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The effects of oestrogens and progestins on the response of mice to barbiturates

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1. Mestranol (oestrogen) prolonged, whilst lynestrenol (progestin) reduced, the duration of pentobarbitone and hexobarbitone sleep in mice, whilst the effects of barbitone were not altered.

2. The effects of these steroids on pentobarbitone sleep were dose-related, did not show tachyphylaxis, and produced optimal effects after only 4 days pretreatment.

3. The effects of lynestrenol were abolished by SKF 525A, whilst those of mestranol were markedly potentiated, suggesting a different mechanism and/or locus of action for mestranol and SKF 525A.

4. Examination of plasma levels of pentobarbitone in mice pretreated with mestranol, lynestrenol or SKF 525A showed that lynestrenol increased whilst mestranol and SKF 525A reduced the rate of clearance of barbiturate from the plasma.

5. The effects of lynestrenol disappeared when pentobarbitone was prevented from inducing hypothermia, whilst some significant prolongation of pentobarbitone sleep persisted in mestranol treated mice. This suggested that the ability to potentiate hypothermia was not the sole mechanism by which the effects of pentobarbitone were enhanced by mestranol.

6. It is concluded these steroids alter the duration of action of pentobarbitone (and hexobarbitone) by changing the rate of barbiturate metabolism. In the case of mestranol, this might be a combination of an effect upon basal metabolic rate (enhancing hypothermia) and a direct effect on the liver. An effect upon renal clearance cannot be excluded by these results.

It has long been recognized that there exists a sex difference in the response of laboratory animals to certain drugs, particularly the barbiturates (Nicholas & Barron, 1932 ; Holck, Kanan, Mills & Smith, 1937 ; Homberger, Efstein & Himwich, 1947). This sex difference may be mediated through differences in the rates of biotransformation of these drugs by the liver (Kato, Chiesara & Frontino, 1962 ; Robillard, D'Iorio & Pellerin, 1954). Similarly, when animals are pretreated with male or female sex hormones, there are also marked changes in the animals' responses to barbiturates, although the direction of these changes is the subject of some controversy. Thus, Quinn, Axelrod & Brodie (1954) showed that male sex

hormone shortened barbiturate sleeping time in female rats, whilst female sex hormone prolonged the effects of barbiturate in male rats. In contrast, although testosterone shortened the effect of hexobarbitone in female mice, the oestrogenic agent stilboestrol also shortened sleeping-time in male mice (Westfall, Boulos, Shields & Garb, 1964). Also, Gessner, Acara, Baker & Edelman (1967) showed that chronic pretreatment of both male and female mice with sex hormones caused opposite effects: pentobarbitone sleeping-time in male mice was prolonged by oestrogens, whilst testosterone shortened sleeping-times in both sexes.

Three things emerge from this earlier work: testosterone consistently shortens sleeping-time, oestrogens have variable effects whilst no work has been carried out with progestogenic agents, despite the availability of a number of synthetic agents during the last few years. Accordingly, we were prompted to examine the effects of oestrogens and progestins on barbiturate sleeping-times in mice.

Methods

Groups of sixteen male or female TO albino mice weighing 20–25 g were used to determine the duration of sleep after the intravenous administration of the sodium salt of pentobarbitone, hexobarbitone or barbitone. Before commencing experiment and during pretreatment, animals were maintained on a conventional 41B cube diet with free access to water; animal house and laboratory temperatures were maintained at 20°–22° C. Mice were pretreated with a steroid or its oily vehicle (controls, 0·1 ml./20 g body weight) for 4 days by subcutaneous injection. On the fifth day and 18 hr after the fourth pretreatment injection, the effect of the barbiturate was determined, sleeping time being measured by a semi-automatic apparatus which recorded on a smoked kymograph drum the point at which an animal regained its righting-reflex. Results are expressed as the group mean sleeping-time (min) ± S.E., with the statistical significance of any observed changes being calculated from Student's *t* test.

