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Adrenal Catecholamines in Rats After Severe Ethanol Intoxication and Acute Withdrawal

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ADAMS, M. A., P. L. PURVIS AND M. HIRST. *Adrenal catecholamines in rats after severe ethanol intoxication and acute withdrawal.* PHARMAC. BIOCHEM. BEHAV. 16(5) 719–724, 1982.—Adrenal levels of adrenaline and noradrenaline were determined in rats subjected to a 96 hour ethanol intoxication and in animals experiencing moderate to severe withdrawal behaviour. Adrenaline content of adrenal glands of animals sacrificed during intoxication and during withdrawal was less than 30% of treatment controls. The concentration of noradrenaline in withdrawing animals was 52% of control. Compensated adrenal weights of intoxicated animals were 80% larger than control values. These findings suggest that "binge-type" ethanol administration has profound effects on adrenal catecholamine levels and on adrenal gland weight.

Ethanol Adrenal medulla Catecholamines Rats Physical dependence Withdrawal syndrome

IN man single small to moderate doses of ethanol cause a short term stimulation of the adrenal medulla [20,23]. Increases in levels of adrenaline (ADR) have been found in urine samples collected within a few hours after oral ingestion, but not in those obtained at later times [1, 12, 20, 23]. Ogata *et al.* [20] have shown increased excretions of adrenaline and noradrenaline (NA) and their metabolites in alcoholic subjects during chronic ethanol ingestion. They further observed, in agreement with others [5,13], even greater levels of urinary catecholamines and metabolites in subjects experiencing severe withdrawal signs and symptoms.

Studies of the effects of ethanol on catecholamine metabolism in laboratory animals show similar increases in adrenergic activity. Short term administration of ethanol to conscious dogs, rats or anesthetized cats has produced substantial increases in urinary adrenaline levels [15,24], or secretion of catecholamines from the adrenal medulla [7,25].

Few studies, however, have examined directly the catecholamine content of adrenal glands exposed to ethanol. Cohen *et al.* [7] showed that a chronic ethanol liquid diet increased total catecholamines and altered adrenal enzyme activities. The liquid diet method has been used in numerous studies of physical dependence to ethanol, but induction is slow and withdrawal signs are not observed consistently [3, 11, 21]. The method of Majchrowicz [16], in which rats are administered ethanol several times a day by intragastric intubation, produces high blood ethanol levels during treatment and obvious, reproducible changes in behaviour during withdrawal. It was considered of interest to determine the adrenal catecholamine content of animals exposed to this treatment, during intoxication and withdrawal.

METHOD

Male Sprague-Dawley rats weighing 344–426 g at the start of the study were housed in individual wire-mesh cages and maintained in a 12-hr light and dark cycle at a controlled temperature (24–25°C). After 2 days of acclimatization, animals were randomly assigned to 4 groups. Group A were four untreated control rats which received Purina rodent laboratory chow and tap water ad lib for five days. In the animals in other groups a Bardic premature infant feeding tube (size 5 Fr.) was introduced into the stomach every 6 hours during a 96-hr period. Group C (7 rats) and Group D (7 rats) received an ethanol solution (25% v/v), the dose being determined by the level of intoxication of the rat at that time, as described by Majchrowicz [16]. Group C were sacrificed after the 96-hr experimental period; Group D underwent a 16–18 hr period of withdrawal from alcohol before sacrifice. Group B (3 rats) received doses of 34.8% sucrose w/v every 6 hours. They were paired with three rats of Group D so that the volumes of the sucrose solution which they received at each interval was equal to the volumes of ethanol solution given to their ethanol-treated counterparts. (It should be noted that a 34.8% w/v solution of sucrose contains the same number of calories as a 25% v/v solution of ethanol.) Thus, the sucrose-fed rats received the same volume of fluid and the same number of calories by gavage as the animals they were matched to. Although ethanol-treated rats ate and

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drank very little beyond what they received by stomach tube, the intake of chow and of tap water of the three paired animals was provided to the sucrose group as a supplement. Both the ethanol and sucrose solutions contained 0.4 ml Poly-Vi-Sol vitamin drops (Mead Johnson, Canada) per 100 ml of intubation solution.

All rats were killed by decapitation, their adrenal glands rapidly removed and placed in Eppendorf test tubes (Brinkman, 1.5 ml) to be frozen by immersion of the tube in a dry ice/acetone bath. They were stored at −75°C. All adrenal glands were weighed as soon after thawing as possible.

