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Hormonal Factors Influencing Liver Catalase Activity in Mice. Testicular and Adrenal Factors

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Greenstein and his associates have shown that the presence of a growing tumour in rats and mice is almost invariably associated with a marked depression in liver catalase activity (Greenstein, Jenrette & White, 1941; Greenstein & Andervont, 1942).

As far as the mechanism of the effect is concerned, evidence has recently been obtained suggesting that the tumour exerts its effect through the medium of some toxic material released into the circulation. Nakahara & Fukuoka (1949, 1950) claimed to have produced depressions of liver catalase activity in mice by the injection of ethanol-precipitated fractions obtained from human tumours. Adams (1950*a*, 1951) has shown that mouse tumours in general contain some factor or factors capable of markedly and rapidly influencing liver catalase activity. The way in which such factors could operate is obscure. Greenstein (1943) looked for a direct inhibitor of liver catalase in tumours without success, and finally concluded that in some way tumours must be interfering with the synthesis of catalase in the liver. The purpose of the present work is to examine the mechanisms which control liver catalase activity in the normal animal as a preliminary to further investigation into the effect of tumours on the system.

During the course of the earlier work (Adams, 1950*a*, 1951) a sex difference in normal catalase activity was observed in all three strains of mice used—males having a level some 30% higher than females. It seemed likely, therefore, that there was some hormonal influence on catalase level. A preliminary report (Adams, 1950*b*) has already appeared on the effects of castration, and castration followed by adrenalectomy, in males, and adrenalectomy and ovariectomy in females. Marked falls in liver catalase level were observed within 48 hr. after all these operations, except ovariectomy in females, where there was no significant change.

Begg & Reynolds (1950) have also recently reported a depression of liver catalase in rats 3 weeks after adrenalectomy.

EXPERIMENTAL

Animals. Young adult mice (weighing 25–30 g.) of a stock albino strain were used. The diet of the animals consisted of rat cubes (supplied commercially) and water *ad lib.*

Estimation of liver-catalase activity. Samples of whole liver homogenates were allowed to react with 0·025 M-H_2O_2 (A.R. quality) in 0·02 M-phosphate buffer (pH 6·8) at 0°, the reaction being stopped after 4 min. by the addition of H_2SO_4. The H_2O_2 remaining was estimated by titration with standard thiosulphate after the addition of excess KI. For details see Adams (1950*a*). Catalase activity is expressed in arbitrary units/mg. N. Nitrogen rather than tissue wet weight has been chosen as a standard of reference for the following reasons: (1) The spread on the catalase activity within groups of animals tends to be reduced. (2) Greenstein *et al.* (1941) and Weil-Malherbe & Schade (1948) used total N and protein N, respectively. (3) There is evidence from starvation studies in rats that the fall in liver catalase activity observed after some days is to some extent paralleled by a fall in liver nitrogen content (Miller, 1947). Reference to N, therefore, will tend to correct for changes in nutritional status. However, from the experimental data obtained, it may be stated that substitution of tissue wet weight for N would have had no effect on the pattern of the results about to be described.

Operative procedures. Castration was carried out through a single median abdominal incision, and adrenalectomy through a single median skin incision over the lumbar spine and two para-median incisions through the posterior muscular abdominal wall. Fat associated with the adrenal glands was removed with them. No special precautions were taken post-operatively with castrated animals, but adrenalectomized animals were maintained at a temperature of approximately 27° (81° F.) and given salt water (0·5%). In all cases where subsequent injections were given an interval of 48 hr. was allowed post-operatively. The absence of adrenal tissue in adrenalectomized animals was checked post mortem.

