Mechanism of the alcohol cyclic pattern: role of the hypothalamic-pituitary-thyroid axis

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Received 14 June 1999; accepted in final form 12 April 2000

CHRONIC ETHANOL FEEDING at a constant rate (10–14 g·kg⁻¹·day⁻¹) causes a cyclic oscillation in the blood and urinary alcohol levels (UALs) (18). Ethanol levels peak at ~500 mg/100 ml and then fall to ~100 mg/100 ml over a six-day cycle. The ethanol elimination rate increases at the time that the blood ethanol levels are falling and vice versa; the ethanol elimination rate decreases when the blood ethanol levels are rising during the cycle (18). This correlation between the ethanol elimination rate and the rise and fall of the blood ethanol level suggests that the cycling is due to fluctuations in alcohol dehydrogenase activity. However, the mechanism that drives these cyclic fluctuations has not been determined. Efforts to demonstrate a role for changes in cytochrome P-450 2E1 levels in the liver using 2E1 inhibitors failed to explain the mechanism for the cyclic fluctuations (2). It is important to determine the mechanism of the cyclic fluctuations since an autoregulatory system that influences the rate of ethanol elimination is probably involved. This autoregulation mechanism, if understood, might be exploited to increase the rate of elimination of ethanol in the blood in a clinical setting when people overdose on alcohol. There is also evidence that the magnitude of the cyclic fluctuations observed correlates with the severity of the associated liver pathology (17). Therefore, the study of the cycling mechanism may provide insights regarding the pathogenesis of alcoholic liver disease (ALD).

While monitoring daily body temperature during the cycle, it was noted that the body temperature increased when the UALs fell and decreased when UALs rose (9). This led to the daily measurement of O₂ consumption, which was also found to cycle in sync with the change in body temperature (10). The present study is designed to answer the question, What are the roles that the swings in body temperature and O₂ consumption rates play in driving the cyclic increase and decrease in UALs during ethanol feeding? Body temperature and the metabolic rate could change in response to changes in circulating endotoxin (1) and the endotoxin-induced increase in circulating interleukin (IL)-1 and tumor necrosis factor (TNF)-α. Endotoxin and TNF-α levels increase in the blood in the intragastric ethanol feeding rat model used here (14) and in clinical ALD (13). Alternatively, the body temperature and metabolic rate could cycle in response to changes in circulating hormones such as T₄. Accordingly, we studied changes in the levels of endotoxin, IL-1, and TNF-α as well as T₄ levels at the peaks and troughs of the urinary cycle. To document this, whole body O₂ consumption rate was monitored daily during the cycle. The rate of O₂ consumption correlated positively with the change in body temperature and negatively with the change in UAL. Since the metabolic rate responds to changes in body temperature, thyroid hormone levels were measured during the UAL cycle. T₄ levels correlated positively with the O₂ consumption rate and negatively with the UALs. In a second experiment using propylthiouracil treatment, UALs did not cycle and a fall in body temperature failed to stimulate an increase in the rate of ethanol elimination. Consequently, rats died of overdose. Likewise, in a third experiment using rats with severed pituitary stalks, UALs failed to cycle and rats died of overdose. From these observations it was concluded that the UAL cycle depends on an intact hypothalamic-pituitary-thyroid axis response to the ethanol-induced drop in body temperature by increasing the rate of ethanol elimination.

Acknowledgments

This study was supported by a grant from the National Institute of Alcohol Abuse and Alcoholism (AA07162).숙주용의 실험은 National Institute of Alcohol Abuse and Alcoholism (AA07162)의 후원으로 수행되었습니다.

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ethanol cycle caused by continuous feeding of ethanol at a constant rate.

