SLEEPING TIME RESPONSES OF MICE—RANDOM BRED, INBRED AND F_1 HYBRIDS—TO PENTOBARBITONE SODIUM

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The sensitivity and the precision of the sleeping time responses to pentobarbitone sodium in 10 per cent ethanol in mice varies with the strain of mouse. In any one strain sleeping time response in animals dosed by weight with pentobarbitone sodium is related to the weight of the animals. As mice aged, the sleeping time for all strains differed between the sexes, the males sleeping longer than the females. If the same mice were reinjected with pentobarbitone sodium the ageing effect was accelerated. It has been confirmed that the responses of F_1 hybrids must be determined experimentally, and may resemble that of either parent, or fall between or outside those of their parents. In choosing animals for the estimation of pharmacological responses, therefore, their strain, weight, age and sex are each of importance, and should be as controlled as their environment before and during tests.

WORK on sleeping time responses in mice was undertaken to investigate some practical questions that arise in pharmacological assay. It had been asked whether when choosing animals for use in the assay of pharmacological responses similar age or similar weight was the more important consideration. It was hoped that the study of one particular reaction in random bred, inbred and hybrid mice using mice of known age, sex and weight might throw further light on the relationship between these variables and indicate which, if any, is the most important.

MATERIAL AND METHODS

Pentobarbitone sodium was chosen as the sleeping drug for it is the hypnotic of choice when long sleep in animals is required. I have followed a usual convention of describing the characteristic central depression which follows anaesthetic doses of this drug as "sleeping time". It was freshly dissolved for each test at a concentration of 5 mg./ml. in normal physiological saline containing 10 per cent ethanol. For the preliminary tests in random bred P/LAC mice a single dose of 60 mg./kg. was used while in all other experiments sleeping times produced by 55 and 66 mg./kg. mouse were measured. The injections were intraperitoneal.

The test animals were bred at the Laboratory Animals Centre and issued soon after weaning so that mice aged 5 weeks $\pm$ 3½ days were available for the first experiments. They were housed not more than five in each metal box 14 × 6 × 4½ in. in size and were fed on diet 41B *ad lib.* from hoppers. They were watered from plastic water bottles with metal drinking tubes and kept in a room thermostatically controlled at 70° F. $\pm$ 2°. For every experiment equal numbers of male and female mice, strictly segregated, were distributed by random numbers into the mouse boxes and usually 10 of each sex were used at each dose of pentobarbitone

TABLE I

RESULTS OF ANALYSIS SHOWING THE EFFECTS OF SEX ON THE WEIGHTS AND ON THE SLEEPING TIMES OF P/LAC MICE INJECTED ON EACH OCCASION WITH 60 MG./KG. OF PENTOBARBITONE SODIUM IN SALINE CONTAINING 10 PER CENT OF ETHANOL

Age	Mice: Normal M	Mice: Normal F	Mice: Treated M	Mice: Treated F	No. of previous treatments	Results of analysis of variance showing sex differences: Between mean weights M>F	Between mean weights P	Between mean log of sleeping times M>F	Between mean log of sleeping times P	Differences of sleeping time adjusted for body weight: M>F	Differences of sleeping time adjusted for body weight: P
5 weeks	28	30			0	−	>0·25	−	>0·25	−	0·6–0·7
6 weeks	18	20			0	++	<0·01	−	>0·25	−	ca. 0·5
			28	29	1	+	<0·05	++	<0·01	+++	<0·001
8 weeks	10	10			0	+++	<0·001	+	ca. 0·02	++	ca. 0·01
			10	10	2	+++	<0·001	+++	<0·001	+++	<0·001
12 weeks	10	10			0	+++	<0·001	−	0·1	++	ca. 0·01
			10	10	3	+++	<0·001	+++	<0·001	+++	<0·001

M = male. F = female.

