AN INCREASED SENSITIVITY TO ALCOHOL IN RABBITS TREATED WITH A CERTAIN FACTOR CONTAINED IN A MICROSOMAL SUBFRACTION OF THE BRAIN

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Cochin and Kornetsky (1) showed that tolerance to the effects of a single injection of morphine in the rat as measured by the hot plate reaction could be demonstrated for periods up to one year following that single injection. Thus, they postulated that tolerance to narcotic drugs might well be an immune phenomenon or a mechanism resembling the antigen-antibody reaction.

Later, Kornetsky and Kiplinger (2, 3) attempted a passive transfer of a hypothetical factor of tolerance in serum from morphine-tolerant animals (rat, dog, monkey and man) to nontolerant animals (mouse). Although they were unsuccessful in transferring the tolerance, they succeeded in demonstrating the presence of certain transferable factors in the serum of morphine-tolerant animals, since under condition of those studies, potentiation of the depressant and an analgesic action of morphine was observed. The role of the potentiating substance in the serum of the morphine-tolerant animal and the mechanism by which it potentiates morphine are as yet unknown.

On the other hand, Kornetsky and Cochin (4) reported that serum from morphine-tolerant rabbits attenuated morphine analgesia in mice and they assumed that the immune mechanisms involved may partially account for some of the phenomena associated with tolerance to morphine. Ungar and Cohen (5) also showed that administration of extracts of the brain taken from morphine-tolerant rats and dogs conferred tolerance on mice.

The presence of the factors in the blood of morphine-tolerant animals that have attenuating or enhancing effects on morphine in nontolerant animals is an interesting finding, although the immune reaction hypothesis is no more than speculation.

During a study of the effect of the treatment with a homogenate of the brain from alcohol-tolerant rabbits, on the duration of alcohol anesthesia following the intravenous injection of a test dose of alcohol, it was noted that an increased sensitivity and/or a decreased or a biphasic change in the sensitivity to alcohol was observed depending on the individual rabbit.

A preliminary report was presented at a meeting of the Japanese Society for Pharmacology, Kyoto (Folia pharmac. jap. 62, 22§, 1966).

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Based on these conflicting evidences the present investigation was undertaken to define the change of sensitivity to alcohol using the normal rabbit as the recipient and the donor species.

**METHODS**

1) **Preparation of primary fractions from the brain**

A total of 109 rabbits was used as the donor animals. The donors were stunned by a blow to the back of the head. The entire brain was rapidly removed, rinsed, weighed and homogenized for one minute in 9 volumes of 0.32 M sucrose (pH 6.3), physiological saline solution or 0.1 M phosphate buffer (pH 6.0), using a Waring Blender.

The homogenate was centrifuged at 10,000×g for 30 minutes to cause sedimentation of the nuclear and mitochondrial fractions (P₁). The supernatant fluid (S₁) was carefully decanted and then centrifuged again at 78,000×g for 60 minutes in a Hitachi ultracentrifuge. The precipitate was designated as the microsomal fraction I (P₂). The 78,000×g supernatant was further centrifuged at 104,000×g for 60 minutes and separated into the microsomal fraction II (P₃) and the final supernatant (S₂). All centrifugations were carried out at -4°C.

Each sediment (P₁, P₂, P₃) was resuspended in a physiological saline solution. The resuspended sediments and the final supernatant (S₂) were stored overnight at -20°C and warmed to 20°C before injection. Preparation of brain fractions from the rabbit is summarized in Fig. 1. Fractionation of liver, kidney, and heart were also performed in the same manner, using about 10 g of fresh weight of tissues (6).

