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The rapid development of functional tolerance to ethanol by mice

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A method is described in which the development of tolerance to ethanol in individual mice can be measured during the inhalation of ethanol vapour. This method has been used with two behavioural end-points, loss of righting reflex and loss of rotarod performance. It demonstrates that, in the adult male, TO Swiss mouse, peak tolerance, in which approximately 2 × the original effective blood ethanol concentration is required to produce the behavioural end-point, can develop in 3–5 h. After this time the ability of the animals to perform normally in the presence of continued high concentrations of ethanol in blood begins to fall. The results are discussed in relation to current concepts of tolerance to central nervous system depressant drugs.

The phenomenon of functional tolerance to centrally acting drugs, in which an organism becomes able to function relatively normally in the face of progressively higher tissue concentrations of the drug, is of considerable interest. Not only is the condition of functional tolerance often considered a prerequisite for the development of physical dependence (e.g. Mendelson 1971) but functional tolerance, implying as it does adaptation of central neurons, is of theoretical and practical importance in its own right. Consideration of the mechanism by which functional tolerance to ethanol is produced is hampered by confusion in the literature about the time course within which it can occur. Most experiments which use repeated administration of ethanol to man or animals (e.g. Majchrowicz & Hunt 1976) report that functional tolerance to ethanol develops slowly, over a period of days, and that it rarely reaches a level in which more than 2 × the original concentration is required to produce the behavioural deficit. However, there are a few reports, which use single administrations of ethanol to animals and man, which suggest that functional tolerance can develop much more rapidly than this (Mellanby 1919; Goldberg 1943; Hurst & Bagley 1972). The kinds of experiment performed by these workers, raised objections, most of which were removed by the elegant work on rats by Leblanc et al 1975. These authors demonstrated conclusively that, 1 h after acute ethanol administration, i.e. on the descending part of the blood ethanol curve, rats were capable of similar performances on a moving belt test at brain ethanol concentrations 2 × that of rats tested 10 min after ethanol administration i.e. on the ascending curve. Their results strongly suggest that in the intervening period functional tolerance to ethanol had developed.

The methods available for studying rapidly developing functional tolerance to ethanol in animals are limited. The methods of Leblanc et al (1975) which involved decapitation and measurement of brain ethanol concentration were necessary to overcome previous objections, but they are extremely wasteful of animals, and make it impossible to achieve a time course for development of tolerance in individual animals. The first objection has been overcome by a method recently published by Tullis et al (1977) in which tolerance to ethanol-induced jumping deficit in an unconditioned avoidance response test is used. This method has been used to measure 'acute tolerance', the difference between performance on the ascending and descending parts of the blood ethanol curve after a single administration of the drug.

One of the major difficulties in obtaining a detailed time course for the development of functional tolerance in animals is the maintenance of a slowly rising blood ethanol concentration, over a period of hours, with conventional methods of administering ethanol. It seemed to us that the use of ethanol administration by inhalation could overcome these problems; we now describe three variations on a method by which functional tolerance to ethanol may be investigated using inhalation. A preliminary report on some of this work has been communicated to the British Pharmalogical Society (Grieve & Littleton 1978). A method using a similar concept, in which mice receive sequential intraperitoneal injections of ethanol, has also just appeared in preliminary form (Gallaher & Goldstein 1978).

METHODS

Animals and materials

Male mice of the TO Swiss strain (20–25 g) were obtained from A. Tuck, Battlesbridge, Essex. Ethanol AR grade was obtained from James Burroughs Ltd. Mice were freely maintained on Spillers modified 41B diet and water before experiments. During periods in the inhalation chamber (see below) no food or water was provided.

Inhalation chambers

Mice were exposed to ethanol vapour in inhalation chambers as described by Griffiths et al (1974). By varying the flow of air into the chamber relative to the flow of air passing through ethanol into the chamber, concentrations of ethanol vapour within can be maintained at a steady level. The ethanol vapour concentration at various points in the chamber is monitored by removing 1 ml samples for gas chromatographic determination. Ethanol vapour leaks out into the surrounding air through small holes in the sides of the chamber. For these experiments an inhalation chamber was modified by inserting a pair of rubber gloves through airtight ports in the roof and by providing a small door with an airlock in the side (Fig. 1).