sodium, or a total of 40 mice per strain for any one test. The mice were ear punched for individual identification. Experimental sleeping time was measured as the time between the animals receiving their injection and that time when they were able to right themselves when placed on their backs. Analyses were made with logarithms of these times and of the weights of the animals used. The possibility of using the reciprocals, the squares or square roots as functions of time in preference to the logarithms was investigated and rejected. A constant temperature sleeping tray was not used, but a tray lined with foam rubber which conveniently took 120 mice and shielded them from draughts was found satisfactory for the comparison of three strains of mice on one day. Repeat sleeping tests were made at intervals of two weeks or longer and not more than four tests were made on any one set of mice. As the temperature was not controlled in the experimental laboratory, comparison could not be made between the absolute values of sleeping times obtained on different days. Mice which died as the result of repeated injections or for any unknown cause were not replaced and unless otherwise stated all analyses were made on the actual numbers used.

RESULTS

The effect of Sex and Body Weight in Random Bred P/LAC Mice on Sleeping Time Responses to Pentobarbitone Sodium

The results obtained in these experiments were analysed as shown in Table I. Preliminary work on the sleeping times of the five week old mice, 28 males and 30 females, had shown that for male mice the correlation coefficient of the logarithms of weight and sleeping times was −0·525, while for female mice it was −0·616, and for five week old mice irrespective of sex −0·564. It therefore seemed reasonable not only to analyse results for the variance between sex, weight (or log weight) and log sleeping time but also to determine the variance between the mean logarithms of the sleeping time of each group of mice adjusted by co-variance for weight. Inspection of Table I shows the advantage of the dual analysis in that differences which appear on analysis of variance are confirmed and become more definite with co-variance analysis. A difference between male and female mean sleeping time arises with age and the ageing effect is accelerated in mice that have received more than one injection.

The Homogeneity of the Variance of the Sleeping Time Response of Groups of Mice including Inbred, Hybrid and Random Bred Strains

In order to determine differences between sleeping time responses for groups of mice by analyses of variance and co-variance it was necessary to determine by Bartlett's test whether the variance of the responses of the groups of mice used was homogeneous. The results obtained indicated much heteroscedasticity within some of the nine groups of mice used. However, the control strain A2G has a large degree of within strain homogeneity of variance at the usual level of significance $P = 0{\cdot}05$, while one

TABLE II

RESULTS OF THE ANALYSIS OF VARIANCE SHOWING THE EFFECTS OF THE SEX AND STRAIN OF MICE AND THE DOSE OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL ON THEIR SLEEPING TIME RESPONSES

GROUPS	Strains M & F	Age in weeks	Significant differences P = 0·001 = +++ Sex	Strain
1	A2G, A	5	——	+++
		8	+++	+++
		11	+++	——
		14	+++	——
3	A2G, A	5	——	——
2	A2G, CBA, LAC Grey ..	5	——	+++
		8	+++	+++
		11	+++	+++
6	A2G, BrCF	5	——	+++
		8	+++	+++
		12	+++	+++
7	A2G, A2DB/1F$_1$	5	——	——
		9	+++	——
		13	+++	——

GROUPS	Strains M only	Age in weeks	Significant differences P = 0·001 = +++ Strain
4	A2G, C57BL, C57Br/cd ..	5	+++
		7	+++
		9	+++
		11	+++
5	A2G, C3H	5	——
		7	+++
		10	——
		13	——
7	A2G, DBA/1, A2DB/1, F$_1$ ..	5	——
		9	——
		13	+++
8	A2G, DB/1A2F$_1$	5	——
		12	+++

TABLE III

MEAN SLEEPING TIMES OF MICE y ADJUSTED FOR THE WEIGHTS OF THE ANIMALS AT TWO DOSES OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL RESPECTIVELY 66 AND 55 MG./KG. MOUSE; ALSO b THE SLOPES OF THE REGRESSION BETWEEN RESPONSE AND DOSE; AND THE PRECISION OF THE RESULTS MEASURED BY λ. GROUPS CORRESPOND WITH THOSE IN TABLE II