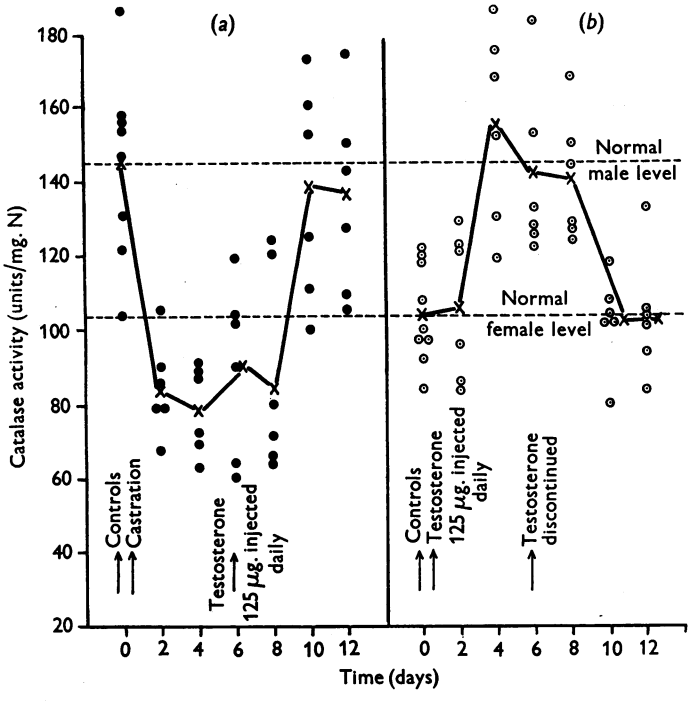

Fig. 1. *a*, Effect of castration and the subsequent injection of testosterone on the liver catalase activity of male albino mice. *b*, Effect of the injection of testosterone and its subsequent withdrawal on the liver catalase activity of female albino mice. ⊙, Individual mice; ×, the arithmetic mean values of the groups in this and subsequent figures.

Hormones and related substances

Testosterone. (Commercially supplied for implantation.)

Cortisone. (*17-Hydroxy-11-dehydrocorticosterone.*) Kindly provided by Prof. Wilson and the Medical Research Council.

Deoxycorticosterone glucoside. (Commercial aqueous solution.)

Progesterone. (Commercial suspension.)

All the above were used in 50% ethanol-water solution and injected subcutaneously. The maximum quantity of solution injected per day was 0·1 ml.

RESULTS

Effect of castration and testosterone administration

A number of male mice were castrated, and the liver catalase activity of small groups was determined every 2 days. Fig. 1 shows that 48 hr. after castration

the catalase activity had fallen considerably, remaining at the new low level on the 4th and 6th day. On the 6th post-operative day the remaining mice were each injected with 125 μg. of testosterone in 50% ethanol, the dose being repeated daily. The control animals received ethanol only. Two days later the catalase level was unaltered, but returned to normal after a further 2 days, and remained so. Fig. 1 also shows the effect on normal females of the injection near, but possibly slightly below, the normal female level. The daily administration of testosterone to normal male mice and its subsequent withdrawal after 6 days had little or no effect on catalase level (Table 1). Fig. 2 shows the effect on normal females of the daily injection of varying doses of testosterone over a period of 5 days. The liver catalase level rises sharply with dose up to about 40 μg./day, but there is little further rise up to 640 μg./day.