The noradrenaline and adrenaline contents of the adrenals were measured by either a gas-chromatographic procedure developed in this laboratory that involved derivatization of extracted catecholamines with heptafluorobutyric anhydride in acetonitrile [14,28] or, later, by liquid chromatography with electrochemical detection.

For liquid chromatographic analysis a weighed adrenal gland was homogenized (Polytron homogenizer, 30 seconds) in a mixture of hydrochloric acid (1.95 ml, 0.1 N) and the chromatographic internal standard, 3,4-dihydroxybenzylamine (50 μl; 4.78 μg), in a polypropylene test tube (15 ml). This was then centrifuged (10 min, 18,000 ×g at 0–4°C), after which the supernatant was percolated through a column of Dowex resin (1.0 ml of a 2 bed volume slurry, AG 50W-X4, 200–400 Mesh, H+ form). After washing the column with water (2×2 ml) and a mixture of water/methanol (2:3, 2 ml), the amines were eluted into a hydrochloric acid/methanol solution (4 N, 2:3, 1.25 ml). A portion (50 μl) of this eluent was mixed with the chromatographic mobile phase (0.95 ml). A small fraction (20 μl) of this solution was analyzed chromatographically.

The apparatus consisted of an Altex 110A pump, a Rheodyne Model 7120 syringe-loading sample injector (20 μl loop) and an Altex LiChrosorb RP 18, 10 μm (4.6×250 mm) stainless steel column (Altex Scientific). A Model LC-2A electrochemical controller was used with CP-O carbon paste as the working electrode (Bioanalytical Systems). The potential was set at +0.8 volts against a silver-silver chloride reference electrode. Chromatographic responses were recorded on a Rikadenki electronic recorder (Model B-24). The mobile phase contained potassium dihydrogen phosphate (0.056 M), octyl sodium sulfate (4.66 mM; Eastman Kodak) dissolved in double-glass distilled water (1.0 litre) to which methanol (300 ml; Fisher Scientific HPLC grade) was added. The pH was then adjusted to 4.5 with hydrochloric acid (4 N). This solution was filtered through a membrane filter (Millipore FHUP, 0.5 μm) prior to use. The pump system maintained a flow rate of 0.7 ml per minute for all analysis. Column temperature was 30°C. The amounts of noradrenaline and adrenaline present in the samples were determined by the peak height ratio method using calibration curves corrected for incomplete recoveries. Validation of noradrenaline and adrenaline peaks was obtained by duplicate runs of standards and samples under two different chromatographic conditions. The GC method was altered by using different columns [14]. The liquid chromatographic conditions were adjusted with changes in pH and methanol concentration.

To assess the relation between levels of intoxication and blood levels of ethanol, samples were taken from some rats one hour before a dose of ethanol and in others one hour after. Samples of blood were obtained from the tails of the intoxicated animals using micro-sampling pipets (Pyrex, 44.7 μl). The blood sample was immediately placed into a mixture of sodium fluoride (15% w/v) and thiourea (25 mM) in distilled water (44.7 μl) and n-propanol (89.4 μl; 1.67 μg) contained in a 1.5 ml Eppendorf micro test tube. Each sample was then mixed thoroughly and centrifuged for 2 min (Eppendorf 3200). The supernatant (100 μl) was placed in distilled water (500 μl) in a rubber-capped glass sampler vial (Wheaton Glass). Samples (5 μl) were injected into a gas-chromatograph (Hewlett-Packard, Model 5750). The column was stainless steel (36 inches × 1/8 inches o.d.) containing Poropak N (50–80 mesh, Waters Assoc.). A pre-column packed with Glassport M (60–80 mesh, Hewlett-Packard) was used to protect the analytical column from the blood contaminants. Chromatographic conditions were: column temperature 150° C, injection port 165°C, flame ionization detector 210°C, air flow rate 300 cc/min, nitrogen 75 cc/min, hydrogen 60 cc/min. The quantities of ethanol were determined by the peak height method relative to the internal standard n-propanol, using standard curves.

The data was evaluated statistically by two-tailed Student's t-tests for paired or unpaired data as appropriate: $p<0.05$ was considered indicative of a statistical difference. Numerical values in the text and figure are of means±standard error of the mean (SEM).

