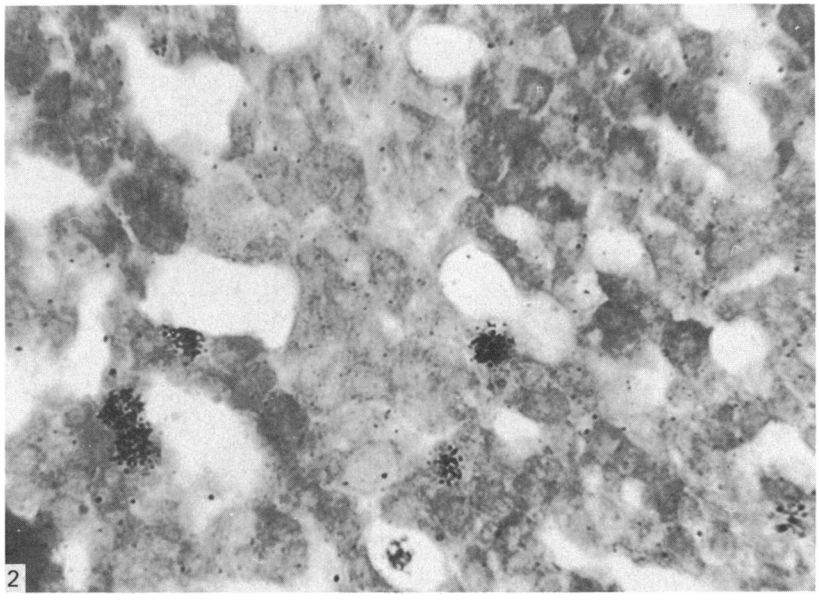

Fig. 2. Autoradiograph of adult adrenal medulla (grains in sharp focus) showing groups of noradrenaline-storing cells (darker) and adrenaline-storing cells (lighter). Giemsa. ×720.

Table 1. *Effect of hydrocortisone on labelling indices (%) of postnatal mouse para-aortic body*

	Unhandled controls	Experimental (full dose)
8 days old	4·1 (0·34), $N = 3$; unobtainable (see text), $N = 7$	8·4 (0·34), $N = 13$
14 days old ($N = 4$, 5 respectively)	indices unobtainable (see text)	
21 days old ($N = 6$, 10 respectively)		
Adult ($N = 7$, 10 respectively)		

Values expressed as mean (standard error of mean in parentheses).
N, number of animals in each group.

Table 2. *Effect of hydrocortisone on volumetric analysis of 8 days old postnatal chromaffin tissue (right and left adrenal medullas combined)*

(units: $mm^3 \times 10^{-3}$)

	N	Volume of para-aortic body	Medullary volume	Volume of para-aortic body as a percentage of medullary volume
Unhandled controls	5	1·2 (0·1)	67 (5·2)	2
Handled controls	14	1·4 (0·1)	55 (3·0)	3
Experimental (full dose)	11	11·0 (0·6)	72 (4·9)	15

Values expressed as mean (standard error of mean in parentheses).
N, number of animals in each group.

Table 3. *Effect of hydrocortisone on adrenal medullary labelling indices (%) and ratio of adrenaline-storing cells: noradrenaline-storing cells in postnatal mice*

	N	Adrenaline-storing cells	Noradrenaline-storing cells	Adrenaline: noradrenaline cell ratio
8 days old				
Unhandled controls	6	2·0 (0·27)	2·3 (0·53)	2·3:1
Experimental (full dose)	8	2·5 (0·37)	2·6 (0·43)	2·2:1
14 days old				
Unhandled controls	4	0·9 (0·17)	0·8 (0·21)	2·9:1
Experimental (full dose)	5	0·8 (0·14)	0·9 (0·15)	3·0:1
21 days old				
Unhandled controls	8	1·2 (0·07)	0·7 (0·07)	2·8:1
Experimental (full dose)	9	1·1 (0·12)	0·7 (0·06)	2·8:1
Adult				
Unhandled controls	8	0·4 (0·03)	0·3 (0·03)	3·9:1
Experimental (half dose)	5	0·3 (0·04)	0·3 (0·02)	4·3:1
Experimental (full dose)	4	0·4 (0·06)	0·2 (0·03)	4·4:1

Values expressed as mean (standard error of mean in parentheses).
N, number of animals in each group.

