Conclusion

Reductive cleavage of three types of the carbamates (I, II, and III) with Raney nickel afforded the corresponding reduction products as follows: I→N,N-diethylformamide, toluene, and hydrogen chloride; II→N,N-diethylmethylamine and toluene; III→N-ethylcyclohexylamine, N-methyl-N-ethylcyclohexylamine, and N,N-diethylcyclohexylamine.

Formation of some of the reduction products indicated an effective participation of ethanol in the processs of reduction of the carbamates. Because most of the products were adsorbed more or less on Raney nickel, resulting in their unsatisfactory recovery, application of this reaction on their quantitative determinations seems to be difficult.

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Mechanism for Interference of Ethinamate in Zimmermann Test for Determination of Urinary 17-Ketosteroids[1]

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The mechanism by which dosage of ethinamate elevates falsely the values for the urinary 17-ketosteroids has been investigated. A specimen of urine collected following oral administration of ethinamate to man was hydrolyzed with sulfuric acid and then extracted with dichloromethane. From the urinary extract two non-steroidal chromogens responsible for the Zimmermann reaction were isolated. The spectral and chromatographic properties indicated that these chromogens would be 1-acetylcyclohex-1-en-4-ol and 1-acetylcyclohex-1-en-5-ol. Their structures were unequivocally characterized by comparison with the synthetic samples. It has been disclosed that during the acid-catalyzed hydrolysis of the conjugates in the process of 17-ketosteroids determination the 4- and 3-hydroxylated metabolites formed from ethinamate are converted artificially into the ketones which give a typical color for the 17-ketosteroids in the Zimmermann reaction.

In the course of running a routine diagnostic laboratory the interferences due to the commonly used medications in the assay procedure have recently become more of a problem. It has been previously shown that after ingestion of ethinamate (1-ethynylcyclohexyl carbamate) (**1**) the urinary 17-ketosteroids values by the Zimmermann reaction are greatly increased.[3] Although the metabolic fate of ethinamate has fully been investigated in man,[4] the metabolites so far identified fail to account for a marked enhancement in the Zimmermann coloration. The present paper describes the mechanism by which dosage of ethinamate elevates falsely the values for the urinary 17-ketosteroids.

A specimen of urine collected following oral administration of an officinal dose of ethinamate to man was hydrolyzed with mineral acid and then extracted with the organic solvent

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[1] Part CIV of "Studies on Steroids" by T. Nambara; Part CIII: T. Nambara, K. Kigasawa, T. Iwata, and M. Ibuki, *J. Chromatogr.*, **114**, 81 (1975).

[2] Location: *Aobayama, Sendai.*

[3] S. Borushek and J.J. Gold, *Clin. Chem.*, **10**, 41 (1964).

[4] *a*) T. Murata, *Chem. Pharm. Bull.* (Tokyo), **8**, 629 (1960); *b*) *Idem, ibid.*, **9**, 146 (1961); *c*) *Idem, ibid.*, **9**, 335 (1961); *d*) R. Preuss and G. Willing, *Arzneimittel-Forsch.*, **13**, 155 (1963); *e*) *Idem, ibid.*, **13**, 234 (1963); *f*) R. Preuss and E. Mayer, *ibid.*, **15**, 747 (1965).

according to the routine procedure for determination of the 17-ketosteroids.[5] The urinary extract was purified by column chromatography on silica gel and the eluate was then subjected to preparative thin-layer chromatography (TLC) using chloroform as a developing solvent. Upon multiple runs two non-steroidal chromogens (I and II) responsible for the Zimmermann reaction could be separated. These two were not obtained crystalline, but substantially homogeneous as judged from the usual criteria.

The principal chromogen I showed the characteristic ultraviolet (UV) and infrared (IR) absorptions due to the α,β-unsaturated ketone system. Inspection of the nuclear magnetic resonance (NMR) spectrum indicated the presence of a hydroxyl group besides the conjugated ketone system. In the mass spectrum a molecular ion peak appeared at *m/e* 140. These evidences together with the metabolic findings lent a support for the assignment of the structure 1-acetylcyclohex-1-en-4-ol to chromogen I.