RESULTS

In general, animals were ataxic throughout the intoxication period. Third-day blood ethanol concentrations ranged between 412 and 471 mg/dl; the level before an ethanol dose was 427±6.8 mg/dl and one hour after was 451±7.8 mg/dl. The mean daily ethanol dose was 8.5±1.22 g/kg, ranging from 7.3 to 10.0 g/kg. Within the 18 hours following the last administration of ethanol all animals exhibited moderate to severe withdrawal characteristics: body extension, body and tail rigidity, generalized tremors and excessive vocalization on touching. Two of the seven animals had tonic-clonic convulsions.

Rats receiving the ethanol treatment ate and drank sparingly. Their daily caloric intake, and hence of the animals given sucrose solution, resulted almost entirely from the intubation solutions, ranging between 51.2 and 70.3 kcal/kg; the mean over the 4-day period was 59.6±6.39 kcal/kg. Of the mean daily water intake of 38.9±9.12 ml over 80% (32.4±1.30 ml) was supplied to the animals by the ethanol or sucrose solutions administered by gavage.

Figure 1 shows that untreated rats gained an average of 10.3±0.23% of their Day-1 body weights over the 4-day experimental period. The mean body weight of the sucrose-treated rats was significantly lower on Day 2 than at the beginning of the experiment and continued to decline, reaching 84.6%±0.45% of the initial weight at the end of the treatment period. Similarly, animals given ethanol showed significantly reduced body weights on the second and subsequent days, reaching 79.6%±0.52% of the initial body weight after the 96 hour intoxication period. After the withdrawal period the body weights were 78.1±0.85% of Day-1 values. The mean weights of random single adrenal glands from all groups are shown in Fig. 2. Those from the untreated rats were not significantly different from those of animals receiving sucrose. In ethanol-treated rats adrenal weights were generally larger than those of the sucrose control group or the untreated animals. The adrenal weights of rats treated for 96 hours with ethanol were significantly higher than those from untreated or sucrose control rats, but not significantly different from those of rats undergoing withdrawal from

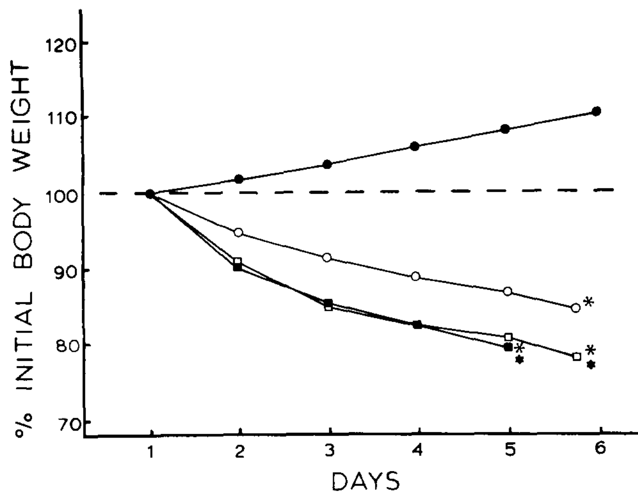

FIG. 1. Percent body weight changes during experimental period in untreated (●), sucrose-treated (○), ethanol-treated (■,□) rats. Significant difference from untreated group (*) and sucrose group (★); ($p<0.01$). (Data are mean±SEM except that error bars do not extend beyond symbols.)

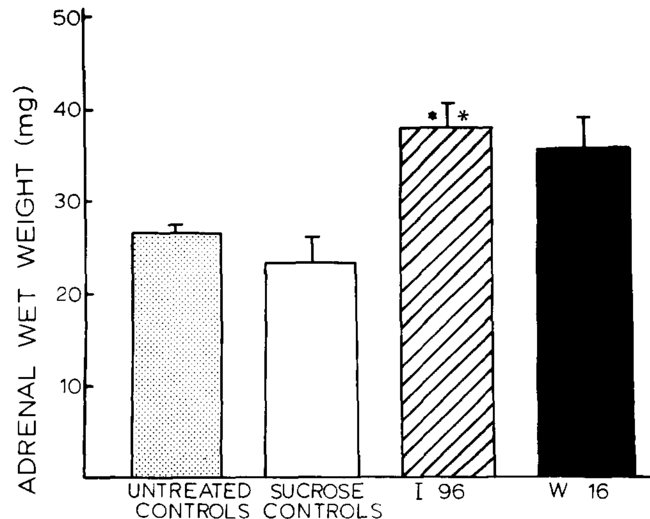

FIG. 2. Single adrenal wet weights of untreated (░) and sucrose-treated (□) controls, and intoxicated (▨) and withdrawing (■) treated rats. Significantly different compared to untreated (*) or sucrose-treated (★) controls. (Data are mean±SEM.)

