Dopamine-Related Tetrahydroisoquinolines: Significant Urinary Excretion by Alcoholics After Alcohol Consumption

Abstract. *Concentrations of dopamine-related tetrahydroisoquinolines (salsolinol and O-methylated salsolinol) were significantly higher in the daily urine samples of alcoholic subjects admitted for alcohol detoxification than in the daily urine samples of nonalcoholic control subjects. Salsolinol concentrations in alcoholic subjects appeared to drop to trace (control) values 2 to 3 days after admission, following the disappearance of ethanol and its reactive metabolite acetaldehyde from the blood. These results indicate that physiologically active tetrahydroisoquinolines increase in humans during long-term alcohol consumption, presumably because of acetaldehyde's direct condensation with catecholamines. The presence of these or similar condensation products in the urine could be useful as clinical indicators of prior blood acetaldehyde concentrations in chronic alcoholics.*

Under some circumstances, catecholamine neurotransmitters, particularly dopamine (DA), may be diverted from normal metabolic pathways through irreversible condensation with aldehydes or α-keto acids. This route, which produces tetrahydroisoquinolines (TIQ's), may become relatively significant in the illnesses related to alcoholism [because of ethanol-derived acetaldehyde (*1*) or biogenic aldehydes (*2*)], in Parkinson's disease during L-dopa therapy (*3*) (because of the elevated concentrations of DA or hydroxylated phenylpyruvates), and in phenylketonuria (*4*) (from increased tissue phenylpyruvate). The TIQ's derived from DA have now been identified and measured in the urine of L-dopa–treated parkinsonian patients (*3, 5*) and of children with phenylketonuria (*6*), but there has been no examination for TIQ's in fluids or tissues of alcoholics.

Because condensation products may have pathophysiological roles in alcohol dependence (*7*) and alcohol preference (*8*), we studied the possibility of their excretion in the urine of chronic alcoholic patients entering an alcohol detoxification program (*9*). Two sensitive and specific chromatographic assays were used—high-performance liquid chromatography with electrochemical detection (*10*) and electron-capture gas chromatography (*11*). We found that during the first 24 hours of detoxification, alcoholic subjects excreted significantly greater quantities of salsolinol, the apparent TIQ derivative of DA and acetaldehyde, than did nonalcoholic controls. Also, nearly five times as much mono-*O*-methylated salsolinol was measured in the urine of alcoholics during the first 24 hours of detoxification than in the urine of controls.

We used as subjects male alcoholics (ages 37 to 54) admitted to the alcohol detoxification unit at Hines Veterans Administration Hospital. Nonalcoholic control subjects were in the same age range

