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Adrenal and Urinary Catecholamines During and After Severe Ethanol Intoxication in Rats: A Profile of Changes

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ADAMS, M. A. AND M. HIRST. *Adrenal and urinary catecholamines during and after severe ethanol intoxication in rats: A profile of changes.* PHARMACOL BIOCHEM BEHAV **21**(1) 125–131, 1984.—Adrenal and urinary levels of adrenaline and noradrenaline were determined in rats subjected to severe ethanol intoxication for periods of up to 96 hours, in rats undergoing withdrawal and in a post-withdrawal period, and in controls. Adrenaline and noradrenaline content of adrenal glands fell markedly to less than eight and twenty percent, respectively, after four days of intoxication. Noradrenaline content, but not adrenaline content, had recovered after a subsequent four day period of recovery. The depletion in adrenal catecholamine levels was coincident with increases in urinary adrenaline and noradrenaline levels over the first 48 hours of intoxication. Urinary catecholamine levels remained higher than control values for the next 48 hours of intoxication. Adrenal glands were larger after 12 hours of intoxication, although there was no increase in dry weight. At later times adrenal enlargement was associated with increased dry weight and protein content. This increase in mass was found to be of cortical origin. These results demonstrate that severe ethanol intoxication promotes an intense stimulation of the rat adrenal gland with enhanced synthesis and release of catecholamines, and cortical hypertrophy.

Ethanol Adrenal gland Catecholamines Urinary excretion

IT has been known for many years that ethanol disrupts the normal functioning of the adrenal gland. Aubertin [5] reported adrenal hyperplasia and hypertrophy in animals exposed chronically to an alcoholic beverage. This was interpreted later as a defensive response of an organism to the toxic effects of ethanol [26,27]. Single doses of ethanol stimulates catecholamine release from the adrenal medulla of animals and man [4, 14, 21, 22, 23]. Changes in adrenal synthetic activity have also been shown in long-term alcohol intoxication [8, 19, 25]. In a recent study it was found that the adrenal catecholamine content of rats that were physically dependent after being severely intoxicated with ethanol for 96 hours was greatly reduced and the glands were enlarged [3]. It was considered of interest to extend this study by assessing in greater detail the time course of the changes, both during the induction phase, during withdrawal and in the post-withdrawal period. In addition, the secretory activity of the medulla was assessed by determinations of urinary catecholamines.

METHOD

Male Sprague-Dawley rats weighing 310–385 g were housed in individual wire-mesh cages and maintained on a 12 hour light:12 hour dark cycle at 23–25°. After two days of acclimatization, during which rats were allowed food and water ad lib, rats were randomly assigned into one of three treatment groups. Each group subsequently received gastric intubations of one of three solutions every six hours for up to 96 hours, as has been previously described [3,15]. Group I (n=11) were designated as intubation controls, receiving tap water (3 ml/intubation) during each intubation session, and at other times had free access to food and water. Group S (n=24) received a sucrose solution (34.8% w/v). The third group, Group E (n=24), received an ethanol solution (20% w/v), the dose of ethanol on the first occasion being 5 g/kg, and at each intubation session the dose of ethanol given was determined by the level of intoxication displayed by the rat at that time, as described by Majchrowicz [15]. Both the sucrose and ethanol solutions contained multivitamin supplement (0.4 % v/v, Poly-Vi-Sol, Mead Johnson, Canada, 1982). The caloric concentration of the sucrose and ethanol solutions was identical and Group S rats were pair-fed with those of Group E, as described in an earlier report [3].

Body weights were recorded once daily for each rat. Urine was collected, from certain rats, into tubes containing hydrochloric acid (1.0 ml, 4.0 N). The urine volumes were measured after each 24 hour period. Urine samples were stored at −75° until analyzed. Rats were killed by decapitation at various times; prior to the first intubation, after 48 hours (48 I) and 96 hours (96 I) of intubations, 16–18 hours (16 W), 48 hours (48 W) and 96 hours (96 W) after with-

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drawal of treatments. Urine was collected from Group I (n=4), Group S (n=6) and Group E (n=6), rats that were assigned to groups killed at the end of the experimental period (96 W).