TABLE 1

SINGLE ADRENAL GLAND WEIGHT PER BODY WEIGHT

Treatment	N	Weight (mg/100 g)
Untreated	4	5.8 ± 0.26
Sucrose	3	7.0 ± 0.29
I96	7	12.6 ± 0.92*†
W16	7	12.5 ± 1.63*

I96=96 hour intoxication; W16=96 hour intoxication plus ethanol withdrawn for 16–18 hours.
*Significantly different from untreated controls ($p<0.01$).
†Significantly different from sucrose control group ($p<0.02$).
Data are mean ± SEM.

ethanol. The proportional adrenal weights (mg of single adrenal tissue/100 g body weight) are presented in Table 1. Again, values from the sucrose-treated animals were comparable to those of untreated rats. Significantly higher values were found in animals that received ethanol ($p<0.01$). The proportional adrenal weights of rats withdrawn for a further 16–18 hours were significantly higher than those of untreated rats ($p<0.02$).

Figures 3a and 3b are chromatograms of samples from adrenal glands of rats from various treatment groups. Figure 3a shows tracings from the gas-chromatographic procedure which yields twin peaks for both adrenaline and noradrenaline. The sum of the areas of these peaks is linearly related to the quantity of these amines. Figure 3b shows chromatograms of adrenal catecholamines using the liquid chromatographic procedure. Both analytical procedures show reduction in total catecholamine content with ethanol intoxication and the data were in good agreement. The liquid chromatographic procedure was much faster and routinely gave less complex chromatograms.

Figure 4 shows that untreated rats had adrenaline and noradrenaline concentrations of 420.6±17.21 μg/g and

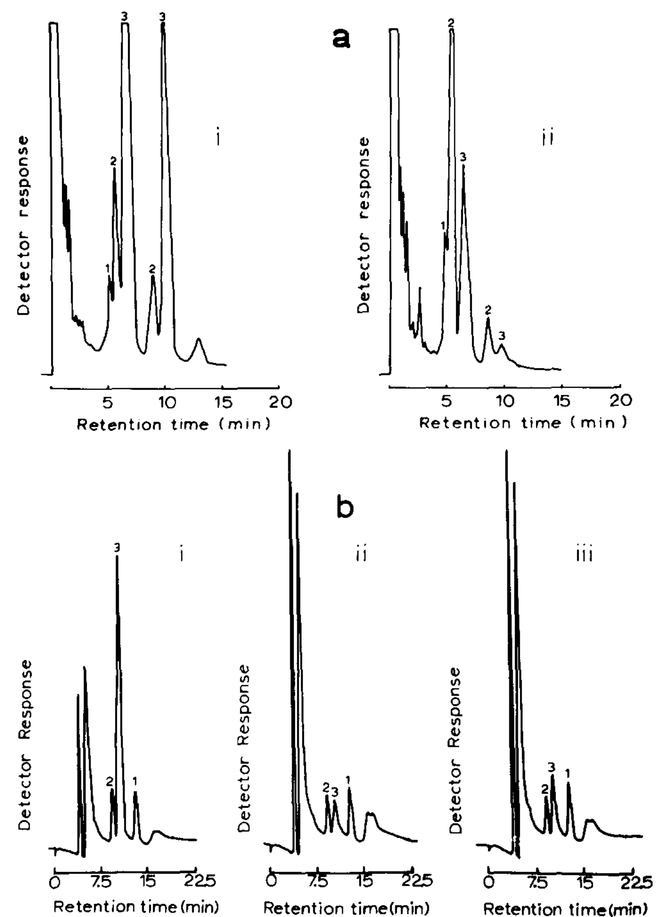

FIG. 3. (a) Tracings from gas chromatographic analysis of adrenals from an untreated (i), and an intoxicated (ii) rat. (b) Tracings from liquid chromatographic analysis of adrenals from a sucrose-treated (i), and intoxicated (ii) and a withdrawing (iii) rat. Peak are identified 1-Internal standard, 2-Noradrenaline, 3-Adrenaline.