Test	Group 1	A2G y	A2G b	A2G λ	A y	A b	A λ
1	M	2·223 1·956	0·256	0·277	2·124 1·856	0·257	0·244
	F	2·243 2·047	0·188	0·507	2·068 1·902	0·159	0·664
2	M	2·247 2·082	0·159	0·436	2·200 1·912	0·181	0·598
	F	2·147 2·059	0·085	0·955	2·102 1·878	0·215	0·354
3	M	2·306 2·126	0·173	0·439	2·320 2·075	0·235	0·415
	F	2·168 1·997	0·164	0·317	2·198 1·823	0·360	0·272
4	M	2·274 2·134	0·134	0·629	2·366 2·072	0·282	0·290
	F	2·138 1·985	0·147	0·551	2·170 2·072	0·094	1·126

Test	Group 2	A2G y	A2G b	A2G λ	CBA y	CBA b	CBA λ	LAC Grey y	LAC Grey b	LAC Grey λ
1	M	2·224 2·081	0·137	0·583	2·232 2·047	0·178	0·295	2·002 1·852	0·144	0·700
	F	2·188 1·994	0·186	0·494	2·172 1·978	0·186	0·248	2·069 1·978	0·087	1·059
2	M	2·182 2·017	0·159	0·470	2·296 2·108	0·181	0·219	2·063 1·949	0·110	0·806
	F	2·051 1·867	0·177	0·280	2·037 1·875	0·156	0·484	1·994 1·831	0·157	0·606
3	M	2·229 2·051	0·171	0·406	2·244 2·112	0·127	0·561	2·170 1·910	0·250	0·425
	F	2·175 1·904	0·260	0·323	2·098 1·940	0·152	0·387	2·031 1·878	0·147	0·637

Test	Group 3	A2G y	A2G b	A2G λ	A y	A b	A λ
1	M	2·167 1·981	0·179	0·397	2·075 1·858	0·208	0·546
	F	2·075 1·877	0·190	0·569	1·981 1·762	0·210	0·612

Test	Group 6	A2G y	A2G b	A2G λ	$BrCF_1$ y	$BrCF_1$ b	$BrCF_1$ λ
1	M	2·353 2·158	0·187	0·356	2·129 2·035	0·090	1·504
	F	2·308 2·048	0·250	0·323	2·227 1·952	0·264	0·472
2	M	2·289 2·146	0·137	0·820	2·191 2·079	0·108	0·646
	F	2·260 1·919	0·328	0·213	2·072 1·835	0·228	0·437
3	M	2·275 2·116	0·153	0·496	2·364 2·116	0·238	0·247
	F	2·218 1·958	0·250	0·349	1·972 1·748	0·215	0·426

Test	Group 7	A2G y	A2G b	A2G λ	$A2DB/1F_1$ y	$A2DB/1F_1$ b	$A2DB/1F_1$ λ
1	M	2·268 2·132	0·131	0·619	2·271 2·040	0·222	0·596
	F	2·397 2·172	0·216	0·305	2·274 2·114	0·154	0·495
2	M	2·246 2·106	0·134	1·005	2·281 2·024	0·247	0·143
	F	2·160 1·958	0·204	0·510	2·081 1·833	0·239	0·427
3	M	2·297 2·168	0·124	0·944	2·376 2·088	0·277	0·287 0·425
	F	2·152 2·004	0·142	0·700	2·061 1·820	0·232	

whole group and portions of seven other groups were sufficiently homoscedastic to be analysed. Only those differences significant at a probability level of 0·001 were allowed.

The Effects on the Sleeping Time Responses of the Dose of Pentobarbitone Sodium and the Sex and Strain of Mice as Determined for Inbred, Hybrid and Random Bred Mice

The results of the analyses of variance showed that there was a significant difference between the sleeping times resulting from doses of respectively 55 and 66 mg. of pentobarbitone sodium per kg. mouse for all strains of mice used.