Immediately after death the rat adrenal glands were removed and cleaned of extraneous tissue. One adrenal gland, randomly chosen, was weighed immediately after excision and prepared for histological analysis, as described below. The remaining adrenal was taken and frozen rapidly to −75°. This was subsequently weighed upon thawing. Dry weight and protein content were determined [2] on the previously frozen single adrenal glands from rats in Group S and Group E killed during the withdrawal phase (16 W). Catecholamine contents were determined, by a procedure involving liquid chromatography with electrochemical detection, in single adrenal glands [1] of all rats killed, and on the 24-hour urine samples [1]. Blood samples were taken from the tails of some Group E rats (96 I, 16–18 W and 48 W) during the second (n=3), third (n=5) and fourth (n=5) days of intoxication, and during the prodromal detoxication phase (n=9), for determinations of blood ethanol levels [3].

Withdrawal severity was scored in all rats in Group E (n=15) and S (n=15) that remained after the intubation period for up to 24 hours after the cessation of the treatment. The behaviour of the rats was monitored during 17 different 5 minute intervals in which each animal was scored according to criteria originally described by Majchrowicz [15] and subsequently modified [1]. Thus, each rat received a scored rating after each observation interval throughout the withdrawal phase, the magnitude of which corresponded to the intensity of withdrawal signs.

The method of adrenal histology used was that developed by Honore [13], a procedure which allows for light microscopic characterization of medullary and cortical zones as well as noradrenaline in the medullary chromaffin cells. Single, fresh, adrenal glands from Group E (n=6) and Group S (n=6) rats killed after 96 hours of intubation were prepared for sectioning (10 μm) using glutaraldehyde fixation and subsequent embedding in a paraffin cube. The staining involved a pre-exposure to a dichromate solution prior to the final immersion in alkaline toluidine blue. The images of the mounted, stained sections were examined under an enlarger and the area of the adrenal medulla and whole gland determined by stereological calculation [6]. Hence, the volume of the adrenal gland and the medulla was determined by reconstructing the dimensions of the gland from a representative proportion of serial sections. The noradrenaline-containing cells could be identified by a visible green coloration, localized in discrete regions of the more darkly stained medulla, which was blue-purple in color. The green coloration resulted from the reaction of the stain with the noradrenaline-glutaraldehyde reaction product. Blood vessels stained an orange yellow and the cortex a lighter blue.

In another experiment, rats (n=15) weighing 230–290 grams were divided into two treatment groups to be intubated with either water or an ethanol solution. In this study, Group I rats (n=5) received a single intubation of water (25 ml/kg) and were killed after six hours. Group E rats were given ethanol by gavage (5 g/kg) and some rats (n=5) were killed after six hours. The remaining group E rats (n=5) were given more ethanol after six hours, the dose being based on characteristics of intoxication indicated above. These rats were killed after twelve hours of ethanol intoxication. Adrenal glands were taken and analyzed for catecholamines, dry weight and wet weight.

All numerical values are presented as means plus and minus standard deviations. A probability level less than 0.05 was considered significant for all data. Data was evaluated using an analysis of variance test based on that of Winer [29] to indicate differences between treatment and time effects. Tests for significance between two means were done using a Student's t-test for unpaired means.

RESULTS

Rats intubated with ethanol were generally ataxic throughout the treatment period. Blood ethanol levels on the second, third and fourth intubation days ranged from 322 to 556 mg%, with a mean of 424±66.1 mg%, determined from samples taken one hour prior to an intubation period. The mean daily ethanol dose was 7.1±0.18 g/kg/day. The caloric intake of Groups E and S was 184±16.9 kcal/kg during the intubation period, coming entirely from the intubated solutions. During the recovery phase (days 6, 7 and 8), all rats were allowed access to food and water ad lib. On the sixth treatment day, sucrose-treated rats, Group S ate and drank substantially more than the corresponding rats in Group E. On subsequent days, however, ethanol withdrawing rats were no longer showing any obvious signs of their treatment and consumed quantities of food and water equivalent to the sucrose-treated rats.