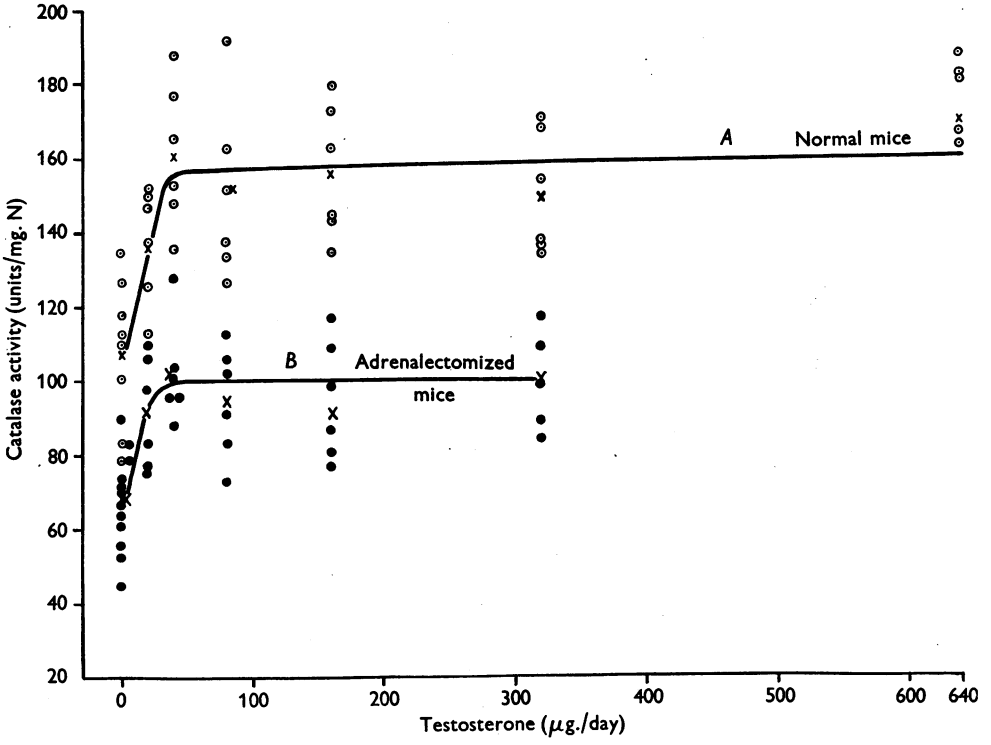

Fig. 2. Effect of the injection of varying amounts of testosterone daily for 5 days on the liver catalase activity of (*A*) normal female mice; (*B*) adrenalectomized female mice.

of the same quantity of testosterone daily and its subsequent withdrawal. Four days from the start of the injections the liver catalase activity rose to approximately the normal male level. On the 6th day testosterone was discontinued, and 4 days later catalase activity returned to normal. It may also be seen from the graph that the castrated male level is

Effect of adrenalectomy and cortisone administration

As shown in Fig. 3 adrenalectomy of female mice resulted in a marked depression in liver catalase activity 48 hr. later, the level remaining low 4 and 6 days after operation. On the 6th day 70 μg. of cortisone in 50% ethanol was injected, the injections

Table 1. *Liver catalase level in male albino mice after the daily injection of testosterone*

Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. Six to eight animals/group.)

Substance	Catalase level Normal controls	Dosage (μg./day)	Catalase level at varying times (days) after commencement of injection 2	4	6*	10	12
Testosterone	145 ± 7·0	125	152 ± 9·9	153 ± 7·3	150 ± 8·0	140 ± 6·6	136 ± 9·0

* Testosterone withdrawn.

being repeated twice daily. This was almost immediately effective, the catalase level returning to normal within 4 days. The effect of injecting adrenalectomized males with varying doses of cortisone daily for 5 days is shown in Fig. 4. A dose

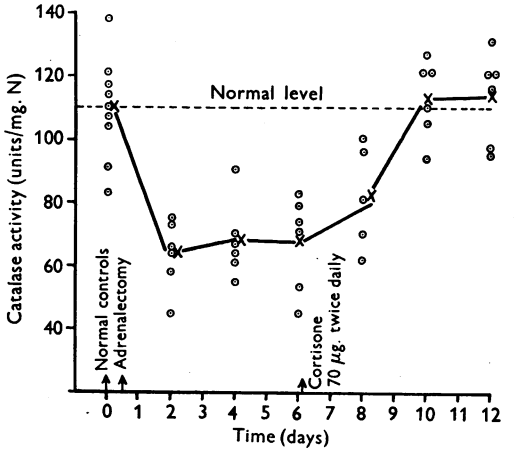

Fig. 3. Effect of adrenalectomy and the subsequent injection of cortisone on the liver catalase activity of female albino mice.

Progesterone and deoxycorticosterone were also injected into adrenalectomized male mice over a period of 5 days. The results appear in Table 2. Progesterone in doses up to 240 μg./day gave no significant rise. Deoxycorticosterone in very high dosage produced a partial restoration of catalase activity. In this experiment only the adrenalectomized controls were given salt water.

To meet the possible criticism that the alterations in catalase level following adrenalectomy might be due to post-operative anorexia, a paired feeding experiment was carried out. As stated previously, adrenalectomized animals are maintained at a temperature of approximately 81° F. Since this alone would reduce calorie requirements it was considered best to maintain the paired-fed controls at this temperature also. For completeness, a group of controls at normal temperature (approx. 70° F.) with unrestricted food intake was included. The results appear in Table 3, and show that no significant difference was observed between the catalase levels of the paired-fed and normal controls. The food intake of the adrenalectomized animals rose steadily over 4 days. On the first day, the food intake of the controls which were subsequently to be paired fed, fell sharply compared with the control animals at the lower temperature.

