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Some endocrinological aspects of barbiturate dependence

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Summary

1. Hypophysectomized rats become dependent on barbitone and show the same withdrawal syndrome as intact animals.

2. Barbitone dependent rats have larger thyroid and adrenal glands, a larger liver, smaller gonads and larger secondary sex organs than untreated animals. The levator ani muscle of the males is smaller.

3. In contrast, dependent female hypophysectomized rats only showed a decreased gonad weight and increased liver weight.

4. Histologically, the thyroid gland of dependent rats appears more active, but the concentration of iodine bound to plasma protein, basal metabolic rate and body temperature are similar in dependent and untreated animals.

5. Resting plasma corticosterone concentration appears to be unchanged in barbitone dependent animals, but stress induced increases in the concentration of corticosterone in plasma are less in dependent animals.

6. Immature barbitone dependent rats grow at a faster rate than untreated animals, but hypophysectomized rats of similar age receiving barbitone do not.

7. The additional body weight gained by barbitone dependent animals is of normal body composition.

8. Administration of growth hormone has an identical growth inducing effect in dependent hypophysectomized animals and in untreated hypophysectomized animals.

9. Barbitone dependent rats do not exhibit the ' frustration effect ' in a double runway. In barbitone dependent rats approach to a potentially ' frustrating ' situation is slower than in untreated animals.

Introduction

Leonard (1966) has shown that adrenal function is depressed in barbitone dependent rats and that there is a large increase in the concentrations of plasma corticosteroid when the barbiturate is withdrawn. The first purpose of the present work was to assess, by using hypophysectomized animals, whether these hormonal changes are involved in the barbiturate dependence mechanism.

It is likely that corticosteroid changes in dependent animals are accompanied by changes in the activity of other endocrine glands and this possibility has been inves-

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tigated. The third purpose of this work was to study how the hormonal changes associated with barbiturate dependence influence the rate of growth, and how they affect the behaviour of rats as illustrated by their response to stress.

Methods

Induction of dependence

Male and female rats of a Wistar strain (Tucks Ltd., Essex) were used. They were housed in individual cages. Barbitone sodium was dissolved in their drinking water, the quantity dissolved being increased once weekly so that each rat received 100 (mg/kg)/day during the first week and 200, 300, 400, 400 (mg/kg)/day during subsequent weeks. The taste of the barbitone was disguised with saccharin sodium (200 mg/l.). The rats were usually 70–80 g when first given barbitone and at the end of this 5-week treatment were dependent on the drug. When the barbitone was no longer dissolved in the drinking water of dependent rats, they became susceptible to audiogenic seizures and underwent a precipitous loss of body weight. The rats were tested for susceptibility to audiogenic seizures in a chamber where they were subjected to a ringing door bell for one minute. Typically, barbitone dependent animals withdrawn from the drug showed a front leg flexor tonic seizure after 30 seconds.

Hypophysectomy

Female rats of 140–150 g were hypophysectomized by a parapharyngeal method. The rats received hormone therapy (dexamethasone, thyroxine, growth hormone) before and after the operation. Postoperatively, they were housed at a temperature of 25°C and were given a 10% glucose solution to drink for several days. They were allowed to recover from the operation for 2 weeks before any experiment was conducted. Any rats showing growth during this period were rejected, and completeness of hypophysectomy was confirmed by examination of the pituitary cavity at the end of the experiment.

General endocrinological methods

Histology. The endocrine glands were stained with Erhlich's haemotoxylin and eosin ; in addition, the pituitary gland was stained by a modification of the periodic acid-Schiff technique using aldehyde thionine (Ezrin & Murray, 1963). Differential cell counts were made from a mid-section of the adenohypophysis.

An estimation of the thyroid gland activity (the epithelial-colloid ratio) was made by projecting a midgland section onto a ground glass screen, tracing it, then cutting out colloid and epithelial areas, and weighing them.

Vaginal smears were stained with methylene blue.

Basal metabolic rate. The method used for recording basal metabolic rate was based on that of Lilienthal, Zierler & Folk (1949). Recordings were made from passive unrestrained animals.

Body temperature. Body temperature was recorded with a thermocouple probe inserted 5 cm into the rectum.