Water-intubated rats, Group I, had stable body weights for the entire experimental period. On the other hand, body weights of Group E and S rats declined significantly over the 96 hour intubation period, falling to 276.3±3.41 g and 291.4±3.12 g respectively from an initial weight of 350.2±3.08 g. Both of these groups continued to lose weight during the first 24 hour period of withdrawal. From this time, however, both groups of rats gained weight. Sucrose-treated rats, feeding ad lib, gained significantly more weight than the corresponding ethanol-withdrawn rats during the remainder of the experimental period. Body weights of Group S rats at the end of the experiment were not significantly different from pre-treatment levels. Group E rats also gained weight during the post-withdrawal phase, but had not regained their starting body weights by the end of that period.

In the short-term study, the group of rats treated with ethanol for only a short period of time (i.e., 6–12 hours) showed a similar decline in weight as their controls. This loss amounted to less than five percent of starting body weight. As shown in Table 1, weights of the adrenal glands of rats intoxicated for 12 hours were 16 percent greater than those from corresponding water-treated rats, although there was no significant enlargement of adrenal glands after six hours. The dry weight of the larger adrenals was similar to that determined in the control group, hence, the percent dry weights of these were significantly reduced. The adrenal gland catecholamine content is also shown in Table 1. There were no significant alterations of the adrenal content of either adrenaline or noradrenaline after six or twelve hours of intoxication.

Within 14 hours of receiving the last administration of ethanol in the longer term study, 12 of the 15 Group E rats exhibited signs of moderate to severe ethanol withdrawal, demonstrating the prior existence of physical dependence. Blood ethanol levels determined 11 to 14 hours after the final dose of ethanol were undetectable in six of nine rats examined, all of these rats showed signs of a withdrawal syndrome. The remaining three rats showed signs of mild intoxication and had blood ethanol levels of 265 to 313 mg%.

TABLE 1

ACUTE EFFECTS OF ETHANOL ON ADRENAL GLAND MEASURES

Treatment	(μg/adrenal pair) NA	ADR	Adrenal Wet Wt. (mg)	Adrenal Dry Wt. (mg)	% Dry Wt. vs. Wet Wt.
6-Hour Water	5.94 (2.103)	21.70 (1.653)	68.52 (2.556)	24.82 (1.590)	36.26 (2.215)
6-Hour Ethanol	4.89 (0.476)	21.48 (1.414)	63.30 (1.983)	21.00* (0.504)	33.20 (0.357)
12-Hour Ethanol	4.60 (0.438)	21.31 (0.512)	79.74* (3.833)	23.56 (1.155)	29.84* (0.851)

*Significantly different compared to water-intubated rats, $p<0.05$.

TABLE 2

ADRENAL GLAND DRY WEIGHT AND PROTEIN DURING WITHDRAWAL†

Treatment	N	Adrenal Wet Wt.	Adrenal Dry Wt.	% Dry Weight	% Protein
Group S	5	27.43 (2.618)	9.77 (0.448)	36.45 (2.614)	13.77 (0.532)
Group E	5	35.03 (3.437)	12.95* (1.204)	37.15 (1.492)	13.47 (0.342)
Change (of Mean)		1.28X	1.32X	1.02X	0.98X

*Significantly different compared to Group S controls, $p<0.05$.
†Sixteen to eighteen hours after cessation of treatment.

Common signs of the abstinence syndrome included: hyperactivity (characterized by running episodes), excessive irritability (vocalization when handled), tail tremor or stiffness, limb and body rigidity, tail and body tremor, "wet body" shakes, and spontaneous or induced convulsions of a tonic clonic nature. Two of the remaining rats showed mild signs of withdrawal during the period immediately before they were killed (16–18 hours of withdrawal). The remaining rat did not exhibit signs of withdrawal during the first 24 hours, however, it remained quite inactive during the second day of withdrawal, an interval during which the other rats were highly active. Nine convulsions, all between 12 and 16.5 hours, were observed during the scheduled observation periods. Numerous spontaneous convulsions were noted in the home cages, but these were not included because the observation of these was neither systematic nor scheduled.