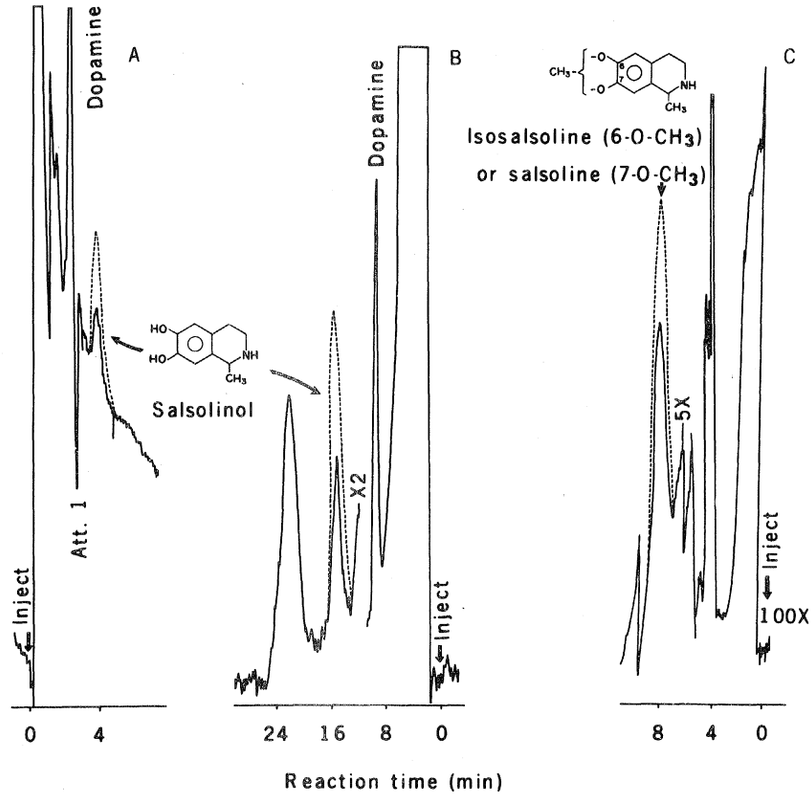

Fig. 1. Chromatograms of urine of alcoholic subjects. Urine was collected in 6*N* HCl (50 ml), refrigerated, and measured into equal portions (10 ml) that were syringed into Vacutainers and frozen until the TIQ and creatinine determinations. The urine (pH $\leq$ 2) was heated for 20 minutes at 80°C to hydrolyze any conjugated TIQ's. Internal standards (salsolinol and isosalsoline) were added to selected tubes prior to hydrolysis, as well as to blank tubes containing aqueous HCl (pH 2). The tubes were centrifuged for 10 minutes and the supernatants decanted and carefully adjusted to pH 7.8 to 8.3 with 1*N* NaOH. Activated alumina (100 mg) was added, the tubes were shaken for 15 minutes, and the supernatant was removed. The alumina was washed three times with distilled H_2O (3 ml) and shaken for 15 minutes with 1*M* acetic acid (HOAc; 1 ml). One half of the filtered HOAc eluate was lyophilized and prepared for chromatographic analysis with heptafluorobutyric anhydride (*11*). The derivatives in ethyl acetate were injected (1 to 2 μl) into a Varian 2100 gas chromatograph equipped with a 6-foot glass column containing 5 percent OV-17 (165°C), a tritium electron-capture detector (d-c), and a CDS 111 integrator. (A) Chromatogram demonstrating the presence of salsolinol (solid peaks) and the potentiation of the endogenous component with added heptafluorobutyric anhydride–derivatized salsolinol (dotted peak). Overall recoveries were 50 to 70 percent. The estimation of salsolinol by high-performance liquid chromatography was made directly on 100 μl of the HOAc eluate from alumina. We used a Vydac (SCX) cation exchange column, a 0.025*M* HOAc + 0.05*M* Na_2HPO_4 buffer (pH 5.1) at a flow rate of 0.8 ml/min, and a carbon-paste electrochemical detector (> 0.75 V). (B) Chromatogram showing the presence of salsolinol (solid peak) and the increase in size due to added salsolinol (dotted peak). Recoveries varied from 45 to 65 percent. To assay for *O*-methylated salsolinol, equal portions of hydrolyzed urine or pH-adjusted supernatants from alumina were diluted with 10 ml of borate buffer (0.5*M* boric acid brought to pH 10.2 with 10*M* NaOH) and extracted with 15 ml of toluene and isoamylalcohol (3 : 2). The two-phase system was centifuged and the aqueous layer allowed to freeze in a Dry Ice and acetone bath. The organic layer was decanted and back-extracted with 1*M* HOAc (1 ml). The HOAc extract was injected (100 μl) onto the Vydac column attached to an electrochemical detector (> 0.85 V) and eluted at 1.1 ml/min with 0.025*M* HOAc and 0.150*M* Na_2HPO_4 buffer (pH 6.7). (C) Chromatogram showing the presence of endogenous isosalsoline and salsoline (solid peak), which is increased by added isosalsoline (dotted peak). Recoveries of isosalsoline were 70 percent. Average concentrations of creatinine in the urine (obtained with a Pierce Creatinine II Rapid-Stat kit) and urine volumes were not significantly different between the two groups.