Iodine bound to plasma protein. The method of Acland (1957) was used for determining protein bound iodine.

Plasma corticosterone. Rats were prepared with intravenous cannulae (Weeks & Davis, 1964) chronically implanted via the lumbar vein into the abdominal vena cava. Such rats were kept under reverse daylight (light 20.00–07.00 h) and were allowed to recover from the operation for at least 2 weeks. The rats were then accustomed to being placed in a large box. During experimental sessions, the implanted cannulae were connected by a long polythene tube to a syringe outside the box. After a 3 h settling down period blood was sampled (23.30 h). In this way, blood sampling was made during the rats' quiescent period under controlled conditions and in complete quiet.

Corticosterone was determined by the spectrofluorimetric method of Zenker & Bernstein (1958).

Growth

Growth hormone and thyroxine administration. Oily and aqueous preparations were made. The aqueous preparation contained 225 μg of growth hormone and 75 μg of thyroxine sodium in 0·4 ml ; 0·2 ml was given subcutaneously twice a day. The oily preparation (0·2ml) was given subcutaneously every afternoon ; this contained 100 μg growth hormone and 40 μg thyroxine sodium.

Body composition determinations. Each rat carcass was weighed after removal of intestinal contents and dried to constant weight in an oven at 90–100° C. The dehydrated carcass was crushed, placed in a paper thimble and the fat extracted with ether in a Soxhlet apparatus. After extraction, the residue was dried and weighed. By this method, water, fat and residual solid matter content of the carcass were assessed.

Behavioural experiments

A double runway similar to that of Amsel & Roussel (1952) was used. In a single runway experiment, the method was based on that of Adelman & Maatsch (1956).

Young male rats were starved for 48 h and then their food intake was controlled so as to allow only a very slow increase in body weight. The rats were then trained to run down 1·5 m long wooden alley for a 100 mg food pellet reward placed in a square box at the end of the alley. The rats were placed in a similar box at the beginning of the experiment and after 15 s a door was opened so that they could run down this alley. In the single runway experiment, a shelf in the reward box allowed the rat to escape. The rats did escape by this means when they were given no food reward.

In the double runway experiment, a door in the reward box was opened 15 s after the rat entered. The rat was then trained to run down a second alley to another box containing a similar food reward. After a period in which the food reward was occasionally omitted from the box at the end of the first alley the rats were only given a reward in this box on 50% of occasions. The speed of running down the first and second alleys was recorded.

Results

Hypophysectomy

Hypophysectomized rats became dependent on barbitone and showed a withdrawal syndrome when barbitone was withdrawn. Of seven hypophysectomized rats which

received barbitone in increasing concentration for 42 days, six became susceptible to audiogenic seizures in the first 72 h of barbiturate withdrawal. Eight untreated hypophysectomized rats did not show audiogenic seizures. The hypophysectomized rats dependent on barbitone which had a mean body weight of 127 g, lost 6 g in the first 48 h after barbiturate withdrawal, whereas untreated hypophysectomized rats increased in body weight 1 g over the same period. This 5% loss of body weight compares with an 8–10% loss normally seen in intact rats. The severity of the audiogenic seizures was similar in hypophysectomized and intact animals.

General endocrinological changes

Histology and organ weights. In Table 1 the organ weights in female, male and female hypophysectomized rats are presented. The weights of the thyroid and adrenal glands were increased by chronic barbitone administration and the regression lines plotted in Figs. 1 and 2 indicate that these changes occurred independently of any change in body weight due to barbitone administration. The thyroid and adrenal glands of hypophysectomized rats did not increase in weight in response to chronic barbitone administration.

Propylthiouracil (10 mg/kg subcutaneously daily for 7 days) accentuated the goitrogenic response to barbitone administration as shown in Table 2.