TABLE IV

VALUES OF y, b AND λ FOR THE MALES OF GROUPS 5, 6, 8 AND 9, WITH 66 AND 55 MG./KG. OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL

		A2G			C57BL			C57Br/cd		
Test	Group 4	y	b	λ	y	b	λ	y	b	λ
1	M	2·354 2·058	0·284	0·278	2·183 1·990	0·185	0·788	2·077 1·784	0·281	0·406
2	M	2·246 2·106	0·134	0·586	2·175 2·007	0·161	0·679	2·016 1·863	0·147	0·739
3	M	2·249 2·120	0·124	0·468	2·081 1·951	0·125	0·993	2·151 1·985	0·159	0·277
4	M	2·286 2·095	0·183	0·374	2·135 1·990	0·139	0·779	2·196 2·017	0·172	0·360
		A2G			C3H					
	Group 5	y	b	λ	y	b	λ			
1	M	2·358 2·151	0·199	0·347	2·221 1·976	0·235	0·544			
2	M	2·396 2·134	0·252	0·172	2·202 1·978	0·215	0·414			
3	M	2·329 2·141	0·176	0·439	2·331 2·152	0·172	0·540			
4	M	2·273 2·012	0·251	0·107	2·382 2·071	0·299	0·416			
		A2G			DBA/1			A2DB/1F$_1$		
	Group 7	y	b	λ	y	b	λ	y	b	λ
1	M	2·270 2·134	0·131	0·619	2·217 2·006	0·203	0·508	2·275 2·044	0·222	0·596
2	M	2·225 2·092	0·128	1·058	2·259 1·940	0·306	0·361	2·263 1·905	0·344	0·325
3	M	2·254 2·146	0·104	1·129	2·193 1·920	0·262	0·488	2·330 2·097	0·224	0·354
		A2G			DBA2F$_1$					
	Group 8	y	b	λ	y	b	λ			
1	M	2·246 2·018	0·219	0·533	2·323 1·881	0·425	0·331			
3	M	2·228 2·048	0·173	0·448	2·313 2·146	0·160	0·409			

The effect of the age and re-injection of the mice is shown in Table II, indicating that with immature mice there is no difference between the reaction of the sexes while with re-injected older mice there is always a difference. Adult males have a greater response to pentobarbitone sodium dosage than adult females whatever the strain of mice. Definite strain differences in sensitivity to pentobarbitone sodium were found in groups 2, 4 and 6, and are also indicated in Table II.

The analyses of co-variance that were made on these groups confirmed the sex and dose differences and elucidated the strain differences. The strains LAC grey, $BrCF_1$, C57BL and C57Br/cd showed less response to pentobarbitone sodium consistently than the A2G strain, as in some tests did strains A and DBA/1. It is therefore clear that the control strain A2G is one of the more sensitive strains.

The Effect of the Strain of Mouse used on the Precision of the Sleeping Response to Two Doses of Pentobarbitone Sodium

Preliminary work indicated a straight line regression between the logarithms of the sleeping time response and the logarithms of three pentobarbitone sodium doses covering the range from 55 to 66 mg./kg. mouse in A2G and C3H males. It was therefore decided to use the index of precision $\lambda = \frac{\text{standard deviation}}{\text{slope of regression}}$ to determine strain differences.

Table III and IV give the sleeping time responses adjusted for the weights of the animals, the slopes of the regression lines, and the index of precision for some of the strains used. For those strains where both male and female mice are considered, only the LAC greys differ consistently from the A2G strain and these are less precise. Where males only are considered C57BL and C3H strains are consistently less precise, and DBA/1 and the $A2DB/1F_1$, strains more precise than the A2G strain.