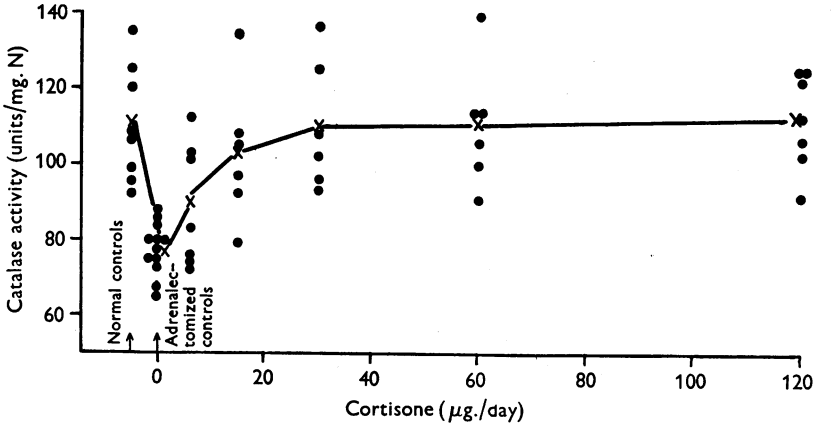

Fig. 4. Effect of the injection of varying doses of cortisone per day for 5 days on the liver catalase activity of adrenalectomized male albino mice.

of 30 μg./day is sufficient to restore the normal level, there being little or no rise with higher doses. In these two experiments the control animals again received injections of ethanol only. However, in a separate experiment, results of which appear in Table 2, the injection over 5 days of up to 0·1 ml. ethanol/day was shown to have no effect on the catalase level of an adrenalectomized animal. The injection of ethanol into control animals was therefore discontinued in subsequent experiments.

Interrelation between adrenal and testicular factors

The results so far have shown that in adrenalectomized males and females the resulting lowered liver-catalase level is restored by cortisone. Testosterone restores the normal activity in castrated males, and elevates the normally lower female level to that of the male. It is therefore of interest to see whether testosterone and cortisone are capable of replacing one another. Testosterone in varying

Table 2. *Liver catalase level in adrenalectomized male albino mice after the injection of varying quantities of progesterone, deoxycorticosterone, or ethanol over a period of 5 days*

(Catalase levels in arbitrary units/mg. N expressed as arithmetic means ± standard errors of means. The figures in parentheses give the numbers of animals in the groups.)

Substance	Catalase level: Normal controls	Catalase level: Adrenalectomized controls	Catalase level 5 days after injection of varying quantities/day			
Progesterone	127 ± 6·3 (12)	75 ± 2·4 (8)	30 μg. 83 ± 6·6 (6)	60 μg. 94 ± 7·6 (6)	120 μg. 86 ± 6·7 (6)	240 μg. 82 ± 4·2 (6)
			100 μg.	200 μg.	400 μg.	1000 μg.
Deoxycorticosterone	113 ± 4·9 (12)	75 ± 4·1 (10)	80 ± 5·9 (6)	84 ± 4·1 (6)	94 ± 6·9* (6)	92 ± 5·5* (6)
			0·05 ml.		0·1 ml.	
Ethanol	141 ± 8·0 (8)	68 ± 4·5 (6)	68 ± 4·5 (6)		74 ± 4·0 (6)	

* These values are just significantly different from the adrenalectomized controls ($t = 2·3$ and 2·4 respectively). ($t > 2$ for $P = 0·05$.)

Table 3. *Liver catalase level in adrenalectomized male albino mice compared with that in paired-fed controls maintained at the same temperature* (81° F.), *and in controls at normal temperature having an unrestricted food intake*

(Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. Eight animals/group.)

	Catalase activity at varying times (days) after commencement				Food intake (g./mouse) at varying times (days) after commencement (averaged)				
	2	3	4	5	1	2	3	4	5
Adrenalectomized	79 ± 4·9	—	81 ± 3·9	—	2·2	3·2	3·6	4·9	—
Paired-fed controls	—	116 ± 6·0	—	127 ± 4·4	3·3	2·2	3·2	3·6	4·9
Normal mice	122 ± 8·2	—	128 ± 10·2	—	6·5	7·7	7·5	6·5	—