METHODS

Animals. Three animal feeding experiments were performed using the intragastric tube feeding model as previously described (19). In experiment 1, six male rats weighing ~270–280 g were fed ethanol at 13 g · kg⁻¹ · day⁻¹ for 2–3 mo. Six control rats were pair fed a dextrose solution that was isocaloric with the ethanol solution fed to ethanol-fed rats. The diet was fed to provide 271 kcal · kg⁻¹ · day⁻¹ in both groups. The diet used was modified to include a salt and vitamin mix (Dyets, Bethlehem, PA) as described by the American Institute of Nutrition Mineral Mix and Vitamin Mix for Optimum Growth of Rats (10). The source of protein was lactalbumin. The diet was supplemented with 500 mg choline and 1 g methionine per liter of diet. The dietary calories derived were 33.3% from fat, 25.9% from protein, 6.7% from dextrose, and 34% from ethanol. In the second experiment, five rats were fed ethanol together with their pair-fed controls for 18 days at 10 g · kg⁻¹ · day⁻¹ ethanol, and at day 19 the ethanol was increased to 11 g · kg⁻¹ · day⁻¹. In this second experiment, propylthiouracil (PTU) was fed at 50 mg · kg⁻¹ · day⁻¹ (12) to both groups. In the third experiment, ethanol (10–11 g · kg⁻¹ · day⁻¹) and the diet were fed intragastrically for 2 wk after pituitary stalks were cut. The procedures were approved by the Research and Education Institute Animal Care Committee in accordance with the guidelines for animal care as described by the National Academy of Sciences (1996).

Experiment 1 was carried out as outlined in Fig. 1. UAL was measured daily. Urine was collected under toluene over 24 h. Stool weight was measured on 24 h collections. Body temperature was measured rectally each day using a thermometer. Body weight was measured daily to determine the dietary calories fed. Blood was drawn monthly (0.6 ml retroorbitally) as well as at some of the peak and trough UALs. Endotoxin levels were determined using endotoxin-free collection tubes and a chromogenic assay kit (Whitaker Bioproducts). UALs were measured using a Radiative Energy Attenuation assay (Abbott AXSYM system; Abbott Labs, Abbott Park, IL). T₄ levels were measured using fluorescence polarization immunoassay (FPIA) (Abbott AXSYM). Alanine aminotransferase (ALT) levels were measured using

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**METHODOLOGY (Experiment #1)**

**Male Wistar Rats**

**Operation**

**Intragastric Cannula**

- Blood Sample Baseline
- Initial Body Weight (270-280 g)

**Diet Regimen**

- Pair fed Diet + Dextrose
- 8% Ethanol + Diet (13 g /Kg/ Day)

**Daily Measurements for 20 Days**

- O₂ consumption Rate (ml/kg/min)
- Urinary Alcohol
- Body Temperature
- Body Weight

**Death After 70 Days**

- Body Weight
- Blood Sample
- Liver Weight
- Liver Histology
- Liver Fast Frozen

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Fig. 1. Experimental design for experiment 1.
The rats were first subjected to pituitary stalk severance with aluminum foil interposed to prevent reestablishment of blood flow from the hypothalamus to the pituitary. They were operated on by Zivic-Miller Laboratories (Porterville, PA). This experiment tested the supposition that the cycle was dependent on the change in body temperature. The drop in body temperature caused by high UALs initiated hypothalamic secretion of thyrotropin-releasing hormone (TRH). The urinary ethanol cycle and body temperature were monitored as before. The blood levels of TSH, T₄, ALT, and ethanol were measured after 2 wk of receiving 10 g ethanol · kg⁻¹ · day⁻¹. At this point, the ethanol dose was increased to 11 g · kg⁻¹ · day⁻¹ as in the PTU-feeding experiment (experiment 2). The UALs and the body temperature were then monitored daily until the death of the rats, which was caused by terminal ethanol toxicity.

RESULTS

Experiment 1. The rats were monitored for 72 days. The rats in experiment 1 gained weight as follows. Starting weight for the ethanol-fed rats (n = 5) was 274 ± 9.9 g and for the pair-fed controls was 266 ± 6.9 g. The ending weight for the ethanol fed rats was 410 ± 9.7 g and for the pair-fed controls was 462 ± 22.5 g. The difference was significant (P < 0.001), indicating that energy wastage had occurred in the ethanol-fed rats, perhaps due to heat generation induced by the ethanol. The liver weights were likewise significantly different in the two groups (P < 0.001). The livers of the ethanol-fed rats weighed 22.3 ± 2.3 g compared with those of the pair-fed control rats, which weighed 14.5 ± 0.8 g.