0036-8075/79/1207-1184$00.50/0

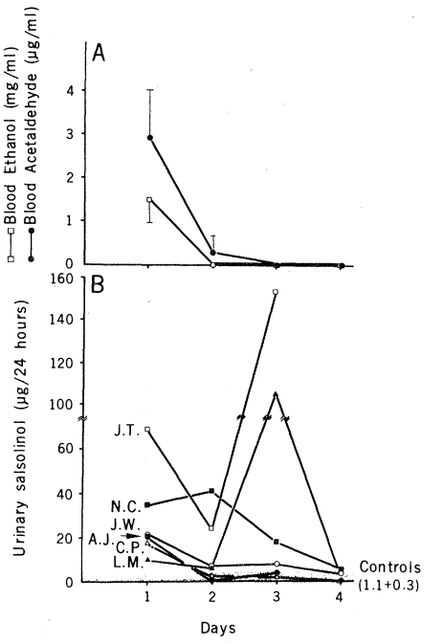

Fig. 2. (A) Blood ethanol and acetaldehyde concentrations in six alcoholic subjects on admission (day 1) and on three successive mornings. Data are expressed as means ± standard errors. (B) Total urinary salsolinol concentrations, corrected for recoveries, in four successive 24-hour collections from six alcoholic patients after their admission for alcohol detoxification, compared to the range (stippled area) for eight control subjects.

and were psychiatric in-patients at the hospital. Both groups were on the same daily menu and between-meal consumption (snacks, beverages) was restricted (*12*). After obtaining their informed consent, we drew venous blood samples from the alcoholic subjects shortly after their admission and once each morning on three successive days. Bloods were analyzed for acetaldehyde and ethanol by flame-ionization gas chromatography using a procedure that minimizes artifactual acetaldehyde production (*13*). Twenty-four-hour urine samples were obtained from both groups, beginning with a sample taken at admission for the alcoholics.

Representative chromatograms for total (conjugated and unconjugated) urinary salsolinol and its *O*-methylated derivatives are shown in Fig. 1. Individual salsolinol excretion patterns were determined for six alcoholic subjects (Fig. 2). The average 24-hour excretion of salsolinol for alcoholics admitted with mean blood acetaldehyde and ethanol concentrations of 3 μg/ml and 1.6 mg/ml, respectively, was 28.8 ± 8.7 μg; this is significantly different ($P < .01$, *t*-test) from the traces of a possible salsolinol component found in control subjects (average, 1.1 ± 0.3 μg/day for eight controls during 23 collection days; range, 0 to 4.8 μg/day). With the exception of two inexplicably elevated salsolinol values on day 3 [in subjects L.M. and J.T. (see Fig. 2), perhaps the result of surreptitious ingestion of alcohol or TIQ-containing foods], the average daily excretion of salsolinol in alcoholics appeared to return to normal as blood acetaldehyde disappeared.

The total excretion of *O*-methylated salsolinol (isosalsoline and salsoline chromatograph as one component) for eight alcoholics during the first 24 hours after their admission averaged 111.4 ± 13.7 μg (Fig. 3)—significantly more ($P < .01$) than the mean amount (20.6 ± 7.1 μg) measured for six nonalcoholic control subjects. Mass spectral analysis of the urinary *O*-methylated salsolinol component derivatized with trifluoroacetyl (TFA) anhydride was carried out on a Finnegan gas chromatography–mass spectrometry system (model 4000). The electron-impact fragmentation pattern of the peak (at 8.2 minutes) demonstrated a molecular ion (M^+) at 384.5 and a major ion ($M^+ - 15$) at 369.5, in agreement with standard TFA-derivatized isosalsoline.

The identity of the salsolinol-like compound in the controls is uncertain, but *O*-methylated salsolinol was a definite urinary constituent in the nonalcoholic subjects (Fig. 3). Others have noted that condensation products, particularly *O*-methylated TIQ's, were present in relatively small amounts in their controls. Sandler *et al.* (*3*) commented that traces of salsolinol appeared to be excreted by normal subjects, and suggested that formation was from endogenously produced acetaldehyde. However, an alternate route to consider is decarboxylation subsequent to condensation of DA with the α-keto acid, pyruvate. Other carboxylated (*O*-methylated) TIQ's originating from DA condensation with phenylpyruvates are present in the brain tissue and urine of normal human subjects and in rat brain tissue (*5, 6*).