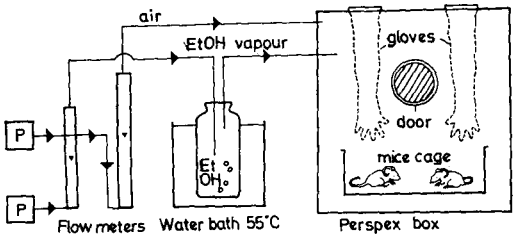

FIG. 1. Ethanol inhalation chamber modified for monitoring the rapid development of cellular tolerance.

Measurement of blood ethanol concentration

The method used routinely for estimation of blood ethanol concentration was to analyse a sample of expired air for its ethanol content (Fig. 2). After intraperitoneal injection there was a direct relationship between expired air ethanol concentration and blood ethanol concentration, as measured by subsequent decapitation of the animal and direct measurement in arterio-venous blood from the neck (see Griffiths et al 1974). The relationship is shown in Fig. 3. This relationship with ethanol in the bloodstream was verified by decapitation and direct measurement in blood on many occasions and there was no discrepancy between the two parameters beyond the level of experimental error shown in Fig. 3 when the precautions described below were observed.

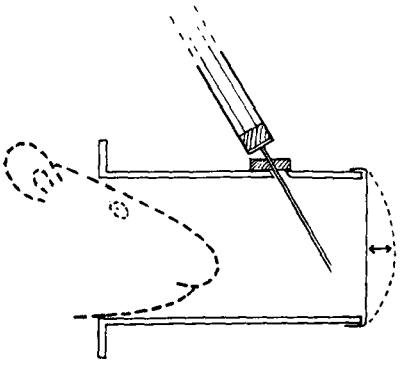

FIG. 2. Rebreathing chamber used for measurement of ethanol in expired air. The mouse is held with its head pressed firmly into the chamber, volume 3 ml, until movements of the diaphragm at the opposite end of the chamber indicate that it is breathing normally and that the system is relatively air-tight. After 30 s a 1 ml sample of air is removed from the chamber via the gas-tight syringe shown protruding through the septum in the upper part of the chamber. The mouse is then removed and the chamber thoroughly flushed with 95% O_2, 5% CO_2 before the next estimation.

When knowledge of blood ethanol concentration is required at a time when the mouse is inhaling high concentrations of ethanol vapour, it was necessary to wait for about 10 min after removal of the animal from the inhalation chamber before the concentration of ethanol in expired air accurately reflected that in blood. Fortunately, since ethanol elimination obeys zero order kinetics, it is possible to extrapolate

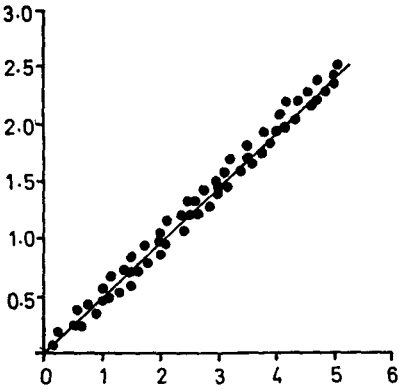

FIG. 3. The relationship between the concentration of ethanol in the expired air and in the blood. The abscissa shows the concentration of ethanol in the blood in mg ml^{-1}; the ordinate the concentration of ethanol in the expired air in mg $litre^{-1}$ min^{-1}.

back from serial estimations made outside the chamber. Fig. 4 illustrates the problem, and demonstrates that elimination of ethanol, as estimated from expired air ethanol concentrations, shows approximately zero order kinetics at a rate similar to that obtained when direct measurements of ethanol in blood are used for the calculation. Elimination of ethanol was investigated frequently throughout these experiments and was always shown approximately to obey zero order kinetics within the range of blood ethanol concentrations found.

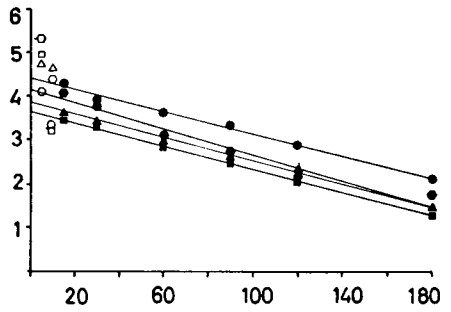

FIG. 4. The concentration of ethanol in the blood (measured by the expired air method) at various times after removal from the inhalation chamber. Different symbols represent results from individual animals. The abscissa shows the time in minutes after removal from the inhalation chamber; the ordinate the concentration of ethanol in the blood in mg ml^{-1}.