The UALs fluctuated between 50 and 500 mg/dl at variable intervals (Fig. 2). The body temperature (Fig. 3) and the O₂ consumption rate (Fig. 4) fluctuated at variable intervals (Fig. 2). The body temperature

In experiment 3, five ethanol fed and five pair-fed control male Wistar rats weighing ~250 g were fed the diet intra-gastrically for 2 wk. Ethanol was fed at 10 g · kg⁻¹ · day⁻¹ for 2 wk. The rats were first subjected to pituitary stalk severance with aluminum foil interposed to prevent reestablishment of blood flow from the hypothalamus to the pituitary. They were operated on by Zivic-Miller Laboratories (Porterville, PA). This experiment tested the supposition that the cycle was dependent on the change in body temperature. The drop in body temperature caused by high UALs initiated hypothalamic secretion of thyrotropin-releasing hormone (TRH). The urinary ethanol cycle and body temperature were monitored as before. The blood levels of TSH, T₄, ALT, and ethanol were measured after 2 wk of receiving 10 g ethanol · kg⁻¹ · day⁻¹. At this point, the ethanol dose was increased to 11 g · kg⁻¹ · day⁻¹ as in the PTU-feeding experiment (experiment 2). The UALs and the body temperature were then monitored daily until the death of the rats, which was caused by terminal ethanol toxicity.

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a clinical laboratory analyzer (a kinetic rate method) on SYNCH RON CX Systems (Beckman Instruments, Brea, CA). TNF-α and IL-1β levels were measured using ELISA kits (Biosource International, Camarillo, CA) per the manufacturer's instructions. O₂ consumption was measured using an OxyMax (Columbus Instruments, Columbus, OH). Basal levels were determined over a 30-min monitoring period at 30-s intervals, and the lowest data obtained was used for statistical analysis using Bonferroni's t-test. Liver tissue obtained at the termination of the experiment was processed for light microscopy. The pathology was scored as reported previously (17).

Experiment 2 was designed to test the role of the thyroid gland in the mechanism of the urinary alcohol cycle. Four groups of five male Wistar rats each were fed the same diet as in experiment 1. In addition, the control group (group 1) was fed dextrose in amounts isocaloric to the ethanol fed in group 4. Group 2 was fed dextrose in amounts isocaloric to ethanol fed in group 4 plus PTU (50 mg · kg⁻¹ · day⁻¹). Group 3 was fed ethanol isocalorically to group 4. Group 4 was fed ethanol (10 g · kg⁻¹ · day⁻¹) and PTU (50 mg · kg⁻¹ · day⁻¹). The ethanol fed was 10 g · kg⁻¹ · day⁻¹ because higher doses, such as 11 g · kg⁻¹ · day⁻¹, killed rats in preliminary studies.

The rats were monitored as shown in Fig. 1 for 19 days, except that body temperature was used as a measure of metabolic rate because body temperature correlated positively with the O₂ consumption rate in experiment 1. On day 19, the dose of ethanol was increased to 11 g · kg⁻¹ · day⁻¹ and the UALs and the body temperatures were monitored daily until death on the 25th or 26th day. Liver and thyroid tissues were obtained at autopsy to determine the histopathology. T₄ was measured by FPIA technology. Thyroid-stimulating hormone (TSH) levels were measured by radioimmunoassay.

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the body temperature fluctuated at the same time intervals as the O\textsubscript{2} consumption (Fig. 5). When the body temperature and the O\textsubscript{2} consumption rates were graphed together, the peaks and troughs were superimposed on each other. This correlation was statistically significant for all five rats fed ethanol (R = 0.894, P < 0.001). When the rate of O\textsubscript{2} consumption was compared with the UAL, the peaks and troughs correlated inversely. (Fig. 4). Likewise, when the UAL went up, the body temperature went down and vice versa (Fig. 3).

When the peaks and troughs of the UALs from all of the ethanol-fed rats were correlated with the corresponding O\textsubscript{2} consumption rates, there was a significant difference between the peak and trough values and between the peak and control values (P < 0.001) but not between the trough and control values (Fig. 5). This indicated that the O\textsubscript{2} consumption rates at the trough of the UALs were not different from the normal O\textsubscript{2} consumption rates. Thus the abnormality occurred at the peaks of UAL. A similar relationship between the UAL peaks and troughs existed with the body temperature, except that the data from the controls was significantly different from both the peaks and troughs (Fig. 6). The total UAL and O\textsubscript{2} consumption rates from individual ethanol-fed rats correlate negatively (R = -0.472; P < 0.001). Likewise, UAL correlated negatively with body temperature (R = -0.921, P < 0.001).