Assay of plasma pentobarbitone levels

The determination of plasma levels of pentobarbitone was made by a method based on those described by Brodie, Burns, Mark, Lief, Bernstein & Papper (1953) and Noordhoek (1968). The blood from four decapitated mice was pooled in a centrifuge tube containing 4 mg of potassium oxalate deposited as a smear over the inside of the tube. The red cells were spun down at 3,000 rev./min for 12 min and 1 ml. of plasma added to a stoppered centrifuge tube containing ether (boiling range 40°–60° C) with 1·5% isoamyl alcohol. The whole was shaken for 20 min and separated by centrifugation at 15,000 rev./min for 10 min. Six ml. of the organic phase was extracted with 4 ml. of phosphate buffer (pH 11·0) by shaking for 4 min; after further centrifugation at 7,500 rev./min for 10 min, the organic phase was removed by aspiration. The absorbance of the clear buffered aqueous phase at 240 m-u. was measured in a SP 500 spectrophotometer using as a zero reading a reagent blank exposed to the above extraction procedure. By determination of plasma barbiturate levels at various times after intravenous administration, the rate of disappearance of barbiturate was shown by a plot of log plasma concentration against time; the relationship was linear after 10 min and the slopes of the various rates of disappearance were examined by regression analysis.

Drugs and other chemicals used

Lynestrenol (19-nor-17α-pregn-4-en-20-yn-17-ol), and mestranol (ethinyloestradiol-3-methyl-ether), were a gift from Messrs. Organon Laboratories, Newhouse, Lanarkshire. They were injected subcutaneously after dissolving in an arachis oil vehicle containing 2% w/v benzyl alcohol. The barbiturates, pentobarbitone, hexobarbitone and barbitone, were each administered as their sodium salt, dissolved in distilled water and uncorrected for pH ; doses quoted refer to the salt. SKF 525A is β-diethyl-aminoethyl-diphenylpropylacetate HCl, and was a gift of Smith, Kline & French Laboratories, Welwyn Garden City, Herts ; it was dissolved in 0·9% w/v NaCl solution, prior to injection. All injections, irrespective of their route of administration, were made in a dose volume of 0·1 ml./20 g body weight.

Results

Duration of barbiturate-induced sleep in mice pretreated with oestrogen, progestin, or a combination of the two

Mice were injected subcutaneously with oestrogen (mestranol, 0·5 mg/kg daily) or with progestin (lynestrenol, 5·0 mg/kg daily) for 4 days ; the duration of sleep after intravenous injection of pentobarbitone (50 mg/kg), of hexobarbitone (60 mg/kg), or of barbitone (300 mg/kg) was then determined on the fifth day. The results are summarized in Table 1.

Mestranol prolonged whilst lynestrenol shortened the duration of sleep induced by pentobarbitone or hexobarbitone, whilst the effects of barbitone were not significantly altered by either type of steroid. The effects of both mestranol and lynestrenol were dose-dependent, and an approximately linear relationship was observed between the duration of sleep and the log. dose of hormone (See Fig. 1).

A number of oestrogens and progestins are used clinically in combination as antifertility agents, so that it was of interest to study the effects of a combination of progestin with oestrogen. The results of such a combination, using various ratios of progestin to oestrogen, are presented in Table 2.

The effect of each combination was determined by the ratio of the two drugs. On a weight for weight basis, mestranol was the more potent, and the effects of the two steroids were cancelled out when they were combined lynestrenol: mestranol, 5 : 1.

TABLE 1. *Duration of barbiturate sleep in male and female mice pretreated with mestranol or lynestrenol*

	Group mean sleeping-time (min ± S.E.), after:			
	Pentobarbitone		Hexobarbitone	Barbitone
Pretreatment	(females)	(males)	(females)	(females)
Vehicle only (controls)	102 ± 9·6	96·6 ± 5·8	19·6 ± 1·9	111 ± 18
Mestranol (oestrogen)	201 ± 23 *	237·1 ± 149 *	51·7 ± 3·5 *	109 ± 17
Lynestrenol (progestin)	37·3 ± 2·3 *	75·4 ± 5·7 *	8·6 ± 0·5 *	109 ± 20

Pentobarbitone (50 mg/kg), hexobarbitone (60 mg/kg) or barbitone (300 mg/kg) were injected intravenously 18 hr after the last of four daily subcutaneous injections of mestranol (0·5 mg/kg), lynestrenol (5·0 mg/kg) or vehicle (5·0 ml./kg). * Significant difference from controls ($P = < 0·05$).