The adrenal gland weights of Groups I and S remained constant throughout the experimental period (Fig. 1). In contrast, however, there was a 1.4 fold increase in the adrenal weights of rats killed after 48 and 96 hours of ethanol intoxication. This enlargement persisted through the withdrawal and post-withdrawal periods. To assess the nature of the adrenal enlargement, dry weight and protein content were determined in adrenal glands from rats killed during the withdrawal phase (Table 2). At this time, adrenal measures

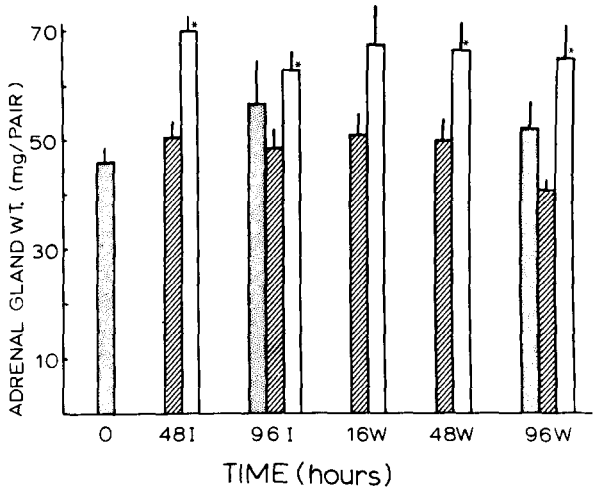

FIG. 1. Adrenal gland pair weight of rats during the intubation (I) period or after withdrawal of treatment (W). The treatments designated are Group I stippled box, Group S striped box, and Group E open box. There was a significant time, $F(5,43)=3.80$, $p<0.01$, and treatment, $F(1,43)=44.26$, $p<0.01$, effect between Groups S and E by analysis of variance.

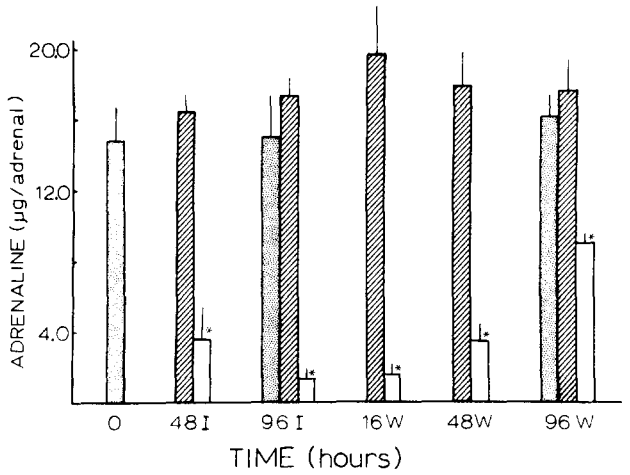

FIG. 2. Adrenal adrenaline content during (I) and after (W) the intubation period. The different treatments are Group I stippled box, Group S striped box, and Group E open box. Adrenaline content of Groups S and E showed a significant time, $F(5,44)=4.92$, $p<0.01$, treatment, $F(1,44)=216.58$, $p<0.01$, and treatment by time, $F(5,44)=11.42$, $p<0.01$, effect by analysis of variance.

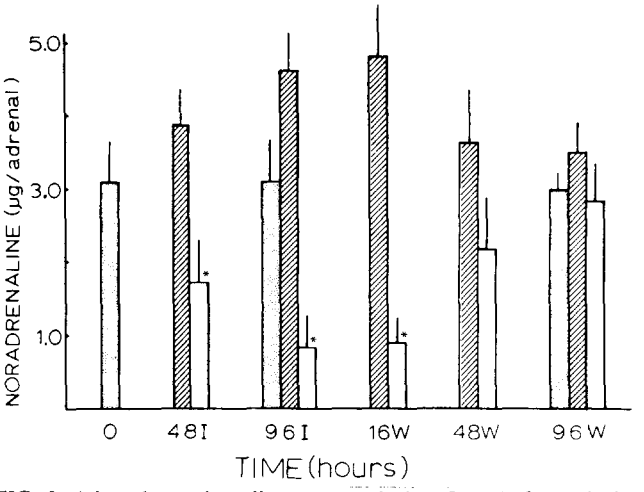

FIG. 3. Adrenal noradrenaline content during (I) and after (W) the intubation period. The different treatments are Group I stippled box, Group S striped box, and Group E open box. Noradrenaline content of adrenals from Group S and E rats showed a significant treatment, $F(1,44)=56.01$, $p<0.01$, and treatment by time, $F(5,44)=6.03$, $p<0.01$, effect by analysis of variance.

from Group E rats were significantly increased above control values. The magnitude of the change in protein content was proportional to the overall hypertrophy of the adrenal gland.