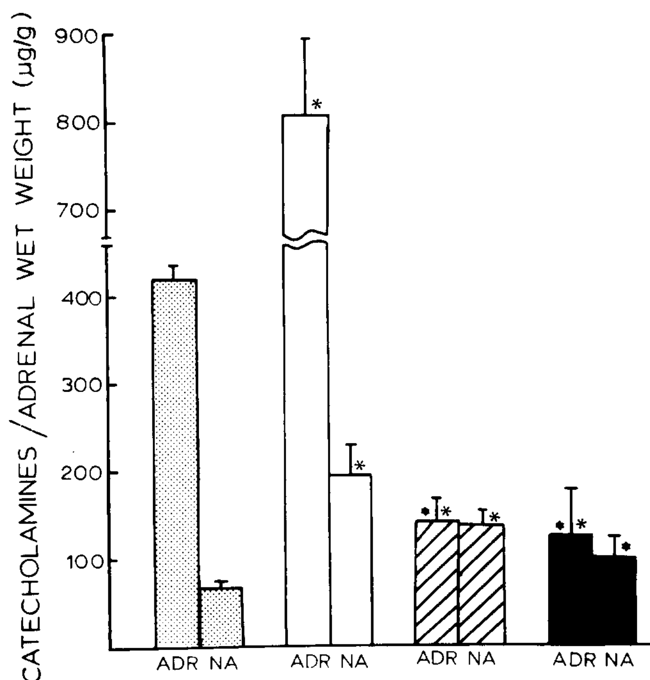

FIG. 4. Catecholamine concentration of adrenals from untreated (░), sucrose-treated (□), intoxicated (▨) and withdrawing (■) rats. (Data are mean±SEM.) *Significantly different compared to untreated rats ($p<0.05$). ★Significantly different compared to sucrose-treated controls ($p<0.05$).

66.8±6.31 μg/g of adrenal tissue, respectively. Control animals which received the sucrose solution for 96 hours and were sacrificed after a further 16–18 hour period had significantly higher adrenal adrenaline (809.3±87.62 μg/g) and noradrenaline (192.2±37.24 μg/g, levels. The adrenaline concentrations were significantly lower in the groups of animals given ethanol, which were found to contain less than 20% of those of the sucrose-treated controls. Levels of adrenaline found in animals experiencing the withdrawal syndrome were not different to those found in intoxicated animals. Although the noradrenaline concentration in ethanol-treated rats was somewhat lower than that in animals receiving isocaloric sucrose, the difference was not significant except in the group (D) withdrawn from ethanol. The noradrenaline concentrations in the two ethanol-treated groups did not differ significantly.

Table 2 shows the adrenaline and noradrenaline contents of the whole adrenal gland. The catecholamine content of sucrose-treated controls was significantly higher than those of other groups. Adrenaline levels were significantly lower in the ethanol-treated animals; the levels in intoxicated and withdrawing animals were only about 27% and 21%, respectively, of those found in the adrenals of sucrose-treated animals ($p<0.001$). The noradrenaline contents of the ethanol-treated groups were not significantly different from those in the adrenals of the sucrose-treated rats, but the adrenal noradrenaline contents of the withdrawing rats was significantly lower than those in intoxicated rats ($p<0.05$).

TABLE 2

CATECHOLAMINE CONTENT OF SINGLE ADRENAL GLANDS

Treatment	N	Adrenaline	Noradrenaline
Untreated	4	10.93	1.77
		(0.656)	(0.169)
Sucrose	3	18.77*	4.38*
		(2.519)	(0.631)
I96	7	5.17*†	5.02*
		(0.974)	(0.564)
W16	7	3.90*†	3.22‡
		(1.448)	(0.624)

I96=96 hour intoxication; W16=96 hour intoxication plus ethanol withdrawn for 16–18 hours.

*Significantly different from untreated controls ($p<0.01$).

†Significantly different from sucrose controls ($p<0.001$).

‡Significantly different from I96 group ($p<0.05$).

Data are mean ± SEM.

DISCUSSION

The development of physical dependence to ethanol requires the maintenance of a critical level of blood ethanol [6] and implies an adequate ethanol intake. In the present study the protocol of Majchrowicz [16] was used to achieve continually high blood-ethanol levels and persistent intoxication throughout the period of ethanol administration. Physical dependence, as demonstrated by signs of moderate to severe withdrawal, was observed in all animals within 18 hours of the final dose of ethanol.