A study was also made of the optimal duration of hormone pretreatment. It was observed that the effect on pentobarbitone sleeping-time increased up to a maximum after pretreatment for 4 days, the effects thereafter being well maintained for the duration of treatment; thus both mestranol and lynestrenol were given to female

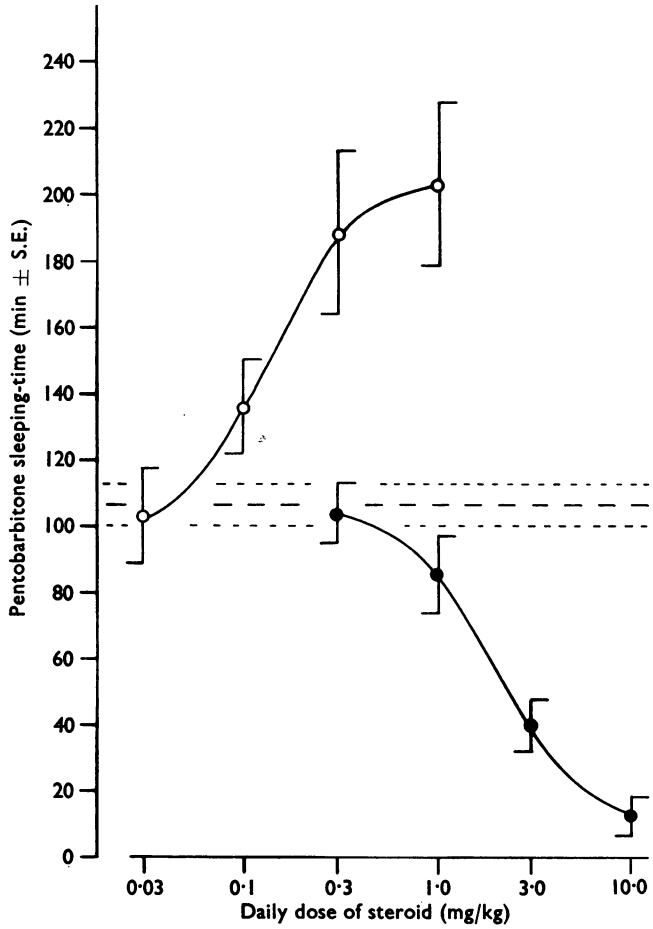

FIG. 1. Duration of pentobarbitone sleep in female mice after pretreatment with various doses of mestranol or lynestrenol. Animals were injected subcutaneously with mestranol (○——○) or lynestrenol (●——●) for 4 days; on the fifth day, they received pentobarbitone, 50 mg/kg intravenously. The dotted lines represent the response in control (vehicle pretreated) animals, group mean ± S.E.

TABLE 2. *Duration of pentobarbitone sleep in female mice pretreated with various combinations of mestranol and lynestrenol*

Pretreatment	Ratio	Pentobarbitone sleeping-time, group mean (min ± S.E.)	
Vehicle only (controls)	—	98·7 ± 6·4	—
Lynestrenol (10 mg/kg) + mestranol (1·0 mg/kg)	10 : 1	68·9 ± 3·9	*
Lynestrenol (5·0 mg/kg) + mestranol (1·0 mg/kg)	5 : 1	102·3 ± 6·0	
Lynestrenol (1·0 mg/kg) + mestranol (1·0 mg/kg)	1 : 1	156·1 ± 9·0	*
Lynestrenol alone (5 mg/kg)	—	32·4 ± 4·0	*
Mestranol alone (1 mg/kg)	—	215 ± 18	*

Pentobarbitone (50 mg/kg) was injected intravenously 18 hr after the last of four daily subcutaneous injection of steroid or vehicle (5·0 ml./kg). * Significant difference from vehicle controls ($P = < 0{\cdot}05$).

mice for periods up to 20 days and their effects persisted. It may be significant that the optimal duration of pretreatment with mestranol or lynestrenol is approximately the same as the oestrus cycle in the mouse, namely four days.