Table 4. *Effect of hydrocortisone on volumetric analysis of postnatal adrenal glands (right and left combined)*

(units: mm^3)

	N	Whole gland	Cortical volume	Medullary volume	Cortico-medullary ratio	Medullary volume expressed as percentage of whole gland
8 days old						
Unhandled controls	5	0·33 (0·03)	0·26 (0·02)	0·07 (0·005)	4:1	20*
Handled controls	14	0·26 (0·01)	0·21 (0·01)	0·05 (0·003)	4:1	21*
Experimental (full dose)	11	0·22 (0·01)	0·15 (0·01)	0·07 (0·005)	2:1	33*
21 days old						
Unhandled controls	7	0·65 (0·06)	0·54 (0·05)	0·11 (0·008)	5:1	16*
Experimental (full dose)	14	0·47 (0·04)	0·36 (0·03)	0·11 (0·007)	3:1	24*
Adult						
Unhandled controls	8	1·65 (0·05)	1·35 (0·05)	0·30 (0·008)	4:1	18*
Experimental (half dose)	6	0·89 (0·03)	0·60 (0·02)	0·29 (0·01)	2:1	32*
Experimental (full dose)	5	0·89 (0·03)	0·56 (0·02)	0·33 (0·01)	2:1	36*

Values expressed as mean (standard error of mean in parentheses).
N, number of animals in each group.
* Values taken from raw data (arbitrary units) before conversion to absolute units (see Materials and Methods).

control group and 8·1 % in the experimental (full dose) group to between 2 % and 2·5 % in 8 days old animals. This fall continued through the 14 and 21 days old values (which, at around 1 %, were similar) to the adult values of between 0·2 % and 0·4 %.

Adrenaline/noradrenaline cell ratios (Table 3)

Hydrocortisone had no effect on the cell ratios of the medulla which increased from just over 2:1 in 8 days old animals to about 4:1 in adults.

Volumetric analysis (Table 4)

The size of the adrenal medulla was in all age groups unaffected by hydrocortisone administration. Growth of the whole animal was reflected in the growth of the adrenal gland: the value for medullary size in 8 days old animals of 0·07 mm^3 rose to 0·11 mm^3 in 21 days old animals and to about 0·3 mm^3 in adults. However, at all age groups without exception, hydrocortisone caused a dramatic reduction in the size of the cortex. In 8 days old animals cortical volume was reduced by about one third, and in adults by over one half. This was reflected by the fall in corticomedullary ratio from 4:1 in control animals to 2:1 in the experimental group. Using light microscopy the zona glomerulosa appeared unaffected, but the zona fasciculata and the zona reticularis were much reduced in size. It was not possible in any specimen to distinguish the cortical X zone.

Hyperplasia or hypertrophy? (Table 5)

In the 8 days old para-aortic body, as in 16 days fetal mice (Monkhouse & Coupland, 1985), the number of cells per unit volume was not changed significantly

Table 5. *Hyperplasia or hypertrophy? Cell numbers per unit area in 8 days old para-aortic body and the effect of hydrocortisone*

	N	Corrected nuclear diameter (μm)	Corrected cell number per 10 grids
Unhandled controls	5	5·9	892 (36)
Handled controls	14	6·1	898 (19)
Experimental (full dose)	11	6·0	881 (15)

Values expressed as mean (standard error of mean in parentheses).
N, number of animals in each group.

by hydrocortisone treatment, confirming the hyperplastic (not hypertrophic) nature of the response to hydrocortisone.