There is a question as to whether or not there was a difference in the liver injury at the peaks and troughs in the cycle of UAL. When the serum ALT levels were measured at the peaks and troughs, a significant increase was found at the trough (P < 0.05) compared with the control and the peaks (Fig. 7). This was substantiated when serum ALT levels were correlated with the corresponding UALs (R = -0.879; P < 0.001).

Histology of livers from the rats fed ethanol for 72 days showed both macro- and microvesicular steatosis in the centrilobular hepatocytes. Apoptosis, megamitochondria, and mononuclear inflammation were also present. Controls appeared without pathological abnormalities.

The histopathology of the livers was significantly different when the pathology scores for the ethanol-fed rats were compared with the pathology scores for the
pair-fed control rats. The pathology scores for the ethanol-fed rats were 5.6 arbitrary units compared with the pair-fed control rats, which showed no pathological change ($P \leq 0.001$). (Pathology score units were 0 to 10.)

Endotoxin levels in the serum were higher at peak UALs compared with the trough values (peak $= 3.1 \pm 0.8$ and trough $= 1.1 \pm 0.2$ EU/ml; $P \leq 0.044$, $n = 18$). (EU unit + endotoxin unit per Limulus Amebocyte Lysate method supplied by BioWhittaker) Plasma TNF-α levels were elevated equally at both the peak and trough of the UAL (control $= 36 \pm 3$, peak $= 112 \pm 36$, and trough $= 100 \pm 25$ pg/ml; $P \leq 0.004; n = 18$). IL-1β levels tended to be elevated but not at significant levels (control $= 27.9 \pm 3.5$, peak $= 38.3 \pm 5.6$, and trough $= 39.5 \pm 8$ pg/ml). Serum T$_4$ levels were decreased in the ethanol-fed rats at 1 mo compared with controls (ethanol-fed $= 1.9 \pm 0.5$ and control $= 5.1 \pm 0.2$ μg/dl; $P \leq 0.001; n = 5–6$ rats). When T$_4$ levels were measured at the peak and trough UALs, they were significantly lower than at the peak and control levels (Fig. 8). There was a negative correlation between the T$_4$ levels and UALs when only the peak UALs were compared ($R = -0.961; P \leq 0.001; n = 10$ rats). When the UAL peak, trough, and control levels of TSH were measured, no significant differences were found (Fig. 9).

**Experiment 2.** Rats fed ethanol and PTU for 19 days had relatively stable UALs, varying between 100 and 200 mg/100 ml. Likewise, the body temperatures fluctuated only slightly (35–36.5°C; Fig. 10). However, over the first 13 days the UAL went from 100 to 200 mg/100 ml and the body temperature fell 1.5°C, suggesting that the body temperature fall was a response to the increase in the level of alcohol. However, when the dose of ethanol was increased to $11 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ on day 19, the UAL increased daily (Fig. 10). When the UAL reached 350 mg/100 ml on day 25, the body temperature began to fall (32°C). As the UALs increased, the body temperature continued to plummet.

![Fig. 7. Graph showing that the alanine aminotransferase (ALT) levels were significantly higher ($P \geq 0.05$) in the trough ($n = 10$) than in control ($n = 5$) or peaks ($n = 10$).](image)

![Fig. 8. T$_4$ levels in serum taken when the UALs were at peak and trough in rats fed ethanol. The values at the peak were significantly lower than the trough ($P \leq 0.03$) and the pair-fed controls ($P \leq 0.003$). Values are means ± SE; $n = 10$ serum samples.](image)

![Fig. 9. Thyroid-stimulating hormone (TSH) levels in serum taken when the UALs were at the peak and trough (TR), from pair-fed controls (C) or pair-fed controls given propylthiouracil (PTU) (CA) or not given PTU (CB), or from ethanol-fed rats given PTU (ROH A) or not given PTU (ROH). PTU treatment increased TSH levels significantly in the control and ethanol-fed rats ($P < 0.001$). Values are means ± SE; $n = 5$ rats.](image)
until fatal levels of UAL were reached (700 mg/100 ml). At this time the body temperature was 25°C. All five rats given PTU showed the same pattern, i.e., the body temperature fell gradually until the UAL reached 300–450 mg/100 ml, at which time the body temperature dropped sharply and the rat died. Thus PTU treatment prevented the increase in metabolic rate normally induced by rising UAL and falling body temperature, presumably because thyroid secretion of T₄ did not increase. PTU treatment, therefore, blocked the UAL-body temperature cycle. Control rats given PTU had stable body temperatures over a 29-day period. T₄ levels were markedly reduced in both the ethanol-fed and pair-fed control rats given PTU. T₄ levels in ethanol-fed rats that were not given PTU were decreased below the levels of the control rats not given PTU (Fig. 11). TSH levels were significantly elevated in the rats given PTU (Fig. 9). The ethanol-fed rats without PTU had reduced levels of TSH compared with pair-fed controls (Fig. 9).