Experimental design

Four experiments were performed. In the first (a) mice were placed in an inhalation chamber breathing ethanol vapour (20 mg l^{-1}) for 6 h, and were then removed and compared with control animals for the blood ethanol concentration required to produce loss of righting reflex after ethanol 3·5 g kg^{-1} i.p. In (b) mice were placed in a chamber for 6 h, and then compared with controls that received ethanol 2 g kg^{-1} i.p. to bring them rapidly to the same state of intoxication as the ethanol-exposed group. The behavioural end-point of loss of righting reflex was then achieved by exposing all animals to a high concentration of ethanol vapour (40 mg $litre^{-1}$) in a chamber. Both experiments were performed to determine whether tolerance to ethanol could be demonstrated in grouped mice after they had been inhaling its vapour for 6 h.

The development of cellular tolerance to ethanol in individual mice was investigated by first administering intraperitoneally a priming dose which almost produced the required behavioural end-point (2·5 g kg^{-1} for righting reflex, 1·5 g kg^{-1} for rotarod performance) then exposing the animal to vapour (40 mg $litre^{-1}$) in a chamber until the animal lost either its righting reflex (experiment c) or its ability to remain on a rotating rod (experiment d). As soon as this occurred the animal was removed for estimation of blood ethanol concentration. For this purpose the means of 3 estimations of ethanol in expired air 15 min after removal from the chamber, and 3 estimations 30 min after removal, were extrapolated back to give an estimate of the blood ethanol concentration of the mouse at the time when it first reached the behavioural end-point. If by this time it had regained righting reflex or rotarod performance it was re-introduced into the chamber and the whole procedure repeated. Increasing concentrations of ethanol in blood at which the animal successively reaches the behavioural end-point are considered to represent the development of functional tolerance. An attempt to explain the procedure by showing representative results for a single mouse appears in Fig. 5.

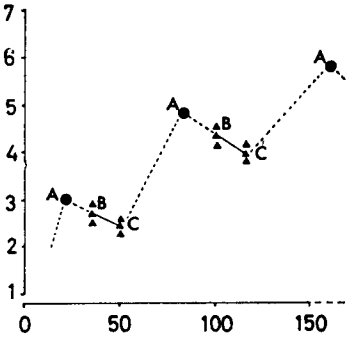

FIG. 5. The development of cellular tolerance to ethanol, representative results for one animal. The concentration of ethanol in the blood was estimated 15 (B) and 30 (C) minutes after removal from the inhalation chamber, by the expired air method. By extrapolation it was possible to obtain the concentration of ethanol in the blood at loss of righting reflex (A). The animal was replaced in the chamber until righting reflex was again lost, and the sequence was repeated. Ths abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1}.

In all these experiments it was attempted to minimize the effects of practice during the experiments on performance by testing animals on several occasions before the experiment began. Testing for loss of righting reflex was by repeatedly placing the animal on its back. Inability to right fully within 30s was used as the end-point. Animals that partially righted themselves were immediately re-tested, if they were unable to right fully on this second occasion they were considered to have reached the end-point. For

loss of rotarod balance mice had to fall from a 2 cm diam. rod, covered in surgical tape, rotating at 0·5 Hz, 30 cm above a sawdust covered cage floor, within 15 s of being placed on the rod.

RESULTS

Experiment (a) Control mice that received ethanol 3·5 g kg^{-1} i.p. without prior exposure lost their righting reflex at an estimated blood concentration of 4·61 ± 0·33 mg ml^{-1} (mean ± s.e., n = 6). Mice that had previously been exposed to ethanol vapour 20 mg litre^{-1} for 6 h lost the righting reflex at an estimated blood ethanol concentration of 7·81 ± 0·32 mg ml^{-1} (mean ± s.e., n = 6) on challenge with ethanol 3·5 g kg^{-1} i.p. Prior exposure to ethanol for 6 h therefore resulted in a significant (P <0·01 in Student's t-test) increase in the concentration of ethanol in blood required to produce loss of righting reflex.

Experiment (b) Control mice, not previously exposed to ethanol, which received a priming dose (2 g kg^{-1} i.p.) followed by exposure to a high concentration (40 mg litre^{-1}) in the modified chamber, lost the righting reflex at an estimated blood concentration of ethanol of 3·68 ± 0·10 mg ml^{-1} (mean ± s.e., n = 4). Mice previously exposed to 20 mg litre^{-1} for 6 h and then exposed to 40 mg litre^{-1} in the modified chamber lost the reflex at an estimated blood ethanol concentration of 5·23 ± 0·23 mg ml^{-1} (mean ± s.e., n = 4). Previous exposure to ethanol therefore results in a significantly higher estimated concentration of ethanol in blood at which the righting reflex is lost (P <0·01 in Student's t-test). These blood concentrations are lower than those estimated in (a) probably because they are reached after ethanol inhalation. Under these conditions blood ethanol rises more slowly and can be more carefully controlled preventing "overshoot" in the measurement at loss of righting reflex.