Discussion

It will be necessary in discussing the responses of mice to injections of pentobarbitone sodium in 10 per cent ethanol that the homogeneity of the variances of the responses of those animals receiving injections should be considered as well as the effects of weights, age, sex and strain of the mice on the response and whether the reaction varies with the dose of pentobarbitone sodium which they receive.

The heteroscedasticity of the groups of mice was such that to make valid deductions from the various analyses, significant difference was defined at a level of probability of 0·001. The possibility of reducing this variance error by using litter mates (Mandl, 1955) or larger groups of mice was found impracticable for inbred strains.

In primarily injected random bred P/LAC mice of similar age the response of the mice and the logarithms of their weights was frequently related. The effect of the weight of mice used on the slope of the dose-response curve, was shown by Young and Stewart (1952) in an extensive analysis of a series of insulin tests in mice. The relation between the

weights of the animals and the response expected cannot be ignored, therefore, even when animals of similar age are used in any one test.

The importance of age as related to sex was found for all mice tested and has previously been noted in the tolerance of rats to barbiturates. Holck, Karan Mills and Smith (1937) found adult female rats to be more sensitive to barbiturates in contrast to the greater sensitivity of male mice. This work was extended by Homberger, Etsten and Himwich (1947), Cameron, Cooray and De (1948), also Brodie (1956) and Edgren (1957). The difference in reaction in rats has been shown to be partially sex hormone dependent, which explains its relation to age.

In work with hexabarbitone in mice, Jay, Jnr. (1958) and Brodie (1956) obtained results which stressed differences in strain responses but did not appear to find marked sex differences. Jay also found that the relation between the responses obtained in different strains can alter with dose, or the precision of the response varied with the strain. I found from a study of the index of precision for males and females that the LAC grey strain was less precise than the control, and from a study of males only that C57BL and C3H strains were less precise and DBA/1 and the hybrid A2DB/1F_1 more precise.

Michie (1955), working with pentobarbitone sodium in mice, described variance differences between strains which led him to advocate the use of random bred F_1 hybrid mice in preference to inbred mice for pharmacological work. In some of his work he used one sex only, and if both were used he considered sexes apart in statistical analysis. But he makes no mention of a sex difference in reaction time. In this work I have considered as significant only differences at the 0·001 level of probability. I have found that adult mice for all strains differ in their sleeping time according to their sex. Also the precision of their response is distributed haphazardly between inbred and F_1 hybrid strains, only the F_1 strains, which were out of DBA/1 mice mated to A2G mice, being more precise than the A2G strain. The random bred strain which I studied was less precise. In all tests the variation between strains emphasised by Chance (1957), and due to environment, was eliminated as far as possible by the manner of their similar treatment.

It was shown by Chai (1960) that the response of an F_1 hybrid to hormone may or may not fall between the responses of its parents. Both F_1 crosses out of DBA/1 and A2G strains resembled the DBA/1 strain more nearly than the A2G strain in their responses to pentobarbitone sodium dosage. The ability to forecast the usefulness of any F_1 hybrid for a particular pharmacological assay will depend on prior knowledge of the responses of its parents, but the confirmatory experimental results may be disappointing, as I have shown with insulin assay (Brown, 1961).

The effect on the responses produced by the route of injection of the substance (Bacharach, Clark, McCulloch and Tomich, 1959), has not been studied in this work and the variance of the responses may well have been more homogeneous with other injection routes.

Hypotheses may be postulated to account for the significant differences in strain response occurring at all ages and in sex response differences

apparent in adult animals. These hypotheses will have some bearing on differences due to weights and ages of the animals. Species, strain and sex differences in the metabolism of hexabarbitone have been shown by Quinn, Axelrod and Brodie (1958). If mice that sleep a shorter time catabolise the pentobarbitone sodium more quickly than others this may be due to differences in liver action (Bunsfield, Child, Basil and Tomich, 1960), liver size, or to the absorption of breakdown products by large deposits of fat in the body (Hong and Cho, 1959). These hypotheses may be tested by experiment.

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