Procedures for inducing ethanol dependence, which involve repeated intubation of the animals with ethanol-water solutions, are similar to most liquid diet methods in that a decline in body weight is seen [3, 4, 18, 21, 22]. Analysis reveals that the untreated animals (Group A) consumed between 20–26 kcal/100 g body weight/day as lab chow whereas those subjected to the intubation procedures (Groups B, C, D) received about one-third as much. It is not then surprising that in the present study the ethanol-treated rats lost some 20% of their body weights over the experimental period. The degree of weight loss in those rats is similar to that reported by Mucha *et al.* [18] and Majchrowicz [16] for intragastric intubation methods. As the ethanol treatment provides the animals with a food deficient in protein and fats it is essential that the appropriate control group for the effects induced by ethanol receive a parallel nutritional exposure, as well as being handled in an equivalent fashion. Pohorecky [27] has identified intragastric administrations of sucrose for control treatments in alcohol studies. In the present study a decline in weight was also evident in the group which received the isocaloric amounts of sucrose. The similar decline in body weight in the treatment groups is a reflection of the reduced caloric and water intake.

The adrenal glands of the intoxicated rats (Group C) were larger than those of the rats given sucrose (Group B). Enlarged adrenals have been reported in animals receiving an ethanol containing diet [26] and also in rabbits and guinea pigs given an ethanol solution as a source of fluid [2]. Earlier studies involving other manipulations which stimulated the hypothalamic-pituitary-adrenal axis have demonstrated hypertrophic changes in the adrenal gland [9, 10, 17]. The adrenal glands of the sucrose-treated rats did not show trophic changes when compared to untreated animals.

The results of the present study show that a program of

ethanol administration which leads to severe intoxication profoundly affects the catecholamine content of the rat adrenal gland. A marked reduction in adrenaline content was evident in all rats receiving ethanol, a difference not considered to reflect a nutritional deficiency, for rats given isocaloric amounts of sucrose had higher levels of adrenaline than the untreated animals. In rats, acute ethanol doses above 4 g/kg, given intragastrically, increased adrenaline output [24]. Klingman and Goodall [15] showed, in dogs, that acute oral administration of high doses of ethanol produced a significant decrease in the content of adrenaline, but not of noradrenaline, in the adrenal glands. The levels of noradrenaline in the present experiments were also changed less than that of adrenaline. The total adrenal noradrenaline content of both sucrose-treated controls and ethanol-intoxicated rats was significantly elevated above the level in untreated animals, but differences between them were not statistically significant. In the group (D) withdrawn from ethanol, the total noradrenaline content was significantly less than that of the intoxicated group (C).

Various investigations have shown that acute ethanol, in moderate to high oral doses, does not affect noradrenaline output from the adrenal gland as much as it influences adrenaline output [15, 23, 24]. Cohen *et al.* [7] found a significant increase in the activities of all the primary catecholamine synthesizing enzymes in the adrenal glands during acute intoxication with ethanol. Calculations of enzyme activities from their data show that dopamine-β-hydroxylase activity was elevated 92% above controls, as compared to a 25–30% increase in other enzyme activities. This finding implies a very rapid induction of noradrenaline synthesis from available substrate pools. Changes in acute catecholamine turnover have been shown to be the result of various short term modulators; activity changes in enzymes [8], reduced feedback inhibition or substrate concentration changes. In the Cohen [7] study, denervation of the adrenal glands did not affect the changes in dopamine-β-hydroxylase activity seen with acute intoxication; therefore, these effects were not considered to be neurally mediated. Chronic intoxication, using a liquid-diet procedure, elevated phenylethanolamine-N-methyltransferase activity, whereas other enzyme activities were unchanged with respect to intact control animals. In the same study, Cohen *et al.* [7] showed that denervation blocked the increase in adrenaline synthesizing enzyme. Analysis of their experiment indicates that control intact rats in the withdrawal group had total catecholamine levels some 2.8 times higher than control intact animals killed eight hours previously. In our experiments catecholamine changes were compared in animals run through an experimental treatment at the same time. Acute or chronic intoxication of animals with even moderate doses of ethanol has been shown to induce a variable stress condition which is often indicative of other experimental conditions (unpublished observations).

In 1960, Munro and Robinson [19] had indicated that neural control of the adrenal medulla provided the most important input for the regulation of catecholamine secretion. The severe depletion of medullary adrenaline found in the present study suggested a rapid and profound neural stimulation of adrenaline secretion during the 96-hour intoxication period. Since adrenaline levels, measured either as concentration or total content, do not fall further during the withdrawal period, it would seem that ethanol treatment has induced either maximal stimulation of adrenaline release or that there is a compensation in the biosynthesis of adrenaline, or perhaps both. Further investigations involving adrenaline and noradrenaline storage and synthesis will help elucidate these questions.

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