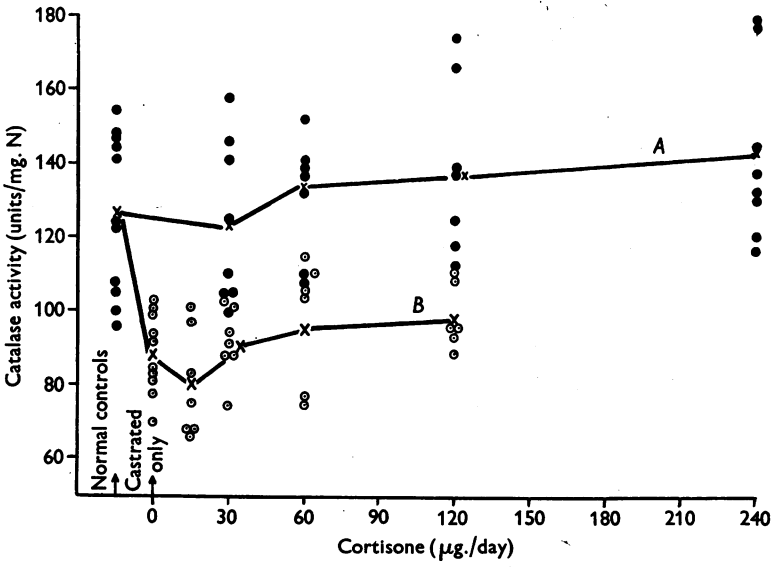

Fig. 5. Effect of the injection of varying doses of cortisone per day for 5 days on the liver catalase activity of (*A*) normal male albino mice; (*B*) castrated male albino mice.

dosage was injected into normal and adrenalectomized female mice. The results appear in Fig. 2. The two curves run approximately parallel to one another, showing that the effect on adrenalectomized mice is no greater than on normals, and therefore that, as far as catalase is concerned, testosterone will not replace adrenal secretion, although, as has adrenalectomy followed by castration. Castration alone, and adrenalectomy alone, produced the usual depressions of activity at 48 hr., the latter greater than the former, and these were unchanged 48 hr. later. Adrenalectomy of the castrated animals resulted in a further fall, but the level was then only very little below that of the mice which had been

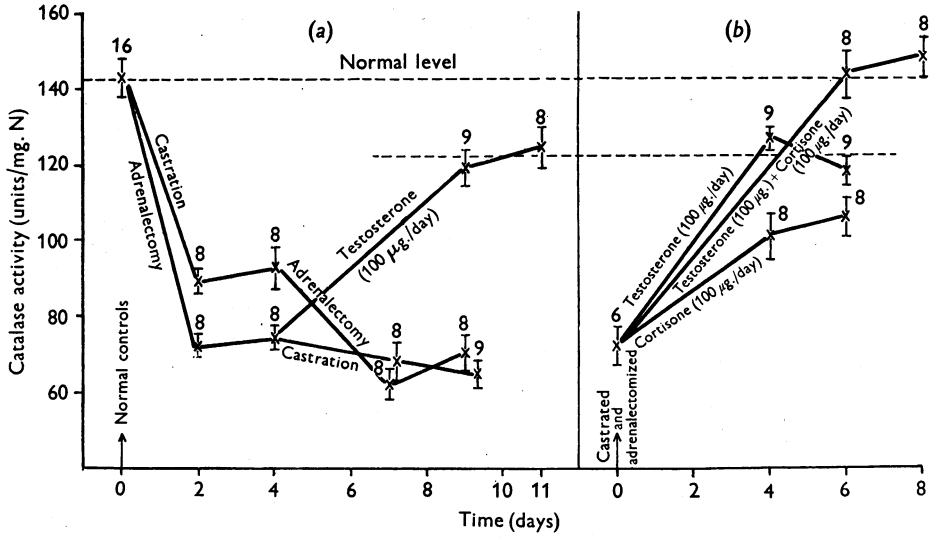

Fig. 6. *a*, Effect of castration or adrenalectomy followed by adrenalectomy or castration, respectively, on the liver catalase activity of male albino mice. Also shows the effect of testosterone injection on adrenalectomized males. *b*, Effect of the injection of testosterone, cortisone, and a mixture of both, on the catalase activity of castrated and adrenalectomized male albino mice. Results are given as arithmetic means (×) ±standard error of means. The number of animals in each group is given by the small figures at the head of the standard error limits.

Table 4. *Liver catalase levels in mice after castration and adrenalectomy*

(Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. The figures in brackets give the number of animals in the groups.)