Histologically, the thyroid glands of female dependent rats had a larger proportion of epithelial tissue to colloid than untreated rats, as shown by Table 3. Histological

TABLE 1. *Endocrine gland weights of untreated and barbitone dependent female, male and female hypophysectomized rats*

	Female		Male		Female hypophysectomized	
Organ	Untreated	Dependent	Untreated	Dependent	Untreated	Dependent
Body (g)	(a) 174±18 (10) (b) 159±17 (18) (c) 184±15 (6)	190±14 (10) 177±25 (12) 192±7 (6)	203±14 (7)	204±13 (6)	145±12 (7)	141±6 (7)
Pituitary (mg)	9·1±1·6 (a)	9·6±1·4	7·0±0·7	6·6±0·5		
Thyroid (mg)	12·8±3·1 (a)	17·3±2·6 †	15·9±2·8	21·0±2·2 †	8·4±1·1	9·4±1·8
Adrenal (mg)	50·3±10·4 (b)	59·8±11·9 *	48·5±3·3	57·4±10·3	10·5±2·1	10·6±2·1
Ovary (mg)	81±7 (c)	71±14			21±4	16±3 *
Uterus (mg)	276±42 (c)	359±46 *			70±7	70±14
Testes (g)			2·4±0·2	2·3±0·3		
Ventral prostate (mg)			202±64	223±78		
Seminal vesicle (mg)			239±46	279±73		
Levator ani (mg)			164±22	134±33 *		
Liver (g)	6·8±0·8 (b)	8·5±1·6 †	9·9±0·1	12·0±0·1 *	4·8±0·6	7·0±0·4 †

Statistical significance of difference between dependent and untreated animals * $P<0·05$, † $P<0·01$. Figures in brackets refer to the number of animals used. Letter in brackets indicate which group of animals yielded the corresponding gland weight in the female rats.

examination of the pituitary gland of these rats revealed a larger concentration of periodic acid-Schiff positive, aldehyde thionine positive cells ; these cells are believed to be thyroid stimulating hormone secreting cells (Ezrin & Murray, 1963). Periodic positive, aldehyde thionine negative (gonadotrophin secreting) and periodic acid-Schiff negative, orange G positive (growth hormone and prolactin secreting) cells were in very similar proportions in untreated and barbitone dependent animals. No cellular abnormalities were observed.

The adrenal gland (medulla and cortex) appeared histologically unchanged in barbitone dependent rats as did the ovary, uterus and testes.

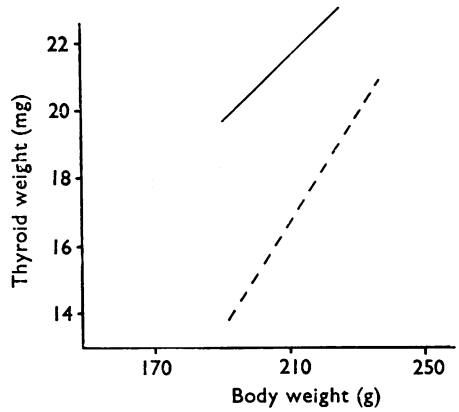

FIG. 1. Regression lines for thyroid gland weight against body weight for barbitone dependent (——, $r=0{\cdot}326$) and untreated (- - -, $r=0{\cdot}728$, $P<0{\cdot}05$) male rats.

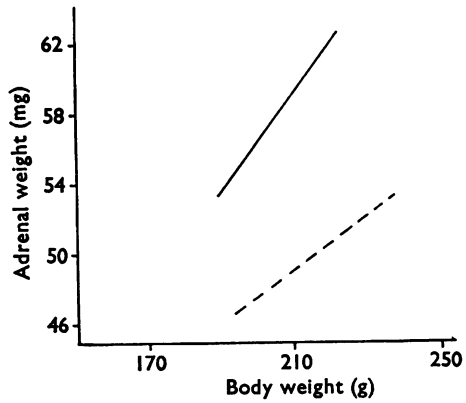

FIG. 2. Regression lines for adrenal gland weight against body weight for barbitone dependent (——, $r=0{\cdot}125$) and untreated (- - -, $r=0{\cdot}698$, $P<0{\cdot}05$) male rats.