Stereological determinations of the glands from histologically prepared sections revealed that the cortical volume of adrenals from the sampled Group E rats (10.06 ± 0.281 mm^3) was 1.3 fold greater than those from the paired Group S controls (7.69 ± 0.913 mm^3). The magnitude of this enlargement was similar to the hypertrophy of the entire gland. On the other hand, there was no alteration in the volume of the adrenal medulla in rats subjected to the alcohol treatment. The medullary volume of adrenals from Group S rats (0.51 ± 0.062 mm^3) did not differ significantly from that determined for the Group E rats (0.44 ± 0.062 mm^3).

In Figs. 2 and 3, adrenaline and noradrenaline content of the adrenal glands are presented as content per gland, in contrast to concentration, which would have produced biased data by incorporating weights arising from cortical changes. There were no significant differences observed in adrenaline content between Group S and Group I rats at any time during the experimental period. Adrenals from ethanol-intoxicated rats contained markedly less adrenaline that those of their controls, with levels falling to 3.64 ± 1.740 µg/gland at 48 hours. At 96 hours of intoxication, and at 16–18 hours of withdrawal, adrenaline content was less than 1.6 µg/gland; levels that represent less than eight percent of those in control animals. Forty-eight hours after the last exposure to ethanol, when there were no obvious behavioural indications of the alcohol treatment, the levels of adrenaline increased. After an additional 48 hours of recovery content rose to 9.04 ± 0.459 µg/gland, but this was still significantly lower than found in the corresponding Group S control rats.

Adrenal noradrenaline content in Group S rats did not differ significantly from those taken from Group I rats over the course of the intubation period (Fig. 3). Adrenals from ethanol-treated rats demonstrated, however, a significant depletion of noradrenaline during the induction phase and in withdrawal. In these groups, noradrenaline declined to less than 20% of content in equivalent sucrose-treated control animals at the end of the induction period; this change extended into the withdrawal period. After 48 and 96 hours from the last administration of ethanol noradrenaline content had risen and did not differ significantly from that found in the corresponding Group S control rats.

Histological examination demonstrated that the processed adrenals of Group E rats, where the contralateral adrenal glands were markedly depleted of noradrenaline content, were devoid of the identifiable green localizations signifying noradrenaline-containing cells. On the other hand, adrenal sections from Group S rats had visible regional distributions of noradrenaline-containing cells, as did those from Group I. Examination of adrenal sections from Group E rats killed in the post-withdrawal period, after 48 and 96 hours, did not show the green stained areas characteristic of noradrenaline-containing cells, although the noradrenaline content of the contralateral adrenal had returned to normal.

The quantities of the major catecholamines found in the daily urine samples from Group S and Group E rats are presented in Fig. 4. The amounts of adrenaline and noradrenaline excreted from water-intubated rats were relatively stable throughout the experimental period. These did not differ from amounts excreted by the sucrose-treated animals over the course of the study. Values ranged from 0.32 ± 0.048 µg/day to 0.67 ± 0.109 µg/day for adrenaline and from 1.08 ± 0.390 µg/day to 2.86 ± 0.982 µg/day for noradrenaline.

Ethanol treatment significantly altered adrenaline excretion. There was a significant, seven-fold increase in urinary levels during the first 24 hours of ethanol intoxication. Enhanced levels persisted throughout the induction phase and into the first day of withdrawal. During the abstinence period the urinary adrenaline content remained nearly twice that of the sucrose-treated rats, but this difference was not significant. The pattern of noradrenaline excretion in the ethanol-treated rats resembled that of adrenaline excretion, although the magnitude of the change was much reduced. In general, there was a two-fold increase in excreted noradrenaline each

day of the intoxication period and in the first day of withdrawal. There were no differences in amounts excreted after this time.