2) **Assay for sensitivity to alcohol in the rabbit**

The assay for sensitivity to alcohol was based on the determination of the duration of alcohol anesthesia; the rabbit with an increased sensitivity would be expected to exhibit a prolonged duration of alcohol anesthesia following the administration of a test dose of alcohol. The challenging doses of alcohol chosen were 1.5 g/kg. Alcohol was administered intravenously as a 20% by volume solution. Injections were given into the marginal ear vein. The time for injection was constant (50 seconds) for all injections. The duration of anesthesia was estimated by determining 1) the reappearance time of the corneal reflex, 2) wink reflex, 3) rearing of head 4) sitting and 5) walking. The reappearance time of the corneal reflex was measured from the termination of the injection of alcohol to the time of recovery of the corneal reflex by using von Fley's technique. Usually, testing was carried
out every week. When alcohol was given once per week, the effect of the repeated administration of alcohol was only slight. Therefore, the development of tolerance to alcohol was not a serious problem in this experiment. There was a relatively little day to day variation for the response to alcohol in a rabbit (see Fig. 2). However, the value for the response to alcohol showed considerable variations from individual to individual (4–65 seconds for the reappearance time of the corneal reflex). Therefore, all subsequent data were calculated as a percentage of the mean original response for the respective test procedure.

3) Assay for a “tolerance-reducing factor” in the brain or liver

The brain or liver fractions were administered intraperitoneally to the rabbit as a single injection. The injection was made into the lower left quadrant of the abdomen.

The presence or absence of a “tolerance-reducing factor”, which would be responsible for the increased sensitivity to alcohol, was determined by a comparison of the duration of anesthesia (sensitivity to alcohol) in the rabbit before and after the treatment.

As our criteria for an “increased sensitivity to alcohol”, it was decided to utilize only the reappearance-time of the corneal reflex listed above. Because this index seemed to be the most reliable. When the reappearance-time of the corneal reflex following the administration of a test dose of alcohol was plotted in percentage of the original response time, approximately 200% (three standard deviations over the original mean response) was taken as the index of an “increased sensitivity to alcohol”.

RESULTS

1) Development and time course of the increased sensitivity to alcohol after a single injection of brain extract

Fig. 3 illustrates a typical time course and magnitude of a case of increased sensitivity
to alcohol in the rabbit treated with brain extract (S₁). It may be observed that there is a distinct prolongation of the duration of anesthesia after the treatment. However, the change in sensitivity to alcohol did not appear immediately after the treatment. An

**Fig. 3.** Development and time course of an increased sensitivity to alcohol in the rabbit.

**Fig. 4.** Subcellular localization of a “tolerance-reducing factor” in brain.
increased sensitivity to alcohol was developed slowly and reached its maximum about one week after the treatment and returned to normal in about four weeks after the treatment. The magnitude of the increased sensitivity to alcohol varied from 80 to 600% of the control values, depending on the individual rabbit.

Increasing the dose of brain extract or multiple injection of brain extract did not produce a corresponding increasing degree of the change of sensitivity or any additional effects. Although a further increase in dosage of the brain extract was not followed by a greater response, a decrease in dosage (half of) was followed by a much less marked rate of effects.

Although the young rabbit is much more sensitive to alcohol than the adult, the weight of the test animal was also not important, with respect to the degree of an increased sensitivity to alcohol following the treatment. Sex differences were also not evident.

It was of interest that only half of the experimental rabbits tested showed such altered sensitivity significantly (see Fig. 4). The relatively rapid return of the sensitivity toward normal was also of interest.

2) Subcellular localization of a "tolerance-reducing factor" in the brain

Since the 10,000×g supernatant (S₃) prepared from the rabbit brain homogenate as well as the entire homogenate was active, it was of interest to determine the subcellular localization of a "tolerance-reducing factor" in the brain. Fig. 4 illustrates that only the pretreatment of rabbits with the microsomal fraction II altered the sensitivity to alcohol in varying degrees. These results clearly demonstrated that a "tolerance-reducing factor"
was located almost exclusively in the microsomal fraction II \( (P_3) \).