The liver histopathology of the rats fed ethanol and given PTU showed a shift in the location of the steatosis to the periportal region of the lobule, and the fat was microvesicular. The thyroid glands of all the PTU-treated rats showed diffuse follicular hyperplasia.

**DISCUSSION**

The pathogenesis of the UAL cycle has eluded investigators since its first description (18). In an early study, it was suggested that the cycling of the urine and blood ethanol levels were due to a two-step induction of cytochrome P-450 2E1 (3). In that study, 24-h UALs were excellent indicators of the blood alcohol levels in the intragastric tube feeding rat model. More recently, it was shown that inhibitors of cytochrome P-450 2E1 did not abolish the cycling of the UALs (2), leaving the mechanism of the cycle unresolved. In the current study, it was noticed that the cycling of the body temperature peaked when the UAL reached the...
trough. Since $O_2$ consumption rates parallel the changes in body temperature, these rates were measured, and it was found that the resulting data correlated positively with body temperature and negatively with the corresponding UALs.

What is the connection between the increased metabolic rate as indicated by the increased rate of $O_2$ consumption and the decrease in UALs? We postulate that the increased metabolic rate increases the ethanol elimination rate by generating NAD$^+$ from the increase in the rate of mitochondrial respiration. The rate of the oxidation of ethanol and acetaldehyde is limited by the availability of NAD$^+$, a cofactor with alcohol and acetaldehyde dehydrogenase. We measured the levels of alcohol dehydrogenase (ADH) apoprotein and cytochrome P-450 2E1 apoprotein by Western blot, and it was found that the levels were not significantly different at the UAL peaks and troughs (unpublished observations). Since ADH and cytochrome P-450 2E1 did not vary, this adds credence to the idea that the level of available NAD$^+$ is the variable that accounts for the difference in the rate of alcohol oxidation at the UAL peaks and troughs. However, most investigators feel that enzyme concentration is more important, since NAD$^+$ has been reported to regenerate at rates greater than necessary for the rates of ethanol metabolism (5, 6).

What is the mechanism of the increased metabolic rate that occurs when the UALs fall? We postulate that the metabolic rate increases as a result of increased blood levels of $T_4$ released from the thyroid, since the body temperature and the rate of $O_2$ consumption are regulated by $T_4$ levels (10). In the present study, $T_4$ increased when the body temperature and $O_2$ consumption rates peaked, as expected. If the $T_4$ cycling was mechanistically involved in the UAL cycle, PTU inhibition of $T_4$ synthesis by the thyroid should then eliminate the UAL cycle. Such proved to be the case. However, the change in $T_3$ between the UAL peaks and troughs is small and, while suggestive, the data are far too limited to conclude that the urine alcohol cycling is due to changes in the levels of $T_4$. Further studies are required. For instance, thyroid hormones are potent and reversible inhibitors of alcohol oxidation catalyzed by isozymes of class I and II ADH (12a). This would suggest that UALs may rise at the trough because the elevated $T_4$ levels inhibit the oxidation by ADH. Against this idea, however, is the finding that when the $T_4$ levels were reduced by PTU treatment or stalk severance, the rate of alcohol oxidation fell from 13–14 g · kg$^{-1}$ · day$^{-1}$ to 10 g · kg$^{-1}$ · day$^{-1}$.