Effects of SKF 525A on the duration of pentobarbitone sleep in mice pretreated with mestranol or lynestrenol

SKF 525A is an inhibitor of hepatic microsomal enzymes, and as such is known to interfere with the metabolism of certain barbiturates (Axelrod, Reichenthal & Brodie, 1954). It was of interest, therefore, to examine its effects in mice pretreated with mestranol or lynestrenol. Mice were pretreated for 4 days with mestranol (0·5 mg/kg) or lynestrenol (5·0 mg/kg); on the fifth day, each mouse received an intraperitoneal injection of SKF 525A (50 mg/kg) 90 min before an intravenous injection of pentobarbitone (35 mg/kg). The duration of pentobarbitone sleep in these mice is recorded in Table 3.

SKF 525A increased the duration of pentobarbitone sleep in all mice, irrespective of whether they were steroid-pretreated or controls. Thus, the effects of lynestrenol were largely overcome, whilst those of mestranol were greatly potentiated. In fact, the duration of sleep in mice pretreated with both mestranol and SKF 525A was far greater than was expected from a simple combination of the effects of the two drugs, and it is suggested, therefore, that the potentiation of barbiturate sleep induced by mestranol and SKF 525A is by different mechanisms and/or at different sites.

Plasma levels of pentobarbitone in mice pretreated with lynestrenol, mestranol or SKF 525A

Female mice were pretreated with lynestrenol (5·0 mg/kg) or mestranol (0·5 mg/kg) for 4 days, and pentobarbitone administered intravenously on the fifth day. Alternatively, mice received SKF 525A (50 mg/kg) intraperitoneally 90 min before the barbiturate. The mice were decapitated immediately or at various intervals up to 90 min after the barbiturate injection. Plasma from groups of four mice was pooled, and assayed for pentobarbitone. The results from three experiments were averaged and are summarized in Fig. 2.

Each line is approximately linear from 10 min onwards when the log. of plasma barbiturate levels is plotted as a function of time; the slope of each line was examined by regression analysis and the significance of difference calculated.

TABLE 3. *Duration of pentobarbitone sleep in female mice pretreated with mestranol or lynestrenol, plus SKF 525A*

Pretreatment	Pentobarbitone sleeping-time, group mean (min±S.E.) after: No further treatment	SKF 525A
Vehicle only for 4 days (controls)	51·4±8·0	260±22
Mestranol (0·5 mg/kg) daily for 4 days	124 ±6·2 *	approx. 600 *
Lynestrenol (5·0 mg/kg) daily for 4 days	15·0±3·1 *	117±24 *

Pentobarbitone (35 mg/kg intravenously) was administered on the fifth day of experiment, 18 hr after the fourth injection of steroid, and 90 min after an injection of SKF 525A (50 mg/kg) intraperitoneally. * Value significantly different from its relevant control ($P = <0·05$).

Mestranol and SKF 525A caused a significant reduction in the rate at which pentobarbitone disappeared from mouse plasma, whilst lynestrenol caused a significant increase in this disappearance.

Possible involvement of body temperature changes as an explanation of altered rates of barbiturate metabolism in steroid pretreated mice

It was frequently observed that mice pretreated with lynestrenol had slightly elevated oesophageal temperatures, whilst animals pretreated with mestranol were slightly hypothermic. It is well known that small changes in deep body temperature