Fig. 5 shows a representative cross section of a pellet of the brain microsomal fraction II \( (P_3) \). Although the microsomal fraction II was not completely free of rough-surfaced endoplasmic reticulum, these studies demonstrated that a "tolerance-reducing factor" was located in particles of "microsomal" size and/or in the smooth-surfaced microsomes (vesicles and tubules).

3) A "tolerance-reducing factor" in various tissues

A "tolerance-reducing factor" was found in the microsomal fraction II \( (P_3) \) from liver as well as from the brain (see Fig. 6). Although the average duration of alcohol anesthesia was somewhat shorter for liver than it was brain, no statistically significant difference exists between these two values. This finding suggests that a "tolerance-reducing factor" was present not only in the brain but also in the liver. In the kidney or heart experiment, however, only a tendency towards an increase in sensitivity was found, which could not be regarded as significant, after the treatment.

4) Chemical and physical stability of a "tolerance-reducing factor" in the liver and the brain

In an attempt to learn something of the chemical and physical nature of a "tolerance-reducing factor," an experiment was done using the supernatant fluid \( (S_1) \) or the microsomal fraction II \( (P_3) \) from the liver or the brain.

The supernatant fluid from the brain or the liver was dialyzed at 5°C for 24 hours against frequent changes of a 0.9% NaCl solution. Then a bioassay for sensitivity to alcohol in the rabbit was carried out in the usual manner. There was no significant difference in the activity of the dialyzed supernatant versus the non-dialyzed supernatant.

Although a "tolerance-reducing factor" in the liver was stable at 38°C for one hour, the activity was completely lost when the supernatant was heated at 50°C for 10 minutes. To test the resistance to trypsin, the supernatant was incubated with trypsin \( (1 \text{ mg/ml}) \) at pH 7.0 for 2 to 24 hours. It was shown that trypsin did not destroy the "tolerance-reducing factor" in either the microsomal subfraction of the brain or liver.

\( n \)-Butanol was added gradually to the supernatant fluid from the liver, while stirring vigorously. After stirring for about 10 minutes the dispersion was centrifuged at 10,000 \( x g \) for 60 minutes. The slight turbid aqueous layer was collected and the excess butanol and the floating precipitate were discarded. A "tolerance-reducing factor" was found in the aqueous layer. After gel filtration of the supernatant fluid on a Sephadex G-25
column, the active substance was found in the eluate.

In order to test the stability of a “tolerance-reducing factor” in different media, diphenhydramine (10⁻¹⁰), \( \alpha \)-tocopherol (0.2 mm) or a chelating agent EDTA (0.2–4 mm) was added to the extractant (0.32 M sucrose). It was shown that no significant reduction in the activity of the microsomal fraction II from the liver was seen (see Fig. 7).

An addition of sodium deoxycholate (0.1%) to the supernatant fluid from the liver did not change the activity of the microsomal fraction either. However, when an increased concentration of deoxycholate (0.3%) was used, there was an invariable loss of activity. These data suggest that the “tolerance-reducing factor” may be a large molecular weight substance such as a polypeptide.

**DISCUSSION**

Our results showed that a greater sensitivity to alcohol was noted in rabbits treated with the respective microsomal fractions obtained from brain or liver. The development of an increased sensitivity to alcohol was slow. Approximately two weeks were required for the full development which continued for about three weeks. Our work demonstrated that about a 2-fold increase in the sensitivity to alcohol was produced by the treatment with the microsomal fraction from the brain. This increased sensitivity to alcohol in the rabbit was best expressed as a change in the duration of disappearance time of the corneal reflex following the intravenous administration of standard test doses of alcohol.

The results obtained showed that a “tolerance-reducing factor”, a substance responsible for the increase in sensitivity to alcohol, was primarily localized in the microsomal fraction of the brain and liver (7).

An increase of the dose or multiple injections of the “tolerance-reducing factor” did not produce a corresponding increase in degree of the change in sensitivity to alcohol. However, the true nature of the “tolerance-reducing factor” remains unknown. Further work is required to determine whether the suspected “tolerance-reducing factor” \( \textit{per se} \) is responsible for the increased sensitivity to alcohol observed or whether it depends on
some nonspecific effects of this factor.