Recent studies (*14*) indicate that there is considerable endogenous salsolinol (4 to 7 μg/g) in the adrenals of normal, untreated rodents. Since adrenal catecholamines are released by acetaldehyde and perhaps by ethanol (*15, 16*), it is possible that a significant portion of the urinary TIQ's in alcoholics are not biosynthesized during ethanol ingestion but are released from potential endogenous stores. However, in our subjects the amounts of *O*-methyl-salsolinol and salsolinol were substantial; for example, total urinary TIQ concentrations were greater than

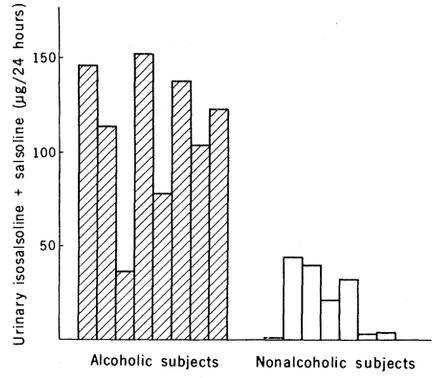

Fig. 3. Total *O*-methylated salsolinol (isosalsoline + salsoline) concentrations, corrected for recoveries, in the urine collected from alcoholics during the first 24 hours of alcohol detoxification and in the daily urine from nonalcoholic subjects.

the values reported for urinary adrenaline or noradrenaline and approached the concentration of urinary normetanephrine found in alcoholics during detoxification. It is inconceivable that the adrenal gland could serve as a greater source of TIQ's than catecholamines when the latter are certain to comprise > 98 percent of the adrenal stores. We conclude that the significant increases in urinary salsolinol and *O*-methylated salsolinol in the alcoholics during the first 24-hour detoxification period is strong evidence that acetaldehyde derived from ingested ethanol sequesters endogenous catecholamines in humans (*17*).

Apart from the two exceptions we mentioned, the decrease in urinary salsolinol was somewhat slower than the disappearance of acetaldehyde from blood in the limited number of alcoholics we tested during the 4-day period. Considering the possible importance of acetaldehyde (*18*) and the difficulties in its precise quantitation (*13*), we suggest that the concentration of urinary or blood TIQ's might be very helpful as an indicator of preceding acetaldehyde and ethanol concentrations in situations involving the abuse of alcohol. This is analogous to the clinical usefulness of hemoglobin A_{1c}, a nonenzymatic condensation derivative of hemoglobin A and aldehyde glucose (or glucose 6-phosphate) whose blood levels are thought to be indicative of plasma glucose control in the diabetic (*19*).

Evidence from animal studies demonstrates that TIQ's are biosynthesized from catecholamines in brain tissue and can modulate neurotransmitter synthesis, metabolism, and dynamics (*7, 20*). However, it has not been established that these effects are related to a specific

aspect of alcoholism. Centrally administered TIQ alters alcohol withdrawal seizures in mice (*21*), which may indicate that TIQ's have a role in the dependence syndrome. Also, results have suggested that amine condensation products may sustain abnormal alcohol-drinking behavior (*8*). A third possible route of involvement, suggested by rat studies in alcohol preference (*22*) and by biochemical and histochemical results with an adrenaline-derived TIQ (*23*), is that TIQ's induce relatively permanent changes in the nervous system. These possibilities take on added meaning in view of our results.

MICHAEL A. COLLINS
WILLIAM P. NIJM
Department of Biochemistry and Biophysics, Loyola Stritch School of Medicine, Maywood, Illinois 60153
GEORGE F. BORGE
GREGORY TEAS
CLARA GOLDFARB
Psychiatry Service, Hines Veterans Administration Hospital, Hines, Illinois 60141