DISCUSSION

The para-aortic body

The effect of hydrocortisone on the 8 days postnatal para-aortic body is similar to that in prenatal and perinatal mice (Monkhouse & Coupland, 1985): it causes a marked hyperplastic response. The results of the control groups show that in the strain of mice used in this work (CS1), the gradual reduction in size of the postnatal para-aortic body has already begun by the eighth postnatal day. The collections of extra-adrenal chromaffin tissue attain their maximum development at or around the time of birth after which they remain more or less unchanged, as in the guinea-pig, or they disperse, as in mouse and man (Coupland, 1960). Viragh & Both (1967) describe degenerative changes in mouse extra-adrenal chromaffin tissue in the first postnatal week, but Coupland (1960) reports a substantially sized para-aortic body until about the twenty first day; this apparent contradiction may relate to strain differences of the animals used. It has been suggested (Goormaghtigh, 1935) that the cells may migrate rather than simply degenerate, but this has not been substantiated by later workers; dispersal of the para-aortic body brought about by the growth of surrounding structures together with some degeneration will adequately explain its disappearance. In an unpublished electron microscopical study associated with this work, the author has seen appearances which can be accounted for by degenerative changes, although such changes were not seen by light microscopy.

The lack of significant effect of hydrocortisone on the 21 days old para-aortic body could be explained on the basis that hydrocortisone administration was not begun until the seventh postnatal day, by which time dispersal of the para-aortic body has already begun. This would accord with the findings of Lempinen (1964) who noted that the later after birth that hydrocortisone treatment was started, the less significant the effect. However, in the 14 days old para-aortic body, where hydrocortisone administration began at birth, there was also little effect on size.

Although intra-adrenal chromaffin tissue is known to be innervated by preganglionic sympathetic neurons (Hollinshead, 1937), the innervation of extra-adrenal chromaffin tissue exhibits species variation. Cholinergic nerve endings have been seen in the frog (Hill, Watanabe & Burnstock, 1975) but innervation is said to be sparse in man (Hervonen, 1971), and absent in the rabbit (Coupland & Weakley, 1970) and the mouse (Mascorro & Yates, 1971). Using electron microscopy, the author has

seen axons in the murine para-aortic body but not distinct nerve endings. Why there should be these differences remains at present unknown. Innervated extra-adrenal chromaffin tissue might well respond similarly to the adrenal medulla, and to comparable stimuli, though the amines released would almost certainly contain proportionately more noradrenaline. This could hardly be the case for extra-adrenal chromaffin tissue without innervation, though it may respond to local or general humoral factors as well as to anoxaemia (Brundin, 1966; Hervonen & Korkala, 1972).

The adrenal medulla

Proliferative activity in the adrenal medulla has been the subject of previous investigations. Mitchell (1948) noted that the mitotic activity of the postnatal rat adrenal was low and he concluded that size increases are brought about by auxetic growth and postnatal cellular hypertrophy. More detailed information about adult rat medullas has been provided by Malvaldi, Mencacci & Viola-Magni (1968) and Benedetti (1976). Their studies showed that fully differentiated cells are capable of mitosis, thus excluding the possibility that it might be the immature cells remaining in the gland during postnatal life that are observed in mitosis. An investigation into the labelling indices of medullary cells was carried out by Malvaldi & Viola-Magni (1972) who obtained adrenal medullary labelling indices in adult male rats of about 1 %, increasing after intermittent exposure to cold. Their figures, however, are at variance with the much lower values they obtained for mitotic indices; hence they postulated that most of the observed labelling was due to metabolic turnover of DNA. Non-replicative DNA synthesis needs to be considered since tritiated thymidine taken up by cells for metabolic DNA repair will be indistinguishable from that incorporated for cell replication.

Pelc (1968) suggested that metabolic DNA consists of extra copies of genes that are active in a cell, regulating and performing the transcription to RNA. These DNA molecules are subject to wear and tear and need to be repaired occasionally. DNA could thus be labelled during the S-phase and during the formation and repair of metabolic DNA. DNA repair in human cells has been studied *in vitro* by Lehmann & Stevens (1980) who found that non-replicative DNA synthesis accounted for between 1 % and 5 % of replicative synthesis in dividing cells. As a source of error in systems with low labelling indices, therefore, it could be a significant factor, especially in those studies which report only isolated labelled cells.