Controls	Castrated	Adrenalectomized	Castrated and adrenalectomized
		Males	
*145±5·3 (16)	91±2·8 (16)	74±2·4 (16)	68±1·8 (36)
141±80 (8)	—	68±4·5 (6)	—
145±7·9 (9)	85±4·1 (18)	—	—
110±5·4 (8)	—	77±1·9 (10)	—
127±6·4 (12)	90±2·9 (10)	75±2·4 (8)	—
113±4·9 (12)	—	75±4·1 (10)	—
		Females	
*110±4·4 (16)	—	66±2·9 (18)	—

* Corresponding batches.

already been shown, cortisone will do so. Nor has cortisone a greater effect on castrated than on normal males, as shown in Fig. 5. There is no sign, in the former, of a return to the normal catalase level. It appears, therefore, that cortisone and testosterone are exerting independent actions on liver catalase. There is, however, a complicating factor in male mice. Fig. 6*a* shows the effect on male liver catalase of castration followed by adrenalectomy some days later, and, in a corresponding experiment, of adrenalectomized, but not castrated. Castration of the adrenalectomized animals produced little or no change in level, contrasting with the large fall obtained on castrating a normal animal. However, although castration after adrenalectomy produced little change, it is evident that the same ultimate level is reached independently of the order in which the operations are performed. A further large group of animals from the same batch were castrated, and adrenalectomized 48 hr. later. Catalase deter-

minations were made in six of these in order to show that the activity had fallen to the level reached in the previous series (the results appear on the left of Fig. 6*b*). The remaining animals were divided into three groups, and testosterone 100 μg./day, cortisone 100 μg./day and testosterone 100 μg./day plus cortisone 100 μg./day injected into each group respectively. Fig. 6*b* shows that the mixed substances restored the normal catalase level, each singly being only partially successful. Although adrenalectomy produced a bigger drop than castration, cortisone gave a smaller rise than testosterone. Fig. 6*a* also shows the effect of giving testosterone, 100 μg./day, to animals adrenalectomized only. There is a rise which falls short of the normal level; the extent of this rise is almost identical with that resulting from the injection of testosterone into a castrated and adrenalectomized animal.

Although there is a considerable variation from batch to batch in the normal male catalase level, Table 4 shows that over a number of batches the levels after castration or adrenalectomy are remarkably constant. The mean levels in adrenalectomized females, and castrated and adrenalectomized males, are also practically identical.

DISCUSSION

Marked and rapid falls in liver catalase level have been shown to follow the operative removal of the testes in males, and the adrenals in both sexes. Post-operative anorexia appears to make no significant contribution to the observed changes. Administration of testosterone or cortisone respectively results in an almost equally rapid rise to normal. It seems clear that the sex difference in normal mice is due to lack of testicular secretion in the females.

It is not claimed, however, that the described effects specifically involve liver catalase. Sex differences in enzyme levels are not uncommon, and, for example, a decrease in rat-liver arginase activity following adrenalectomy has been reported (Folley & Greenbaum, 1947; Kochakian & Vail, 1947). However, the probability that other liver components may follow a similar pattern is not of great significance from the point of view of the present work.

Cortisone will not restore the catalase level of a castrated male to normal, and testosterone has no greater effect on an adrenalectomized than on a normal female. Further, a mixture of cortisone and testosterone is required to restore the normal activity in a castrated and adrenalectomized male, neither alone being adequate. This seems to indicate that each hormone exerts its own effect on the liver. Nevertheless, as Fig. 6 and Table 4 show, the total system in males is more complicated. Adrenalectomy produces a greater fall in male catalase level than castration. Adrenalectomy of a castrated animal results in a further significant depression, but castration of an adrenalectomized animal does not. In view of the abnormal metabolism of an adrenalectomized animal and the complexity of the known interrelations between the various hormone systems, the evidence is not sufficiently clear cut to show whether the secretion of testosterone is under adrenal control. However, as far as the system under observation is concerned, it does appear that in an adrenalectomized male, either the testes are not producing the normal quantity of testosterone, or the testosterone which is produced is incapable of exerting its normal effect. The further observation that 100 μg./day of testosterone raises the level in an adrenalectomized animal to the same point whether the testes are present or not, does suggest that adrenalectomy interferes in some way with testicular secretion.

Neither progesterone nor deoxycorticosterone will replace cortisone in restoring the catalase level of an adrenalectomized mouse, although high doses of the latter appear to have a slight effect.

The level of activity observed in castrated or adrenalectomized males is relatively constant, whereas average levels in different batches of normal mice fluctuate more widely. This suggests that the fluctuations are due to differences in the hormonal stimuli.

No work has yet been done on the possible influence of the pituitary gland on the system, but this point will be investigated.