References and Notes

1. G. Cohen and M. Collins, *Science* **167**, 1749 (1970); Y. Yamanaka, M. J. Walsh, V. E. Davis, *Nature (London)* **227**, 1143 (1970); M. A. Collins and M. G. Bigdeli, *Life Sci.* **16**, 585 (1975).
2. V. E. Davis and M. J. Walsh, *Science* **167**, 1005 (1970).
3. M. Sandler, S. B. Carter, K. Hunter, G. Stern, *Nature (London)* **241**, 439 (1973); J. L. Cashaw, K. McMurtrey, H. Brown, V. E. Davis, *J. Chromatogr.* **99**, 567 (1974).
4. J. Robbins, *Clin. Res.* **16**, 554 (1968).
5. C. J. Coscia, W. Burke, G. Jamroc, J. M. Lasala, J. McFarlane, J. Mitchell, M. M. O'Toole, M. L. Wilson, *Nature (London)* **269**, 617 (1977).
6. J. M. Lasala and C. J. Coscia, *Science* **203**, 283 (1979).
7. G. Cohen, *Biochem. Pharmacol.* **25**, 1123 (1976); M. Hirst, M. G. Hamilton, A. M. Marshall, in *Alcohol and Opiates: Neurochemical and Behavioral Mechanisms*, K. Blum, Ed. (Academic Press, New York, 1977), pp. 167–187.
8. R. D. Myers, *Alcoholism Clin. Exp. Res.* **2**, 145 (1978).
9. A preliminary account of this work has been published. See W. P. Nijm, P. Riggin, G. Teas, P. Kissinger, G. Borge, M. A. Collins, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 334 (1977).
10. R. M. Riggin and P. T. Kissinger, *Anal. Chem.* **49**, 530 (1977).
11. M. Bigdeli and M. A. Collins, *Biochem. Med.* **12**, 55 (1975).
12. Dietary control was exercised in order to diminish the possibility that dietary sources of TIQ's could cause significant differences in TIQ excretion between groups or individuals. Although TIQ's (salsolinol) have been detected in some common foods such as bananas [R. Riggin and P. Kissinger, *J. Agric. Food Chem.* **24**, 189 (1976)] and cocoa products (R. Riggin, M. McCarthy, P. Kissinger, *ibid.*, p. 900), the precise effect of diet on urinary TIQ's has not been ascertained.
13. Measurement of acetaldehyde in human blood by headspace gas-chromatography techniques has been complicated by artifactual generation of acetaldehyde during deproteination and incubation [C. Eriksson, H. Sippel, O. Forsander, *Anal. Biochem.* **80**, 116 (1977); A. Stowell, R. Greenway, R. Batt, *Biochem. Med.* **18**, 392 (1977)]. Control studies showed that the artifact could be minimized by using low incubation temperatures and thiourea [W. Nijm, G. Borge, T. Origitano, G. Teas, C. Goldfarb, M. Collins, *Res. Commun. Chem. Pathol. Pharmacol.* **20**, 187 (1978)]. The blood acetaldehyde values we obtained are in approximate agreement with some studies of uncontrolled drinking in alcoholics but several times higher than those obtained in recent studies involving controlled alcohol administration.
14. P. Purvis and M. Hirst, in preparation.
15. F. Schneider, *J. Pharmacol. Exp. Ther.* **177**, 109 (1971).
16. M. Ogata, J. Mendelson, N. K. Mello, E. Majcrowicz, *Psychosom. Med.* **33**, 159 (1971).
17. Other reasons for the elevated urinary TIQ concentrations during early detoxification were ruled out. Excess acetaldehyde (0.2 to 0.8*M*), DA (0.2*M*), or the acetaldehyde-trapping agent cysteine (0.2*M*), added separately to selected acidified urine samples before storage and hydrolysis, failed to change salsolinol concentrations significantly. Thus, artifactual condensation during the isolation procedures did not contribute to the values we obtained. Also, assuming that the 6-*O*-methylated salsolinol isomer (which, unlike salsolinol, is not formed from acetaldehyde condensation under physiological conditions) is substantial, the presence in urine of this enzymatic derivative of a nonenzymatic product (salsolinol) requires that the uncatalyzed condensation occur in vivo. Finally, although the two groups were not treated identically (at the time of our study, the alcoholics were receiving chlordiazepoxide and vitamin supplements and five of the controls were being treated with an antidepressant, phenelzine sulfate), it is unlikely that this difference could be a major factor in the TIQ excretion results.
18. K. O. Lindros, in *Research Advances in Alcohol and Drug Problems*, Y. Israel, F. Glaser, H. Kalant, R. Popham, W. Schmidt, R. Smart, Eds. (Plenum, New York, 1978), vol. 4, pp. 111–176.
19. R. Koenig, C. M. Peterson, R. L. Jones, C. Saudek, M. Lehrman, A. Cerami, *N. Engl. J. Med.* **295**, 417 (1976); R. Graf, J. B. Halter, D. Porte, *Diabetes* **27**, 834 (1978).
20. C. Weiner and M. A. Collins, *Biochem. Pharmacol.* **27**, 2699 (1978); G. Cohen, in *Drugs of Abuse*, C. W. Sharp and L. Abood, Eds. (Liss, New York, 1979), pp. 73–90; M. Hamilton, K. Blum, M. Hirst, *Alcohol Clin. Exp. Res.* **2**, 133 (1978).
21. K. Blum, J. D. Eubanks, J. Wallace, H. Schwertner, W. W. Morgan, *Ann. N.Y. Acad. Sci.* **273**, 234 (1976).
22. R. Myers and C. L. Melchior, *Pharmacol. Biochem. Behav.* **7**, 381 (1977).
23. I. Azevedo, W. Osswald, M. Vasques, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **30**, 139 (1977).
24. We thank the staffs of Hines Veterans Administration Hospital buildings 53C and 51B for clinical assistance, and J. Hannigan, T. Origitano, C. Weiner, and L. Bauman for technical aid. We also thank P. Kissinger for his initial participation and R. Reed of the Clinical Chemistry Division at Hines Veterans Administration Hospital for chromatographic assistance. Isosalsoline HCl was a gift from S. Teitel of Hoffmann-La-Roche, Inc. Supported by Public Health Service grant AA00266 and the Veterans Administration Medical Research Service.