TABLE 2. *Thyroid gland weight (mg) after propylthiouracil administration to untreated and dependent rats (means ±standard deviation)*

	Untreated rats	Dependent rats	Significance of difference
Female	24·7±1·7	40·2±5·8	$P<0{\cdot}01$
Male	17·9±1·1	29·2±3·3	$P<0{\cdot}01$

TABLE 3. *Effect of barbitone dependence on pituitary and thyroid gland histology*

			Pituitary				Thyroid	
				Cells per view				
	Rats	Body weight (g)	Gland weight (mg)	PAS+ aldehyde thionine−	PAS+ aldehyde thionine+	PAS− orange G+	Gland weight (mg)	Epithelial–colloid ratio
Barbitone depressant	1	222	9·0	0·4706	4·7647	32·26	18·8	1·1024
	2	168	8·5	1·3462	2·5485	14·77	13·8	1·8583
	3	181	9·3	1·7941	1·8235	23·89	18·3	1·9998
	4	186	7·6	1·3793	4·2414	32·44	19·0	1·5461
	Mean			1·2475	3·3420	25·84		1·6266
Untreated	1	158	7·0	0·4762	1·8571	25·73	11·0	1·2737
	2	156	8·4	2·4000	2·7600	27·42	11·3	1·1044
	3	162	9·3	0·5484	0·5484	21·30	13·5	1·2436
	Mean			1·1415	1·7218	24·82		1·2072

Ovarian weight appeared to be slightly decreased and uterine weight was significantly increased during barbitone dependence. Figure 3 shows the regression line of uterine weight against body weight. Female hypophysectomized rats, made dependent on barbitone, did not show any increase in uterine weight but ovarian weight was significantly reduced when compared with the untreated hypophysectomized animals. The gonads and secondary sex glands from intact male animals tended to show similar weight changes as intact females. However, the weight of the levator ani muscle was decreased by chronic barbitone administration. Liver weight was greatly increased in female, male and female hypophysectomized rats.

In a group of twelve young female rats receiving barbitone and twelve untreated rats, daily vaginal smears were made for 17 days after the vaginas opened, which was when the dependent rats were 118 ± 13 g and the untreated rats were 100 ± 15 g (mean $\pm$ S.D.). It was found that a period of prolonged anoestrus in the dependent rats was followed by approximately 1 day longer ($P<0{\cdot}05$) cycle time due to an extension of the anoestrus stage of the cycle.

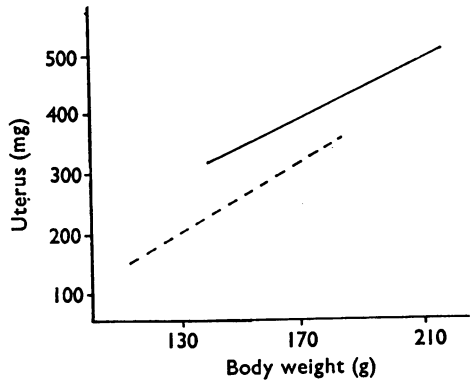

FIG. 3. Regression lines for uterine weight against body weight for barbitone dependent (——, $r=0{\cdot}261$) and untreated (- - -, $r=0{\cdot}426$, $P<0{\cdot}01$) rats.

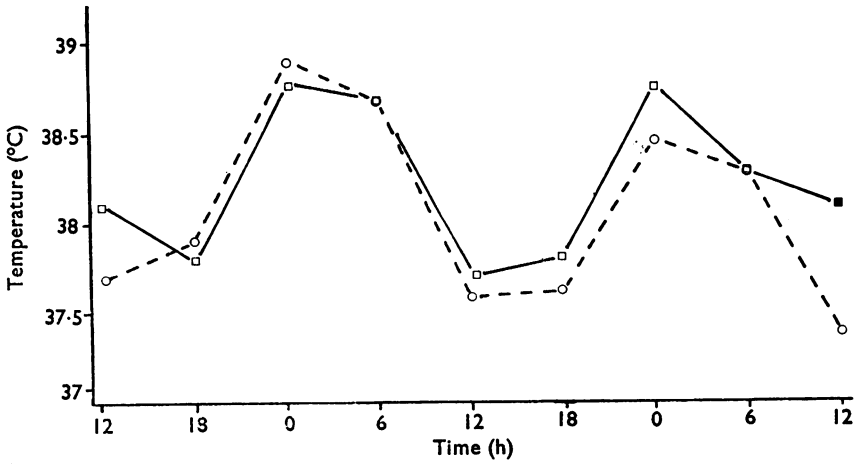

FIG. 4. Daily variation in temperature of barbitone dependent (——) and untreated (- - -) female rats. ○- - -○, Untreated rats; ■——□, barbitone dependent rats; ■ different from untreated value $P<0{\cdot}05$.