What is the reason for the increase in $T_4$ blood levels during the cycle? $T_4$ levels are regulated by hypothalamic neuronal receptors, which are sensitive to cold and heat (11). Therefore, we postulate that when the UALs become elevated during the cycle to the point at which the rat becomes hypothermic, the cold temperature-sensitive neurons in the hypothalamus are stimulated to secrete TRH into the portal circulation of the pituitary stalk. To test this idea, rats with their pituitary stalks severed were subjected to ethanol feeding intragastrically. This would interrupt the cycle by disconnecting the hypothalamic thermoregulator mechanism and thus prevent UAL cycling, just as the PTU treatment did. This theory depends on the fact that ethanol, like other strong sedatives, depresses the body temperature by reducing the reactivity of the hypothalamus temperature controller (11) when the blood ethanol levels become sufficiently elevated during the cycle, i.e., $-400$ mg/100 ml UAL. Severing the stalk did prevent the cycling. Therefore, we suggested that the UAL cycle was initiated by a drop in body temperature caused by rising UALs.

As the UALs rise, the body temperature slowly falls over several days. When it drops to 36.5°C or lower, it abruptly increases to peak within 1 day to $-38°C$. Then the cycle begins again. It appears that this abrupt and dramatic increase in body temperature initiates the fall in the peak UAL. If the hypothalamic-pituitary-thyroid axis is interrupted by cutting the stalk or by giving PTU, then the blood ethanol levels continue to rise and the body temperature continues to fall. The rat then dies of ethanol overdose. These findings support the idea that ethanol overdose would respond to therapeutic hyperthermia, but this has not yet been tested with the intragastric tube feeding rat model.

There are questions left unanswered regarding these hypotheses. First, why does the cycle take several days and why is there a delay in onset between the peak UAL and the increase in metabolic rate at the trough UAL? The answer to both questions requires further experiments.

The second question yet to be answered is, What is the proof that NAD$^+$ levels fluctuate during the cycle? Ethanol metabolism causes a shift in the redox state when the NAD$^+$/NADH ratio changes to a reduced state. This is further worsened by the centrilobular hypoxia induced by chronic ethanol ingestion (7). However, it remains to be shown that the ratio changes between the UAL peaks and troughs in a cyclic manner. Data on the levels of NAD$^+$ in freeze-clamped livers taken at the UAL peaks and troughs indicate that the levels of NAD$^+$ are higher at the troughs (unpublished observations).

The UAL cyclic phenomenon may have some relevance to the pathogenesis of ALD. For example, the serum ALT levels were higher at the troughs of UAL compared with the ALT levels when UAL peaked, indicating that the liver injury may be occurring at a time when $O_2$ consumption is increased. Israel et al. (12) reported that PTU prevented liver necrosis when ethanol-fed rats were subjected to hypoxia, suggesting that the metabolic rate was increased in the ethanol-fed rats and that this increase made the livers susceptible to damage caused by hypoxia. High blood ethanol levels did increase the vulnerability of the liver to hypoxia when the intragastric tube feeding rat model was used (8). This may be how PTU therapy worked in patients with ALD, since hypothyroidism had to first be established before survival improved. (15). Of course, PTU may protect the liver from injury through
a number of other mechanisms, such as inhibiting myeloperoxidase or preventing free radical damage (16). PTU protection of the liver in the ethanol-fed rats was apparent, in that the fatty liver was microvesicular and not macrovesicular as was seen in the ethanol-fed rats to which no PTU was fed.

Initially, the fluctuation in body temperature during the UAL cycle was suspected to be in response to endotoxin. Endotoxin injected into the portal vein induced an increase in O2 consumption (1) and body temperature elevation, probably through an increase in the serum IL-1 and TNF-α levels. Endotoxin levels are increased in the serum in ALD, as are the IL-1 and TNF-α levels (13). The serum endotoxin levels were higher, however, at the peaks rather than the troughs of the UAL cycles in the present study. Also, the serum TNF-α levels were elevated equally at the peaks and troughs of the UAL cycle. The IL-1 serum levels were not significantly elevated either at the peaks or the troughs of the UAL cycle. It was concluded that the UAL was not due to oscillation of the serum endotoxin levels.

It is concluded that UAL cycling requires an intact hypothalamic-pituitary-thyroid axis. The signal that initiates the reversal of the rising UAL may be the fall in UAL by stimulating the cold-sensitive neurons in the hypothalamus.

We acknowledge the assistance of Adriana Flores, who typed the manuscript, and Charles Peoples, who prepared the glossy prints.

This study was supported by a National Institute of Alcohol Abuse and Alcoholism Grants AA-8116 and AA-P50–11999.

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