DISCUSSION

The results of this study demonstrate that treatment of rats with divided doses of ethanol can sustain high blood ethanol levels for 96 hours, leading to the development of physical dependence. In contrast, the control rats of Group S did not exhibit any notable behavioural changes during the intubation procedure, or in the subsequent recovery phase.

As has been previously noted [3], repeated intubations of ethanol or sucrose solutions caused a decline in body weight. The loss of weight observed in this investigation resulted primarily from the reduction in caloric intake. The degree of weight loss in these animals was similar to that previously reported for intragastric intubation of alcohol [15,18] and liquid-diet methods of inducing physical dependence [7,20]. The control group for ethanol treatment received a calorically equivalent diet of sucrose [24].

In the short-term study adrenal enlargement was evident at twelve hours after the initial administration of ethanol. Dry weight determinations, however, revealed that this was not due to an increase in dry tissue mass, which suggests that the short-term weight change was associated with an increase in water content. In the longer duration study, however, adrenal dry weight and protein content were significantly greater after more than four days of treatment. These increases were proportional to the extent of adrenal enlargement. Continuous, severe ethanol intoxication appears to induce adrenal tissue hydration followed, within four days, by the formation of protein and a corresponding elevation of the dry tissue weight. Examination of adrenal sections showed that the cortex, rather than the medulla, was enlarged; the degree of cortical expansion paralleling adrenal gland hypertrophy. As there was no evidence of medullary expansion, results from the adrenal catecholamine determinations were presented to emphasize content per gland rather than concentration, which would erroneously incorporate weight arising from the cortical changes.

Adrenal cortical hypertrophy has been demonstrated using various experimental manipulations [10, 11, 16]. Mendelson *et al.* [17] showed that chronic ethanol ingestion in human alcoholics was associated with cortical activation, primarily through the hypothalamic-pituitary-adrenal axis. Ethanol induced a release of ACTH from the hypophysis, thereby, promoting adrenal activation. In the present investigation the observed cortical enlargement may be indicative of a similar elevation in anterior pituitary release of ACTH.

In 1956, von Euler estimated that adrenaline and noradrenaline are excreted in the urine in amounts which seem to be proportional to the quantities produced by the adrenal medulla and the sympathetic nerves. Various investigators [4, 14, 21, 22, 23, 28] have shown that administration of ethanol increases catecholamine excretion in several species of mammals, including man. Klingman and Goodall [14] further suggested that the magnitude of the alteration of urinary catecholamine levels correlates to the severity of the intoxication. Ogata and his colleagues [19] similarly demonstrated, in "spree" drinking alcoholics, that there was a parallel relationship between the severity of the intoxication and the increase in catecholamine excretion, thereby, implicating adrenal medullary stimulation.