30 March 1979; revised 31 July 1979

Temperature-Dependent Sex Determination in Turtles

Abstract. *The sex of hatchling map turtles is determined by incubation temperature of eggs in the laboratory as well as in nature. Temperature controls sex differentiation rather than causing a differential mortality of sexes. Temperature has no effect on sex determination in a soft-shelled turtle.*

Genotypic sex determination is nearly ubiquitous in tetrapod vertebrates. In many species genotypic control is manifested in morphologically distinct sex chromosomes (mammals, birds, some reptiles), but genotypic control is also known in species that lack detectable sex chromosomes (amphibians) (*1*). There are some reptiles, however, in which genotypic control of sex determination has not been demonstrated and in which incubation temperature of the eggs affects the sex ratio of hatchlings in the laboratory (four turtles, one lizard) (*2, 3*). Possibly, temperature is the sex determining agent in these species (*3*), but evidence from incubation under natural conditions is lacking. We present data obtained in the laboratory and field which indicate that temperature is a natural determinant of sex in map turtles but not in a soft-shelled turtle.

Previous laboratory studies of turtles have shown that constant incubation temperatures of 31°C and above produce female hatchlings, cooler temperatures (24° to 27°C) produce males (*2, 3*), and, in the snapping turtle (*Chelydra*), even cooler temperatures (20°C) again produce females (*3*). It is likely in *Chelydra* that temperature is controlling sex differentiation rather than causing differential mortality of the sexes because more than 80 percent of the eggs hatch (*3*), but data for the other species do not warrant such a conclusion. There has been little work on sex determination in turtles under natural conditions. Results from artificial incubation at constant temperatures do not necessarily apply to field situations because nest temperatures fluctuate in some species (*4, 5*). A laboratory study in which eggs of *Emys* (European pond turtle) were incubated at fluctuating temperatures in fact produced males and females (*6*). Field incubation of *Emys* eggs produced a male-biased sex ratio in accord with low soil temperatures (*5*), but the study included partial laboratory incubation at low temperatures, and the eggs were buried at sites not necessarily representing the parental population. Thus, uncertainties remain in interpreting the existing studies.

In 1978 we initiated a comparative study to survey several species of turtles for temperature effects on sex ratios of hatchlings, to observe whether the mechanism was due to sex determination or differential mortality, and to contrast results of incubation in field and laboratory settings. We performed three types of incubation experiments: experiment 1, constant temperatures; experiment 2, controlled, fluctuating temperatures; and experiment 3, natural, field, incubation. Each experiment consisted of two temperature regimes, one warm and one

0036-8075/79/1207-1186$00.50/0