The mean values for indices obtained in this work in postnatal adrenal medullas (Table 3) were never more than 2·6 % and in adults they were 0·4 % or less; comment must therefore be guarded. Isolated mitotic figures were occasionally seen and so at least some of the tritiated thymidine was taken up for replication. One can assert with reasonable confidence, however, that hydrocortisone has no effect at any age on the labelling indices of either adrenaline- or noradrenaline-storing cells. These results in untreated adrenal medullary cells are comparable with those of Diderholm & Hellmann (1960) who obtained a labelling index (4 hours after tritiated thymidine administration) in 21 days old male rats of between 1 and 2 %. Jurecka, Lassmann & Hörandner (1978) performed a similar study on normal 2 weeks old and adult mice, although they did not distinguish between cell types, and neither did they define a labelled cell. Their value for the index in 2 weeks old mice one hour after tritiated thymidine administration was about 9 %, considerably greater than in this study (after two hours) of about 1 %; their index for adult mice was under 1 %, a value with which the present results agree even though, as has already been discussed, caution

must be exercised in making comparisons. Other reasons for caution are that Jurecka, Lassmann & Hörandner counted fewer cells ("a minimum 1000 cells per mouse") than in this work (over 4000), and also that they used semithin Epon embedded sections; to assume that labelling indices from tissues embedded in different media are comparable is unjustified (Monkhouse, 1985).

The lack of response of hydrocortisone of 8 days postnatal and older adrenal chromaffin tissue is in contrast to the results in perinatal animals (Monkhouse & Coupland, 1985). It suggests that at some time during the first week of postnatal life, the intra-adrenal chromaffin cells become unresponsive to corticosteroids. In the 8 days postnatal age group the indices for the two control populations (intra- and extra-adrenal) are quite different. This could well be a reflection of the fact that by this age the medulla is, and has been for some time, exposed to so great a quantity of endogenous corticosteroids that administration of exogenous steroids, coupled with ACTH feedback effects, makes little overall difference.

In the postnatal adrenal gland, it is obvious that hydrocortisone is quite without effect on the medullary volume or on the adrenaline:noradrenaline cell ratio, although its atrophic effect on the cortex is notable. Similar cortical effects of exogenous steroids are reported by Garvey, Migally, Sullivan & Sullivan (1983) who find that dexamethasone leads to a fall near term in fetal adrenal weight and fetal adrenal corticosteroid levels. Inspection of Table 4 shows that the corticomedullary ratio in unhandled postnatal mice, about 4:1, undergoes no marked changes after the eighth postnatal day. The ratio in perinatal control mice is 8:1 (Monkhouse & Coupland, 1985) which indicates that most change takes place during the first postnatal week. These results are in broad agreement with those for the albino rat reported by Jackson (1919) who also found no differences in this ratio between the sexes or as a result of inanition.

In conclusion, then, it can be seen that the hyperplastic effect of hydrocortisone on the mouse para-aortic body becomes much less marked during the first two weeks of postnatal life and that it is absent from older animals. Its previously reported hyperplastic effect in 16 days fetal and perinatal adrenal chromaffin cells (although less marked than in the para-aortic body) does not persist into postnatal life. These results are compatible with the concept of the development in maturing chromaffin tissue of a non-responsiveness to glucocorticoids which is modulated in the two different situations, intra- and extra-adrenal, by factors or substances as yet undefined.

SUMMARY

Hydrocortisone administration *in vivo* to neonatal mice for seven days led to a significant increase in both the size and the labelling index of extra-adrenal chromaffin tissue (as represented by the para-aortic body) of 8 days old mice. In untreated animals at this age, the para-aortic body was in most cases too small to obtain a valid labelling index. In the para-aortic bodies of 14 days old, 21 days old and adult mice, the extra-adrenal chromaffin tissue was too dispersed to obtain values for either volumetric analysis or labelling indices, and hydrocortisone was without significant effect in promoting a hyperplastic response.

In the postnatal adrenal medulla at all ages studied, hydrocortisone had no effect on the medullary size or on the labelling indices of either adrenaline- or noradrenaline-storing cells, although it led to a marked diminution of adrenocortical volume. The relative proportion of adrenaline-storing cells increased between the values for 8 days

old animals and those for adults; this was unaffected by hydrocortisone. The cortico-medullary ratio remained unchanged from the eighth postnatal day onwards.

The results are discussed and related to those of other workers. It is suggested that factors as yet unknown might modulate the response to corticosterioids of developing intra- and extra-adrenal chromaffin tissue.

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