Basal metabolic rate and body temperature. Basal metabolic rate was recorded between 10–13 h after both dependent and untreated female rats had been starved overnight. The consumption of oxygen (ml O_2/min)/100 g body weight was 2·18 ± 0·16 for four untreated rats and 2·19 ± 0·18 (means ± S.D.) for four dependent rats. In the body temperature recordings, six dependent and six untreated female animals had very similar temperature when recorded 6 hourly throughout a 48 h period. Only at one time were the dependent rats observed to have a higher temperature ($P<0·05$) than the untreated animals (Fig. 4).

Iodine bound to plasma protein. This was determined in dependent and untreated female and female hypophysectomized rats, as recorded in Table 4. No change in the concentration of iodine bound to plasma protein was caused by barbitone administration.

Plasma corticosterone. The concentration of venous plasma corticosterone was determined in four untreated female rats and three barbitone dependent female rats. In the untreated rats, the resting corticosterone concentration was a mean of 9·5 μg corticosterone % (individual figures 15·0, 4·0, 15·0, 4·0) and in the barbitone dependent rats a mean of 16·0 μg corticosterone % (individual figures 12·5, 11·5, 24·0).

In a subsequent experiment, 2 weeks later, the same animals were stressed by ringing a bell for 12 s in every minute for a period of 30 min before sampling. The untreated rats yielded a mean corticosterone concentration of 47·5 μg corticosterone % (individual figures 45·0, 20·0, 47·5, 77·5) and the dependent rats a concentration of 16·3 μg corticosterone % (individual figures 15·0, 17·5, —).

Growth hormone and thyroxine administration. Growth hormone and thyroxine were administered to seven hypophysectomized rats and seven hypophysectomized rats made dependent on barbitone by administration of the drug for 4 weeks. Over the period of hormone administraton (14 days), the barbitone dependent rats increased in weight by 21·0 ± 5·6 g and those which did not receive barbitone by 22·3 ± 5·3 g (means ± S.D.).

When the administration of growth hormone and thyroxine was stopped no susceptibility to seizures or weight loss was observed in either dependent or untreated hypophysectomized animals.

Growth of young rats receiving barbitone. When female rats received barbitone during their period of rapid growth (as described in **Methods**) they grew at a faster rate than untreated animals. Figure 5 illustrates this growth promoting effect in female rats initially 150 g in weight and maintained on barbitone for 142 days (concentration maintained at 400 mg/kg after 4 weeks). Both body weight and skeletal length as measured by the nose–anus distance were increased by barbitone administration. Since barbitone was administered until the rats reached their growth plateau, the growth advantage was maintained even after barbiturate withdrawal.

TABLE 4. *Effect of barbitone administration on the concentration of iodine bound to plasma protein*

	Number of rats per group	Dependent rats	Untreated rats
Female rats (a)	6	9·82 ± 0·09	10·8 ± 1·55
Female rats (b)	7	10·61 ± 1·40	11·07 ± 1·66
Hypophysectomized	7	3·00 ± 1·29	2·28 ± 0·51

All figures mean ± standard deviation as μg protein bound iodine 100 ml of blood plasma.

Male rats showed a similar weight increase whereas hypophysectomized rats did not (Fig. 6).

Body composition determinations. In female barbitone dependent rats the composition of the additional weight gained over the mean body composition of the untreated rats was 5·21 g fat, 22·24 g of fat-free body weight of which 69·2% was water. Fat formed 16·2% of the weight gained. In untreated rats fat formed 10% of body weight and water formed 72% of fat-free body weight. The additional weight gained by rats receiving barbitone was therefore of normal composition.