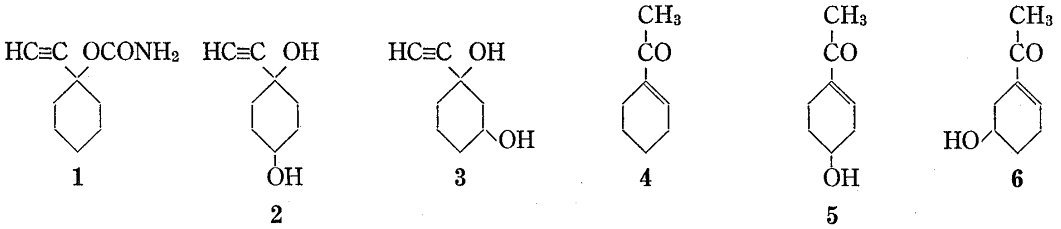

Chart 1

In order to characterize the structure definitely the preparation of the authentic sample was then undertaken employing the method of Jones, *et al.*[6] First, cyclohexane-1,4-diol was transformed into the monobenzoate which in turn was oxidized with chromium trioxide to 4-benzoyloxycyclohexanone. The Grignard reaction with ethynylmagnesium bromide and subsequent removal of the benzoyl residue by treatment with lithium aluminum hydride afforded a mixture of epimeric 1-ethynylcyclohexane-1,4-diols (**2**). Being refluxed with formic acid and then with water, **2** underwent elimination of the *tert*-hydroxyl function and hydration of the ethynyl group successively to provide the desired compound. Upon direct comparison with the synthetic sample chromogen I was unambiguously identified as 1- acetylcyclohex-1-en-4-ol (**5**).

The minor chromogen II also seemed to have the α,β-unsaturated ketone structure as evidenced by the UV spectral properties. The mass spectral data were indicative of the existence of a hydroxyl group and chromogen II being a positional isomer of chromogen I. The synthesis of the authentic sample was then carried out in a similar fashion employing cyclohexane-1,3-diol as a starting material. The 1,3-diol monobenzoate was oxidized with chromium trioxide to the ketone, which on the Grignard reaction with ethynylmagnesium bromide was led to a mixture of epimeric 1-ethynylcyclohexane-1,3-diols (**3**).[4d] Subsequent treatment with formic acid and water afforded the desired 1-acetylcyclohexenol in a fairly good yield. In the NMR spectra there was seen no spin-spin coupling between proton attached to carbon bearing a hydroxyl function and vinyl proton. On the basis of these data the location of the hydroxyl group in the 1-acetylcyclohex-1-ene system was established at C-5 rather than at C-3. The identity with the synthetic sample permitted unequivocally to assign the structure 1-acetylcyclohex-1-en-5-ol (**6**) to chromogen II.

The mechanism for the Zimmermann reaction of the ketosteroids has recently been elucidated.[7] Therefore it is evident that these chromogens having an active methyl group

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[5] A. Kambegawa, *Rinsho Byori*, **19**, 301 (1971).

[6] E.R. H. Jones and F. Sondheimer, *J. Chem. Soc.*, **1949**, 615.

[7] O. Neunhoeffer, K. Thewalt, and W. Zimmermann, *Z. Physiol. Chem.*, **323**, 116 (1961); T.J. King and C.E. Newall, *J. Chem. Soc.*, **1962**, 367; T. Nambara and M. Katō, *Chem. Pharm. Bull.* (Tokyo), **13**, 78 (1965); *idem*, *Chem. Ind.* (London), **1967**, 1090.

are responsible for the Zimmermann reaction. In actuality both chromogens exhibited the characteristic coloration having an absorption maximum at 520 nm with the Zimmermann reagent and their contamination apparently elevated the 17-ketosteroids value. In addition, unchanged ethinamate was similarly converted into 1-acetylcyclohex-1-ene (4), when treated with mineral acid under the same conditions as the above. From these results it has been disclosed that during the acid-catalyzed hydrolysis of the conjugates in the process of 17-ketosteroids determination the 4- and 3-hydroxylated metabolites principally formed from ethinamate[4] undergo the transformation yielding artificially the ketones which give a typical color for the 17-ketosteroids in the Zimmermann reaction.

Experimental[8]

Thin-Layer Chromatography (TLC)——TLC was carried out on a Silica gel HF_{254} (E. Merck AG, Darmstadt) plate employing the following systems: TL-I = $CHCl_3$–AcOEt (10: 1), TL-II = acetone–$CHCl_3$–cyclohexane (3: 3: 10).

Gas-Liquid Chromatography (GLC)——The apparatus used was a Shimadzu Model GC-3BF gas chromatograph equipped with a hydrogen flame ionization detector and a "silanized" U-shaped glass tube (3 mm i.d.) packed with 1.5% OV-1 on Shimalite W (100—120 mesh) (length 3 m) (condition GC-I) or 1.5% OV-17 on Shimalite W (60—80 mesh) (length 2.1 m) (condition GC-II). Temperatures of column and injection chamber were kept at 110° and 130° for GC-I or 120° and 140° for GC-II, respectively. N_2 was used as a carrier gas at flow rates of 35 ml/min for GC-I and 80 ml/min for GC-II. Retention time relative to phenanthrene (GC-I 7.4 min and GC-II 10.5 min), t_R, was given.

Administration of Ethinamate——Five hundred milligrams each of ethinamate was orally administered to three normal adult males before sleeping and the first morning specimen of the next day was collected.