SUMMARY

1. Castration of young adult male albino mice produces a depression in liver catalase activity. The level is restored by the injection of testosterone. Injection of testosterone into female mice elevates their lower normal level to that of the male.

2. Adrenalectomy in both sexes results in a depression in liver catalase activity. Cortisone, but not progesterone or deoxycorticosterone, restores the normal level.

3. Cortisone has little effect on the liver catalase level of castrated or normal mice, and testosterone has no more effect on adrenalectomized than on normal female mice.

4. Although adrenalectomy of castrated males results in a further fall in liver catalase level, castration of adrenalectomized males does not. The ultimate level is the same in whichever order the operations are done.

5. A mixture of cortisone and testosterone is needed to restore to normal the liver catalase activity of a castrated and adrenalectomized male, neither alone being adequate. Testosterone has the same effect on an adrenalectomized as on a castrated and adrenalectomized male.

I am grateful to Prof. S. P. Bedson, F.R.S., for his interest, and to Dr M. H. Salaman for his suggestions and advice. I am also indebted to Dr G. H. Bourne and Dr C. J. O. R. Morris for helpful discussions, to Mr L. J. Hale, Miss B. L. DeBoise and Mr K. Goodall for skilled technical assistance, and to Mr G. Downes for his care of the animals. The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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Exogenous and Endogenous Cytochrome *c*

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Keilin (1930) first suggested that extracted cytochrome *c* might be different from the cytochrome *c* existing in the living cell. Later, Keilin & Hartree (1940) showed that exogenous cytochrome *c* is much less active catalytically than the endogenous form. This has been re-emphasized by Keilin & Hartree (1945, 1949) and by Slater (1949*b*, 1950*b*). Keilin & Hartree (1940) also pointed out that their heart-muscle preparation contained a cytochrome system in a very high degree of organization comparable to that existing in the living cell, and this was later demonstrated quantitatively by Slater (1949*b*). In the present investigation, the quantitative difference in catalytic activities between exogenous and endogenous cytochrome *c* is further examined, a qualitative difference between the two forms is demonstrated and it is also shown that by suitable means the soluble exogenous form of cytochrome *c* can be transformed into a bound form which is identical with the endogenous form in every respect. Some of the findings have been briefly reported elsewhere (Tsou, 1951*c*).

MATERIALS AND METHODS

Cytochrome c of iron contents of 0·34 and 0·43% was prepared by the methods of Keilin & Hartree (1945). Unless otherwise specified, the preparation with 0·34% iron was used.

Dihydrocozymase. Cozymase was obtained by an unpublished method of Ochoa and reduced enzymically as described by Slater (1950*a*).

Light absorption in the visible and ultraviolet regions was measured with a Beckman photoelectric spectrophotometer.

Heart-muscle preparation containing the complete cytochrome system and the enzymes responsible for the reduction of cytochrome *c* by succinate and dihydrocozymase was prepared according to the method of Keilin & Hartree (1947) as previously described (Tsou, 1951*a*).

Extraction of cytochrome c *from washed heart-muscle mince.* Samples (6 g.) of heart-muscle mince, well washed with water until haemoglobin-free, were weighed into a series of test tubes to which were added 6 ml. portions of the extracting solutions of known concentrations. The water content of the same washed muscle mince was determined by drying a separate 6 g. sample to constant weight at 100°. The mixtures were allowed to stand at room temperature overnight, filtered, and the cytochrome *c* content of the filtrates was determined as follows: to 3 ml. of the filtrate was added 0·1 ml. of diluted (1:10) heart-muscle preparation in 0·25 M-phosphate buffer, pH 7·3, thoroughly mixed, and the optical density (D_o) at 550 mμ. read in a 1 cm. cell against a blank containing 3 ml. of 0·1 M-phosphate buffer, pH 7·3, and 0·1 ml. of the same diluted heart-muscle preparation; 0·1 ml. of a solution containing 0·4 M-succinate and 0·05 M-cyanide was then added to both the cytochrome *c*-containing solution and the blank. After mixing, the optical density was again read until a steady value (D_r) was reached. The concentration of cytochrome *c* in the extract was given by the formula

$$\frac{D_r(3\cdot2/3\cdot0) - D_o(3\cdot1/3\cdot0)}{1\cdot92} \times 10^{-4}\text{M},$$

in which the difference ($D_r - D_o$) is corrected for dilution by the added reagents and divided by the difference between the

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