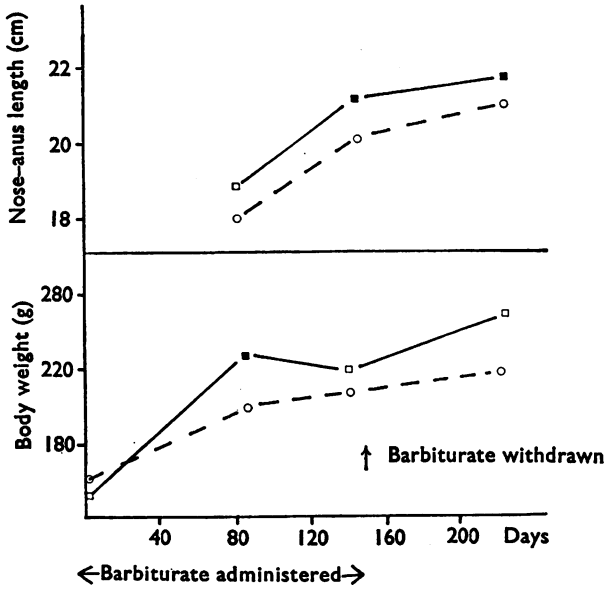

FIG. 5. Body weight and skeletal length of female rats receiving barbitone and of untreated rats. □——■, Rats receiving barbitone ; ○- - -○, untreated rats ; ■, value for rats receiving barbitone significantly ($P<0·05$) different from value for untreated rats.

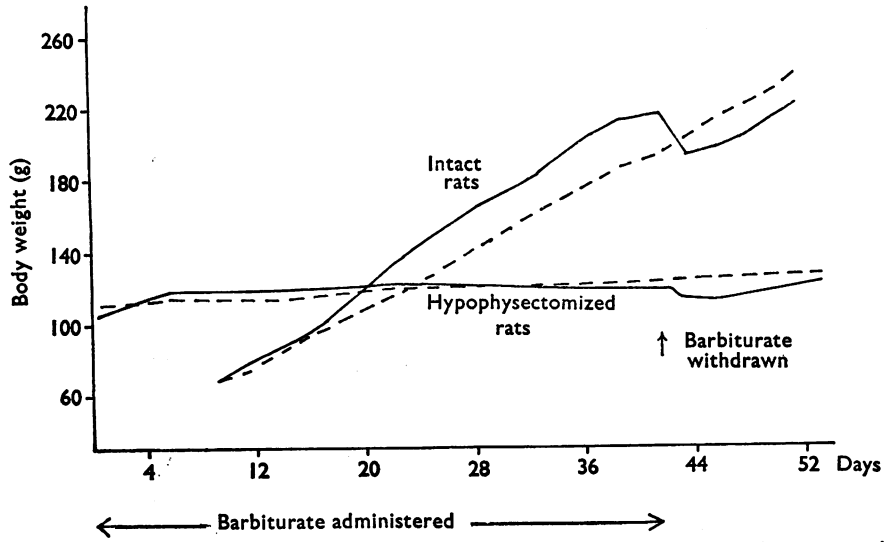

FIG. 6. Body weight changes of intact male and hypophysectomized female rats receiving barbitone (——) and when untreated (- - - -).

Behavioural experiments

When rats received a food reward on only 50% of the occasions they entered the first goal box, the seven untreated male animals doubled their speed of running down the second alley. This did not occur in the seven male barbitone dependent animals, as is shown in Fig. 7. In part A of this figure the speed of running down the second alley is compared with the speed in the first alley when the animal was