Experiment (c) mice lost the righting reflex for the first time after 20–60 min in the chamber. The estimated mean blood ethanol concentration at which the righting reflex was first lost was 2·90 ± 0·2 mg ml^{-1} (mean ± s.e., n = 4). Subsequently there was an upward trend in the estimated blood concentrations at loss of righting reflex. This continued until 240–300 min in the chamber when the trend was reversed. The maximum estimated blood concentration at which mice lost their righting reflex was 6·06 ± 0·34 mg ml^{-1} (mean ± s.e., n = 4). Repeated exposure in the chamber therefore results in a significant increase (P<0·01 in Student's t-test) in the estimated blood ethanol concentration at loss of righting reflex. The results are shown graphically in Fig. 6.

For experiment (d), qualitatively similar results were found to those described (see Fig. 7). Mice first lost the ability to remain on the rotarod at an estimated blood ethanol concentration of 1·80 ± 0·2 mg ml^{-1} (mean ± s.e., n = 4). The maximum blood concentration at which rotarod performance

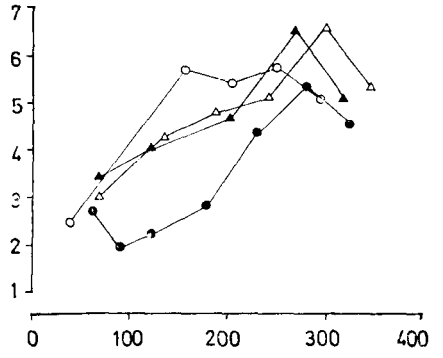

FIG. 6. The development of cellular tolerance to ethanol in TO mice, using an end-point of loss of righting reflex. Connected symbols represent values for an individual mouse. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1}.

was lost after repeated exposure was 3·19 ± 0·34 mg ml^{-1} (mean ± s.e., n = 4). It occurred between 200–320 min after first exposure to ethanol. Once again, the increase is significant at the P< 0·01 level.

In both these experiments the slope of the ethanol elimination curve appeared to change after repeated administration. The slope increased 2–4 h after first exposure to ethanol, and then returned toward the original value after about 6 h. This may be an artifact, in which case the error produced in the extrapolated blood ethanol concentrations is small (an

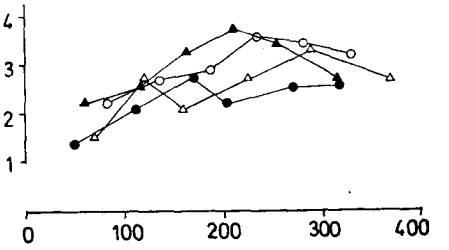

FIG. 7. The development of cellular tolerance to ethanol in TO mice, using an end-point of loss of rotarod balance. Connected symbols represent values for an individual mouse. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concencentration of ethanol in the blood in mg ml^{-1}.

overestimate of about 10%) but there is reason to believe that the change is real (see Discussion).

DISCUSSION

These experiments show that young, male mice of the TO Swiss strain exposed to ethanol by inhalation for a period of hours require higher blood ethanol concentrations to produce the same degree of central nervous system depression than animals not previously exposed to ethanol. This occurs independently of the behavioural end-point used, and independently of whether the final concentration of ethanol is reached by intraperitoneal injection or by inhlation. Experiments on individual mice of this age and strain suggest that the maximum effect is shown within 3–5 h of beginning ethanol administration when 1·5–2 × the originally required concentration in blood is needed to produce loss of righting reflex or rotarod performance.

There are several possible interpretations of these results. The findings could be artifactual, produced by our method of estimating the concentration of ethanol in blood from that in expired air. However, we frequently verified that the concentration in expired air bore a direct relationship to that in mixed arterio-venous blood from the neck on decapitation. Approximately zero order kinetics for ethanol elimination was always found, arguing that estimation by extrapolation is also justified. That similar results were obtained whether the final blood concentration was obtained by injection or inhalation also suggests that our method of measurement is accurate.