The severe reduction in adrenal catecholamine content

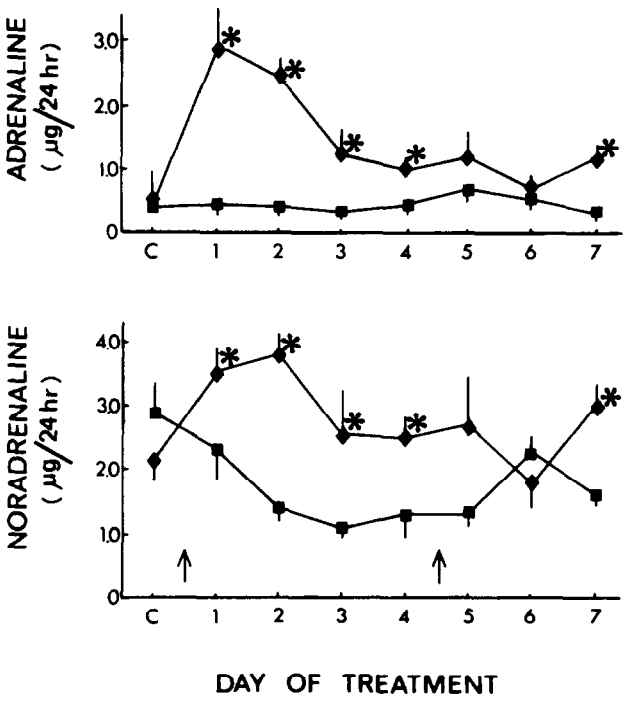

FIG. 4. Twenty-four hour urinary excretion of adrenaline and noradrenaline during the intubation and withdrawal periods. Arrows indicate the intubation phase, ◆ refer to the ethanol-treated and ■ the sucrose-treated groups. Adrenaline excretion showed a significant treatment, $F(1,10)=10.04$, $p<0.001$, time, $F(7,70)=12.00$, $p<0.01$, and treatment by time, $F(7,70)=5.85$, $p<0.001$, effect using analysis of variance. Noradrenaline excretion showed a significant treatment, $F(1,10)=27.32$, $p<0.001$, time, $F(7,70)=2.96$, $p<0.01$, and treatment by time, $F(7,70)=5.85$, $p<0.001$, effect using analysis of variance.

observed after prolonged ethanol intoxication would have led to a proportional increase in catecholamine release. This was reflected in the increases in urinary catecholamine levels. As Cottet-Emard *et al.* [9] have reported that dietary constituents can significantly influence urinary levels of catecholamines, the pair-fed rats of Group S were the control group for the rats receiving ethanol. Severe ethanol intoxication produced significant elevations in adrenaline and noradrenaline excretion: urinary adrenaline levels were more than six times greater than in controls during the first two days of intoxication, times during which adrenal content underwent the greatest degree of depletion; noradrenaline levels in the urine were increased during the same period, paralleling also the reduction in medullary content. Excretion of the catecholamines remained elevated throughout the remainder of the induction period, accompanying the further reduction in medullary content to even lower levels. Even so, more adrenaline was excreted in the final two days of ethanol intoxication than was present in the urine samples of control rats. This indicates that the ethanol exposure induces a persistent and extensive increase in catecholamine biosynthesis in the adrenal medulla. Taken together with the medullary content data, severe intoxication with ethanol is characterized by adrenaline release that exceeds the capacity of compensatory synthetic and uptake processes.

After the period of induction, rats withdrawn from ethanol exhibited signs of a moderate to severe abstinence syn-

drome. Adrenal catecholamine content in these rats did not differ from values determined at the end of the induction period. In the post-withdrawal phase, 48 and 96 hours after the last dose of ethanol, a time when rats are no longer showing signs of abstinence, noradrenaline content had increased and did not differ from controls. On the other hand, the recovery of adrenaline was not complete. Adrenaline content increased into the post-withdrawal period, but was still significantly depressed even 96 hours after the cessation of ethanol administration. Continued disruptions of the medulla were further indicated by the lack of appearance of localizations of noradrenaline-containing cells, even though the content of this amine had returned to normal in the post-withdrawal phase.

Urine samples were obtained from rats exhibiting signs of a severe ethanol withdrawal syndrome. The excretion of catecholamines, during the interval corresponding to the phase of the abstinence syndrome, exceeded control levels, but did not differ from quantities excreted on the final day of intoxication. There were subsequent reductions in levels of urinary catecholamines on the next day and increases further into recovery. While these may be associated with alterations in medullary synthetic and release processes occurring after severe intoxication, this data is confounded by the fact that the previously intoxicated rats consumed more food on the last day, thereby providing more substrate for catecholamine synthesis [9].

Studies that have investigated the influence of ethanol on peripheral sympathetic activity have concentrated on effects resulting from a single application of ethanol [8, 14, 21, 22, 23] or following long-term exposure [8, 19, 20]. The short-term effects of ethanol treatment have been mentioned previously. Chronic exposure studies have shown alterations in adrenal function after a prolonged period, but the establishment of physical dependence is left in doubt. Nonetheless, long-term studies have shown small increases in medullary adrenaline content and synthetic capability, indicative of a compensatory process.

In the present investigation, prolonged, severe ethanol intoxication produced a sustained release of adrenal medullary catecholamines and cortical hypertrophy during an interval in which unequivocal physical dependence developed. The medullary changes persisted through the period in which behavioural disturbances associated with the withdrawal syndrome were evident. Following this, the recovery of adrenaline content was significantly slower than that of noradrenaline, which may indicate differential compensation by the medullary chromaffin cells in the synthesis, uptake, storage and/or release of catecholamines after exposure of rats to severe ethanol intoxication.

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