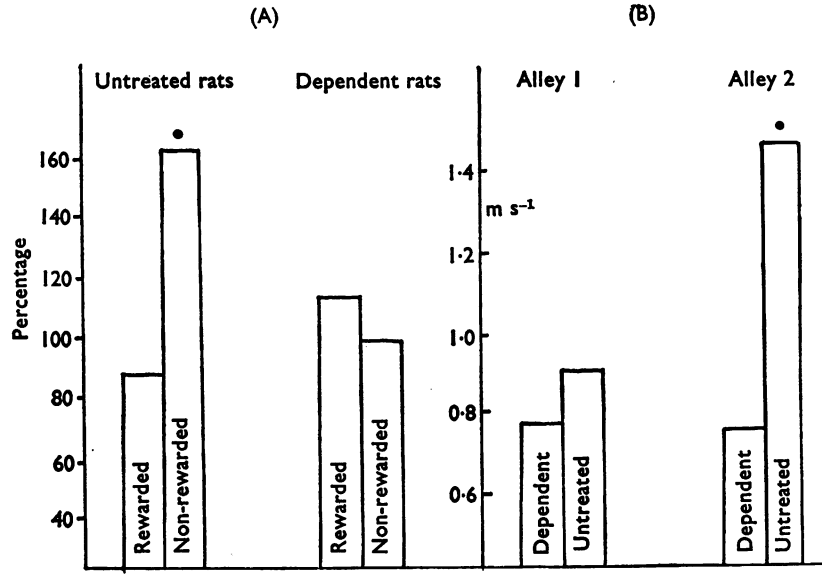

FIG. 7. Effect of non-reward at the end of the first alley on the speed of running down the second alley. (A), Ratio of performance in the second alley to that in the first alley expressed as a %; (B) absolute running speeds in the first and second alleys when the animals were not rewarded at the end of the first alley; (●), significant difference ($P<0{\cdot}05$) between pairs.

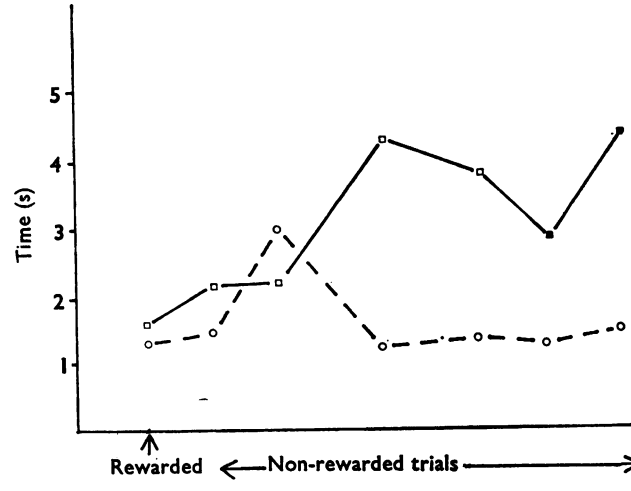

FIG. 8. Effect of barbitone dependence on running times when the rats were rewarded at the start of the experiment and subjected to a series of non-rewarded trials. (□——■), Rats receiving barbitone; (○- - -○), untreated rats, (■), figure for rats receiving barbitone significantly ($P<0{\cdot}05$) different from value for untreated rats.

rewarded and not rewarded in the box between the alleys. The huge increase in speed when the untreated rats were not rewarded is termed the 'frustration effect'. In part B of Fig. 7 is presented the absolute speed in both alleys of the dependent and untreated rats when they were not rewarded in the first goal box. Again the frustration effect in the untreated animals is very evident but absent in the dependent animals.

In the single runway experiment, it was found that four male barbitone dependent animals took significantly longer ($P<0{\cdot}05$) to escape from the box at the end of the alley when non-rewarded than four untreated male rats. However, when subjected to a series of non-rewarded trials the dependent animals approached the box which normally contained a food reward slower than did the untreated animals (Fig. 8). It would therefore appear that receiving no reward deterred the dependent rats from running more than the untreated animals.

Discussion

Hypophysectomized rats are capable of becoming dependent on barbitone and showing audiogenic seizures when the drug is withdrawn. Hormonal changes dependent on the pituitary gland do not appear to be involved in the barbiturate withdrawal syndrome. However, several hormonal changes do appear to occur during chronic barbitone administration and these may have effects on the physiological function, development and behaviour of the animal. For example, the thyroid gland increases in weight and development in barbitone dependent animals. There is occasionally some increase in body temperature but basal metabolic rate and the quantity of circulating thyroid hormones as measured by serum protein-bound-iodine is unchanged. That there is an increased stimulation of the thyroid gland via the pituitary is suggested by the greatly increased goitrogenic response to propylthiouracil which is found in dependent rats. Possibly, this increased activity of the thyroid gland compensates for an increased peripheral destruction of thyroid hormones. It is also possible that barbitone has a direct action on the thyroid gland as suggested by Premachondra & Long (1965). Whatever the primary action of barbitone on thyroid function may be the enlargement of the thyroid gland involves the pituitary because no such change was found in hypophysectomized rats.