Assuming that the findings are not artifactual, they can still be explained in several ways. The increasing concentrations of ethanol required to produce the behavioural end-point in individual mice inhaling ethanol vapour could be regarded as a "learned" compensation for the effects of ethanol. However, this seems unlikely since similar results were obtained in the first experiments where groups of control and ethanol-exposed animals were compared. Here each group was tested approximately the same number of times under the influence of ethanol so that the ethanol-exposed groups did not have a greater opportunity to learn the specific response.

A lag in the disposition of ethanol from plasma to brain during the inhalation of ethanol has been suggested as a possible explanation for these results, and this cannot be fully refuted. It seems unlikely however, since our method for estimation of blood ethanol gives a value which is related to arterio-venous blood from the neck, and one would expect rapid equilibration between ethanol in the blood supply to the brain and the brain itself (Crone 1965). The experiments of Leblanc et al (1975) were designed specifically to meet this point, and they obtained results very similar to ours by measuring ethanol directly in brain tissue. Results that we have obtained subsequently (Grieve & Littleton 1979) which show differences in this phenomenon in mice of different age and strain, suggest strongly that it is not due to some simple barrier to diffusion of ethanol into the brain, or to some depot effect on ethanol disposition. We prefer the explanation that this phenomenon represents the development of functional tolerance to ethanol, in that the brain is able to develop resistance to the depressant effects of ethanol during the time course of these experiments.

These results compare with those previously published on tolerance to ethanol after its acute administration (e.g. Leblanc et al 1975) and with the recent work of Greizerstein (1977) on goldfish and Gallaher & Goldstein (1978) on Swiss Webster mice. Thus Leblanc et al showed that rats have acute tolerance to the two-fold level in about 1 h. Greizerstein (1977) reports that goldfish adapt to high environmental concentration of ethanol in 3 h and Gallaher & Goldstein (1978) find rapid development of ethanol tolerance (to about 1·7 × the original effective level) in Swiss Webster mice using a stationary rod performance test. The last named paper gives results extremely close to those reported here. In our findings, rotarod performance was first lost at a blood ethanol concentration of 1·8 mg ml^{-1} and maximum tolerance was reached between 3 h and 5 h after first exposure to ethanol, at this time, mice lost rotarod performance at an ethanol concentration in blood of 3·2 mg ml^{-1}. In Gallaher and Goldstein's experiments mice were initially able to remain on the rod at 1·8 mg ml^{-1} in the bloodstream, and reached maximum tolerance at 3 h when the end-point was achieved at 3·0 mg ml^{-1} ethanol. Given the small differences in techniques this degree of concurrence is surprising. There seems little doubt that mice derived from the TO Swiss or Swiss Webster strains show development of functional tolerance to ethanol within hours.

There are two incidental observations made during the course of these experiments which are of interest. One is the tendency of functional tolerance to fall during prolonged exposure to high concentrations of ethanol; the other is the observation that ethanol elimination rate initially increased during repeated exposure, but then returned toward normal levels. The reason for the decline in cellular tolerance is

unknown, it may be that exposure to the extremely high tissue concentrations obtained produces permanent cell damage which cannot be overcome, or perhaps the form of cellular adaptation responsible for rapid tolerance cannot be maintained for more than a few hours. The phenomenon is interesting and deserves further investigation. The increase in ethanol elimination rate was at first thought to be an artifact. However, subsequent experiments (in preparation) have established that this is unlikely. The results are supported by the observation of Thurman et al (1979) whose experiments suggest an increase in ethanol oxidation by the isolated perfused rat liver 2·5 h after administration of ethanol. This increase is also relatively short-lived. Clearly, if this change in ethanol elimination rate is real, it may represent another important rapid mechanism for ethanol tolerance.

The results described demonstrate that the mammalian brain seems able to adapt rapidly to the continuous presence of a central depressant drug, ethanol. The results of Maynert & Klingman (1960) suggest that a similarly rapid adaptation can occur to other central depressant drugs, including general anaesthetics and barbiturates. Since cross-tolerance is thought to exist between all these drugs, the mechanism of adaptation to the class of general central depressant drugs may be similar. Hill & Bangham (1975) proposed that adaptation in neuronal cell membrane fluidity may underlie all depressant drug tolerance. In this connection, it is of interest that the alteration by ethanol of synaptosomal phospholipid composition reported from our laboratory (Littleton & John 1977) has a time course which is sufficiently rapid to play some part in the development of functional tolerance. It remains to be seen whether this is coincidental, or whether a causal relationship exists.

Acknowledgement

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