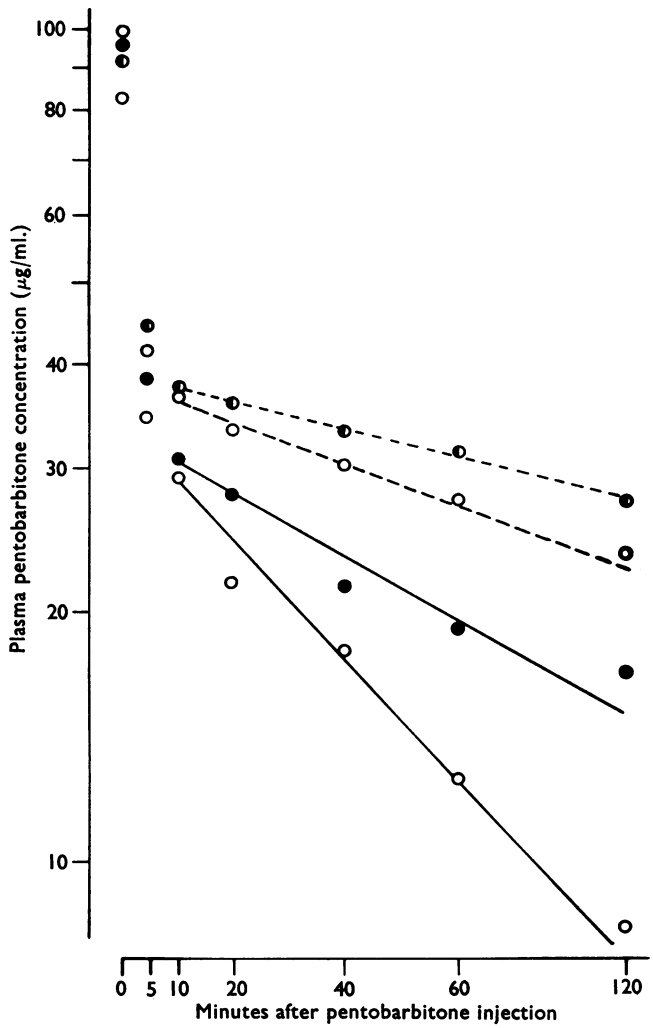

FIG. 2. Effects of pretreatment with mestranol, lynestrenol or SKF 525A on plasma pentobarbitone levels in female mice at various intervals after its injection. Animals were pretreated as follows: mestranol, 0·5 mg/kg subcutaneously, for 4 days (○- - - -○); or lynestrenol, 5·0 mg/kg subcutaneously for 4 days (○——○; or SKF 525A, 50 mg/kg intraperitoneally, once only 90 min before the barbiturate (◐ - - - - ◐) or the vehicles only (controls) (●——●).

are associated with marked alterations in the rate of metabolism of certain drugs (see, for example, Fuhrman & Fuhrman, 1961), and it was possible, therefore, that the changes observed in this study might be due to alterations in body temperature.

The effects of pentobarbitone (50 mg/kg intravenously) were determined in control mice, and in mice pretreated with mestranol (0·5 mg/kg) or lynestrenol (5·0 mg/kg) for 4 days, oesophageal temperatures being measured by the method of Brittain & Spencer (1964) before and after administration of pentobarbitone. The results are recorded in Fig. 3.

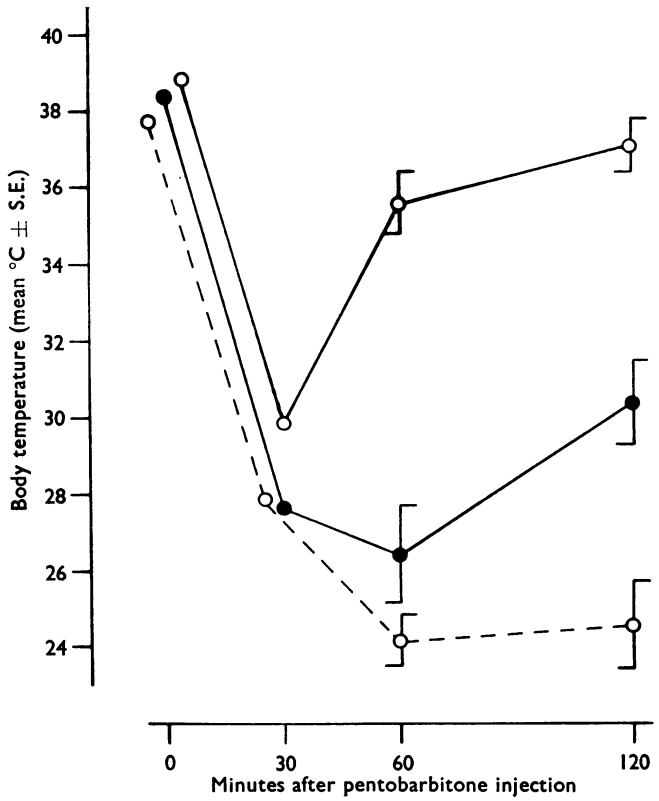

FIG. 3. Effects of pentobarbitone on the oesophageal temperatures of female mice pretreated with mestranol or lynestrenol. Animals were injected subcutaneously with mestranol 0·5 mg/kg for 4 days (○- - - -○); or lynestrenol 5·0 mg/kg for 4 days (○——○); or the vehicle only (controls) (●——●).