Separation and Identification of Urinary Zimmermann Chromogens——To the specimen of combined urine (1500 ml) was added conc. H_2SO_4 (90 ml), heated at 100° for 15 min, and then extracted with CH_2Cl_2. The organic layer was washed with 8% NaOH and H_2O, dried over anhydrous Na_2SO_4, and evaporated. A gummy residue (125 mg) was chromatographed on silica gel (10 g) and eluted with benzene–AcOEt (3: 2). The eluate was submitted to preparative TLC on Silica gel HF_{254} using $CHCl_3$ as developing solvent. The chromogen was located by spraying the edges of the plate with 2% *m*-dinitrobenzene in EtOH and 30% KOH in 50% EtOH. Elution of the material (^5Rf 0.16) exhibiting the positive Zimmermann test with AcOEt gave chromogen I (7 mg) as colorless oil. IR $\nu_{\max}^{CHCl_3}$ cm^{-1}: 3460—3440 (OH), 1670 (conjugated C=O), 1650 (conjugated C=C). UV $\lambda_{\max}^{MeOH}$ nm: 230. Mass Spectrum *m/e*: 140 (M^+). NMR (1.7% solution in $CDCl_3$) δ: 1.85 (1H, s, –OH), 2.35 (3H, s, $-COCH_3$), 4.00 (1H, m, $>C\underline{H}OH$), 6.70 (1H, m, $-HC=C<$). TLC: TL-I ^9Rf 0.35, TL-II ^4Rf 0.36. GLC: GC-I 0.77, GC-II 0.40. Chromogen I proved to be identical with the synthetic sample of 1-acetylcyclohex-1-en-4-ol[6] in all respects. Elution of the material (^5Rf 0.14) exhibiting the positive Zimmermann test with AcOEt gave chromogen II (1 mg) as colorless oil. UV $\lambda_{\max}^{MeOH}$ nm: 230. Mass Spectrum *m/e*: 140 (M^+). TLC: TL-I ^9Rf 0.30, TL-II ^4Rf 0.35. GLC: GC-I 0.77, GC-II 0.40. Chromogen II proved to be identical with the synthetic sample of 1-acetylcyclohex-1-en-5-ol by TLC, GLC and mass spectral comparison. Both chromogen I and II exhibited the purple coloration having an absorption maximum at 520 nm with the Zimmermann reagent.

Synthesis of 1-Acetylcyclohex-1-en-5-ol (6)——1-Ethynylcyclohexane-1,3-diol (3)[4d] (108 mg) was refluxed with 98% formic acid (3 ml) for 1 hr. After removel of formic acid by evaporation under the reduced pressure the residue was diluted with H_2O (3 ml) and refluxed for 1 hr. The resulting solution was evaporated *in vacuo* and the residue (79 mg) obtained was submitted to preparative TLC on Silica gel HF_{254} using $CHCl_3$–AcOEt (10: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^9Rf 0.30) with AcOEt gave **6** (38 mg) as colorless oil. UV $\lambda_{\max}^{MeOH}$ nm: 230. Mass Spectrum *m/e*: 140 (M^+). NMR (1.3% solution in $CDCl_3$) δ: 1.60 (1H, s, –OH), 2.28 (3H, s, $-COCH_3$), 4.00 (1H, m, $>C\underline{H}OH$), 6.85 (1H, m, $-HC=C<$). 2,4-Dinitrophenylhydrazone: dark red plates (from AcOEt). mp 228°. $[\alpha]_D^{15}$ 0° ($c=0.10$ in $CHCl_3$). *Anal.* Calcd. for $C_{14}H_{16}O_5N_4$: C, 52.49; H, 5.04; N, 17.49. Found: C, 52.58; H, 5.05; N, 17.21.

Transformation of Ethinamate into 1-Acetylcyclohex-1-ene (4)——To a suspension of ethinamate (1 g) in H_2O (1000 ml) was added conc. H_2SO_4 (100 ml) and treated in the manner as described above. The crude product obtained was submitted to preparative TLC on Silica gel HF_{254} using benzene as developing solvent.

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[8] All melting points were taken on a micro hot-stage apparatus and are uncorrected. UV and IR spectral measurements were run on Hitachi Model 124 and JASCO Model IR-S spectrophotometers, respectively. Mass spectra were obtained on a Hitachi Model RMU-6E spectrometer. NMR spectra were recorded on a Hitachi Model R-20A spectrometer at 60 MHz or a JEOL PS-100 spectrometer at 100 MHz. The chemical shifts are quoted as ppm downfield from tetramethylsilane used as an internal standard. Abbreviation used s = singlet and m = multiplet.

Elution of the adsorbent corresponding to the spot (*Rf* 0.12) with AcOEt gave **4** (300 mg) as colorless oil. NMR (20% solution in $CDCl_3$) δ: 2.27 (3H, s, $-COCH_3$), 6.86 (1H, m, $-HC=C\langle$). Oxime: colorless needles (from aq. MeOH). mp 56—58°. Semicarbazone: colorless plates (from MeOH). mp 208—213°. The transformation product proved to be identical with the authentic sample of 1-acetylcyclohex-1-ene prepared by a different route[9] in all respects.

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9) F. Ebel and M.W. Goldberg, *Helv. Chim. Acta*, **10**, 677 (1927).