The experiments of Leonard (1966) in this laboratory have shown that chronic barbitone administration reduces the concentration of corticosterone in the blood. However, these experiments were probably made under conditions of stress since the rats were decapitated and the corticosterone concentrations in the untreated animals were high (35 μg % corticosterone). The depressant effect of barbitone on the corticosteroid response to environmental stimuli has been shown by Barrett & Stockham (1965). In this respect, barbiturates share a property with many drugs in damping the release of ACTH to stressful stimuli (Gaunt, 1961 ; Royce & Sayers, 1958). Although the number of experiments carried out in the present work is small, the results confirm the idea of Barrett & Stockham (1965) that basal corticosteroid concentrations are not reduced by barbiturate administration and that as shown by these workers and by Leonard (1966) the response to environmental stress is greatly reduced in barbitone dependent animals. The present work has shown that the liver is increased in size in barbiturate dependent rats and it is known that barbiturates induce liver enzymes so that the metabolism of corticosteroids is

increased (Conney, Schneidman, Jacobson & Kuntzman, 1965). The adrenal gland is heavier in barbitone dependence and it is likely that this is in compensation for the increased destruction of corticosteroids in the liver.

Ovarian weight tended to be reduced in barbitone dependent rats. This change is apparently not dependent on the pituitary gland because hypophysectomized rats showed a significant reduction in ovarian weight. Histologically no changes were noted in the gonads. It has been found that the secondary sex organs, particularly the uterus, increase in weight due to chronic barbiturate administration. However, there was a reduction in weight of the levator ani muscle, therefore contrary to what would be expected from the secondary sex organs, it would seem that testosterone secretion is probably reduced in dependent male animals. Vaginal cytology showed that the anoestrus stage of the oestrus cycle was lengthened, indicative of a reduced oestrogen secretion in the female animal but contrary to the uterotrophic action of the barbiturate. The contradictory factors may be explained by an increase in quantity of steroid metabolites released into the blood stream by the increased catabolic activity of the liver. These compounds are not necessarily inactive (Conney *et al.*, 1965) and could conceivably have androgenic and not anabolic properties and uterotrophic but not other oestrogenic actions.

Young rats grow at a faster rate when receiving barbitone. The present work confirms the extensive data of Turnbull (1966) that barbitone dependent rats are heavier, longer and of similar body composition as compared with untreated animals. Young hypophysectomized rats receiving barbitone do not increase in weight and since the removal of the pituitary gland does not prevent rats becoming obese (Kennedy & Parrot, 1958) hyperphagic effects which can occur with barbiturates (Baile & Mayer, 1966) do not take place in barbitone dependent animals. In addition, adult rats do not increase in weight with barbiturates (Turnbull, 1966 ; Essig, 1966). An attractive explanation for these effects is that the release of growth hormone releasing factor from the hypothalamus is increased. This would result in increased growth of young but not old animals (Pecile, Muller, Falconi & Martini, 1965). Growth hormone administration to hypophysectomized animals has shown that barbitone dependence does not alter the growth promoting action of growth hormone. A peripheral action of barbitone on tissue responsiveness to growth hormone is therefore ruled out.

It is also possible that the reduction of stress induced increases of corticosterone concentrations may result in increased growth rate of young animals. Barrett & Stockham (1963) have shown that under normal laboratory conditions corticosteroid concentrations are elevated during a considerable portion of the day. Since this effect is antagonized by barbiturates, it is likely that on average the corticosterone concentration is lower in dependent animals. There is much indirect evidence that corticosteroids inhibit growth (Marx, Simpson, Li & Evans, 1943 ; Bellamy, 1964 ; Krulich & McCann, 1966) and that a reduction of plasma corticosteroids results in increased growth (Weininger, 1954, 1956 ; Christian, 1960 ; Rosen, 1964).

Barbiturates have been cited as drugs which reduce fear and frustration (Miller, 1964) and in the present work it has been shown that rats respond to stressful situations less readily than do control animals.

It is well known that adrenocortical hormones are necessary for normal responses to stress to occur and the failure of the rats to respond to stress in the present

experiments is probably directly related to the finding that stress induced increases in corticosterone levels are inhibited.

While the present study demonstrates that endocrine changes are probably not involved in the mechanism of barbiturate dependence, they do indicate that endocrine changes are induced and that these in turn modify growth and behaviour.

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