TABLE 4. *Duration of pentobarbitone sleep in mestranol-pretreated and lynestrenol-pretreated female mice maintained at a laboratory temperature of* 22° *C or* 30° *C*

Pretreatment	Pentobarbitone sleeping-time, group mean (min±S.E.) at: 22° C	30° C
Vehicle only daily for 4 days (controls)	98·7±9·2	24·9±1·6
Mestranol (0·5 mg/kg) daily for 4 days	237·7±19·7 *	40·7±2·7 *
Lynestrenol (5·0 mg/kg) daily for 4 days	37·6± 3·4 *	24·9±1·3

Pentobarbitone (50 mg/kg intravenously) was administered on the fifth day of experiment, 18 hr after the fourth pretreatment injection of steroid. * Value significantly different from its relevant control ($P<0·05$).

Over a period of 120 min, pentobarbitone induced marked falls in the body temperature of mice ; this hypothermia was slightly more marked in animals pretreated with mestranol, whilst lynestrenol-pretreated mice became significantly less hypothermic than with either of the other two groups. It is not possible from this experiment alone to determine whether the different degrees of hypothermia were responsible for differences in duration of pentobarbitone effect, or vice versa. Therefore, a study of sleeping times was carried out at an elevated room temperature, namely a laboratory environment of 30° C instead of 22° C. A comparison of the effects of pentobarbitone at these two temperatures is summarized in Table 4.

At 30° C pentobarbitone failed to induce significant hypothermia in any of the groups of mice, irrespective of their pretreatment. Examination of Table 4 shows that under these conditions, the sleeping time of controls is only one quarter that of controls allowed to become hypothermic. Similarly, sleeping-time in mestranol pretreated mice is reduced from nearly 4 hr (at 22° C) to 40 min when hypothermia is prevented. With lynestrenol pretreated animals, there was a slight reduction in the already short sleeping-time, but now the duration of sleep was identical with that of the controls. Despite the marked reduction in the degree of potentiation, mestranol still produced a significantly longer sleeping-time under the raised environmental temperature than that seen in the controls.

Discussion

The data presented in this report shows clearly that in mice mestranol and lynestrenol, two synthetic female sex hormones with respectively oestrogenic and progestogenic activity (*World Health Technical Report*, 1965), greatly altered the duration of action of pentobarbitone and hexobarbitone, but not of barbitone. These effects occurred in both female and male mice, were dose-related, and were persistent, their effects continuing for as long as their administration was continued. Yet, peak effects with either steroid were produced after only 4 days of pretreatment, a period approximately equal to the mouse's oestrus cycle. Pilot experiments were also carried out with another oestrogen (ethinyloestradiol) and other progestins (norethynodrel, megestrol acetate and norethisterone acetate) ; in each case, the oestrogen was associated with a prolongation, whilst the progestins were associated with a reduction, of the pentobarbitone effect. The effects of a combination of oestrogen with progestin were also studied: thus, when a number of oestrogen/progestin combinations were examined, their effects were determined by the ratio of mestranol to lynestrenol ; at a ratio of 1:5, the effects of both steroids effectively cancelled out one another.

There are several explanations of these steroid-induced changes in the sensitivity of the mouse to certain barbiturates ; there may be changes in the inherent sensitivity of the cerebrospinal axis to these drugs ; or, there may be changes in the tissue distribution or plasma binding of the barbiturates ; renal handling of the barbiturates may be affected ; or finally there may be changes in the ability of the mouse to metabolize barbiturates. The inability of oestrogens or progestins to alter the sensitivity of the mouse to barbitone largely excludes the first three possibilities, since the modes of action and physio-chemical properties of the three barbiturates studied are qualitatively the same. One area in which they are known to differ is in their susceptibilities to biotransformation ; barbitone remains largely un-metabolized in

most mammalian species (Maynert & van Dyke, 1950), so that little or no change in the effects of barbitone would be anticipated from a quantitative change of the rate of hepatic metabolism. Previous workers' findings confirm this conclusion: thus, long-term treatment of mice with testosterone shortened the duration of hexobarbitone but not that of barbitone hypnosis (Gessner, Acara, Baker & Edelman, 1967). The same workers observed that oestrogens prolonged the action of hexobarbitone, a drug known to be extensively metabolized by the liver in both male and female animals (Quinn, Axelrod & Brodie, 1954).