The mechanism for the increased sensitivity of a moderate degree to alcohol remains to be determined. It should be noted that, in our experiments, an intravenous administration of the standard test dose of alcohol was employed as the means of assay for the sensitivity to alcohol in the rabbit. Since the literature shows that the duration of anesthetic action of a single intravenous injection of alcohol is determined on the basis of its distribution and redistribution to body tissues and also since alcohol is not rapidly destroyed in the tissues, the duration of alcohol anesthesia may not be a reliable measure of the rate of detoxication. One must assume here that an increase in the sensitivity to alcohol following the treatment with brain or liver extract must be caused largely by some other factor or factors than the inhibition of the degradation of alcohol.

Although the blood-brain barrier may be altered under a variety of conditions, an explanation for the change in distribution of alcohol in tissues as a result of the treatment with brain or liver extract may not be readily available, because alcohol penetrates tissues rapidly by simple diffusion.

The mechanism whereby this increased sensitivity to alcohol could occur is mere speculation, but at least two mechanisms come to mind: 1) depletion in the brain of noradrenaline, serotonin, or dopamine, or by some combination of changes in these amines. 2) biochemical alteration of the receptor site in the brain cell (5).

It was of interest that the time course of the change in sensitivity to alcohol in the rabbit somewhat resemble the type of supersensitivity in the nictitating membrane observed after chronic decentralization (preganglionic denervation) (8). Supersensitivity after decentralization develops slowly and reaches its maximum about two weeks after the severance of the preganglionic nerve. Supersensitivity after decentralization is of a moderate degree, is nonspecific, is independent of the presence or absence of the norepinephrine stores, is unaccompanied by subsensitivity to indirectly acting amines, and requires time for its development. However, there is no clear evidence for a suspected mechanism of decentralization type of supersensitivity to catecholamines.

The nonspecific character of the decentralization type of supersensitivity suggests that it is due to some change in the physiology of the responding cells rather than to a change in the receptors.

An increase in an animal's sensitivity to barbiturates has been demonstrated after ablation of cerebral tissue by several investigators (9). Lesions in the septal area of the forebrain or in the dorsomedial tegmentum of the brain stem produce a 3-fold prolongation of thiopental-induced sleep. Roth and Harvey suggested that chemical changes occurring in the remaining portions of the brain after production of subcortical lesions might be responsible for the occurrence of increased thiopental-induced sleep. It may be possible that the subcortical lesions have affected neurochemical functions which are also affected by barbiturates, and this in turn is responsible for an increased action of thiopental.

Thus, the neurochemical changes in the cerebral cortex may also be a key to the development of the increased sensitivity to alcohol after the treatment with the microsomal
fraction from the brain or the liver.

Finally, it may be proposed from our experiments that some kind of immune reaction induced by the treatment with microsomes can play some role in these phenomena (10).

No specific mechanism for the altered sensitivity to alcohol in the present study can be advanced at this time. In order to pursue this point further, additional experiments are in progress.

SUMMARY

The development of an increased sensitivity to the anesthetic effect of alcohol in the rabbit treated with the microsomal subfraction obtained from brain or liver was demonstrated in the present paper.

The sensitivity to alcohol was determined by the duration of disappearance of the corneal reflex following an intravenous injection of a test dose of alcohol.

The increased sensitivity to alcohol in the rabbit occurred slowly and appeared to persist for three weeks after treatment with the brain microsomes.

Some experiments were also done to gain some knowledge of the physico-chemical nature of a "tolerance-reducing factor" responsible for the increase in sensitivity to alcohol in the rabbit.

It is felt that these results indicate that some sort of immune mechanism induced by the treatment with microsomes may play a role in this phenomena.

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REFERENCES