The actions of the microsomal enzyme inhibitor, SKF 525A, on pentobarbitone hypnosis were also studied. Whilst the effects of lynestrenol were completely reversed by SKF 525A, those of mestranol were greatly prolonged. The prolongation of pentobarbitone by a combination of mestranol plus SKF 525A was far greater than was predicted from a summation of their individual effects, and also far greater than that achieved with the largest doses of mestranol used in the earlier dose-response experiment. It appears, therefore, that the loci and/or mechanisms by which mestranol and SKF 525A inhibit pentobarbitone metabolism are different, despite the view expressed by Kuntzman, Jacobson, Schneidman & Conney (1964) that the barbiturate hexobarbitone and the hormones oestradiol-17 β and testosterone may be metabolized by a common hepatic microsomal-located enzyme system, which would be presumably susceptible to SKF 525A.

A study of plasma pentobarbitone levels supports the conclusion that these steroids, like SKF 525A, alter the rate of metabolism of pentobarbitone. Noordhoek (1968) stated that the elimination of a barbiturate, (hexobarbitone in his study), proceeds essentially by diffusion into tissues, by liver metabolism and renal excretion. A similar picture can be assumed for pentobarbitone. In this study, not only the controls, but also animals pretreated with mestranol, lynestrenol or SKF 525A exhibited an approximately linear relationship between the logarithm of plasma pentobarbitone concentration and time, from about 10 min after injection. This suggests that diffusion processes from plasma into other compartments had ceased by this time (Dost, 1953), and that only hepatic and renal sites of removal would remain. Little is known about the renal handling of barbiturates. In the dog, excretion of unchanged hexobarbitone is negligible (Bush, Butler & Dickison, 1953), whilst barbitone is known to be re-absorbed in the renal tubule (Giotti & Maynert, 1951). It is concluded that an effect on hepatic metabolism, admittedly at perhaps different sites, is a common explanation for the changes in plasma clearance of pentobarbitone seen in animals pretreated with oestrogen, progestin or SKF 525A.

There may be one further explanation of the altered rates of metabolism observed in steroid-pretreated mice. It is well known that changes in the ambient temperature, and more specifically in an animal's core temperature, can markedly alter the rate of metabolism of certain drugs (see, for example, Fuhrman & Fuhrman, 1961 ; Morris, 1963). Specifically, Morris (1963) observed that the duration of pentobarbitone sleep was reduced whilst that of barbitone sleep was unaffected, when the ambient temperature was raised 10° C ; although no direct measurement of body temperature was made in that study, the mice were taken well above their critical temperature so that at least hypothermia would have been prevented. As well as sleep, but perhaps a consequence of it, pentobarbitone and other medium-to-long-acting barbiturates may produce marked falls in body temperature, and it is likely such a fall in body temperature will drastically affect the rate of metabolism of the

pentobarbitone. It has also been observed that progestin raises whilst oestrogen depresses the human body temperature (Israel & Schneller, 1950), and in this present study slight, though statistically not significant, falls and rises in resting body temperatures were observed in mice given only mestranol and lynestrenol. It was, therefore, of interest to see whether or not pentobarbitone sleep was accompanied by greater or smaller degrees of hypothermia in steroid-pretreated mice. The speed of onset of hypothermia was not affected by steroid pretreatment, but the final depth and duration of hypothermia were significantly altered (see Fig. 2), suggesting that the actual duration of pentobarbitone sleep might be significantly affected by the hypothermia. To test this hypothesis, the experiment was repeated at a laboratory temperature of 30° C instead of the usual 22° C. At this temperature, pentobarbitone did not induce hypothermia, and control animals slept for only a short period of time which was identical to that of lynestrenol-pretreated mice. The effects of mestranol were also greatly reduced although there was still a significantly longer sleeping-time in these animals than in the controls. In some way, perhaps by a reduction in basal metabolic rate, mestranol increases the duration of pentobarbitone hypothermia in mice, but this does not completely explain the observed reduction in the rate of pentobarbitone metabolism observed in mestranol-pretreated mice, and it seems likely that some direct effect upon the liver is involved.

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