ELSEVIER

Journal of Chromatography B, 693 (1997) 297–305

JOURNAL OF CHROMATOGRAPHY B

# Determination of acetaldehyde in biological samples by gas chromatography with electron-capture detection

Hiroaki Ohata, Masato Otsuka, Shinji Ohmori*

*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1-1-1, Okayama 700, Japan*

Received 21 February 1996; revised 22 November 1996; accepted 23 December 1996

**Abstract**

A simple specific assay was developed for the determination of acetaldehyde in biological samples. Acetaldehyde was derivatized to 2,4-dinitrophenylhydrazone, which was determined by gas chromatography with electron-capture detection. The use of this detection method is an important device to which no one drew notice. This procedure was very simple and so sensitive that as little as 500 fmol of acetaldehyde could be measured in aqueous solution. The calibration curve of acetaldehyde was linear at least up to 40 $\mu M$. Its recoveries from human plasma and rat liver homogenate were 96.5 and 95.7%, respectively.

*Keywords:* Acetaldehyde; 2,4-Dinitrophenylhydrazine

## 1. Introduction

We have studied alcohol metabolism in mammals to elucidate biochemically the hangover, which is a commonplace event. We established a determination method for $C_4$ compounds, such as diacetyl, acetoin and 2,3-butanediol, which are metabolites of acetaldehyde [1,2]. Using these methods, the interconversion and accumulation of these $C_4$ compounds in rat tissues were investigated [3]. During these studies, a specific, sensitive and simple method for the determination of acetaldehyde in biological samples was required. However, no such method has yet been reported in the literature. Thus, the purpose of this study was to develop a simple assay method for determining minute amounts of acetaldehyde in biological samples.

## 2. Experimental

### 2.1. Materials

Acetaldehyde was purchased from Merck (Darmstadt, Germany) and was distilled before use. 2,4-Dinitrophenylhydrazine (DNPH), from Wako (Osaka, Japan), was used after recrystallization from methanol. *n*-Heptane and *n*-hexane for gas–liquid chromatography (GLC) with electron-capture detection (ECD) were purchased from the same company. 2,4-Dinitrophenylhydrazone of acetaldehyde (ADPH) was synthesized in the usual manner in ethanol under acidic conditions. Recrystallization

*Corresponding author.


*PII* S0378-4347(97)00065-0

from ethanol; m.p., 167°C; yield, 89%; (the reported m.p. is 168°C [4]). Butyraldehyde and aldrin were purchased from Wako. The former was distilled before use and used as an internal standard and the latter was used as an external standard for ADPH determination.

### 2.2. Standard curves

Various concentrations (1 to 5 μ*M*) of ADPH in *n*-hexane containing 25 ng of aldrin per ml were prepared. A 1-μl volume was injected into the gas chromatograph and the areas of the ADPH and aldrin peaks were calculated with a Shimadzu C-R5A Chromatopac integrator. The ratios ($y$) of peak areas of ADPH to that of aldrin were plotted against the ADPH concentration ($x$).

### 2.3. Calibration curve of acetaldehyde

Ice-cold 1 m*M* acetaldehyde (10 to 40 μl) and 100 μl of ice-cold 2,4-dinitrophenylhydrazine–6 *M* HCl solution (4 mg/ml) containing 20 nmol of butyraldehyde were placed in a 5-ml vial. Ice-cold water was then added to a volume of 1 ml. The mixture was reacted at 40°C for 60 min and extracted twice with 2 ml of *n*-heptane. The heptane layer (3.5 ml) was placed in a 5-ml vial and evaporated using a Savant Vac concentrator (Model SVS-100H, New York, NY, USA) at room temperature for 20 min. The residue was dissolved in 1 ml of *n*-hexane containing 25 ng of aldrin. The areas of the two peaks corresponding to the *syn*- and *anti*- forms of the hydrazones derived from acetaldehyde and butyraldehyde were automatically calculated. In order to make a calibration curve for lower acetaldehyde concentrations, aliquots (10 to 100 μl) of 0.1 m*M* acetaldehyde solution were derivatized to the hydrazone and analyzed by GLC.

The reaction rates of the formation of hydrazones from aldehydes were calculated from the standard curve that was made using aldrin as a standard.

In the case of the determination of acetaldehyde in biological samples, aldrin was also added, because the peak area of aldrin was used as an index for the appropriate injection volume.

### 2.4. Analytical procedure for the determination of acetaldehyde in human blood plasma

Human blood plasma (1 ml) was mixed with 1 ml of 1.2 *M* $HClO_4$ in a 5-ml vial with a tightly fitting cap with a Teflon-coated rubber insert and was allowed to stand at 4°C. The mixture was centrifuged at 1500 *g* for 10 min. The supernatant (1 ml) was transferred to a fresh 5-ml vial with a screw-cap and was mixed with 2,4-dinitrophenylhydrazine (0.4 mg) dissolved in 0.1 ml of 6 *M* HCl containing 20 nmol of butyraldehyde.

The mixture was reacted at 40°C for 60 min and extracted twice with 2 ml of *n*-heptane. The heptane layer (3.5 ml) was placed in the 5-ml vial and evaporated using a Savant Vac Concentrator. The residue was dissolved in 1 ml of *n*-hexane containing 25 ng of aldrin.

### 2.5. Analytical procedure for the determination of acetaldehyde in rat liver

Fresh rat liver was perfused with ice-cold 0.15 *M* KCl. The liver (2 g) was homogenized at 4°C in 8 ml of 0.15 *M* KCl with a Potter-Elvehjem homogenizer with a Teflon pestle and then centrifuged at 4°C and 6000 *g* for 15 min. A 1-ml volume of the supernatant was placed in a 5-ml vial, mixed vigorously with 1 ml of 1.2 *M* $HClO_4$ and allowed to stand at 4°C for 30 min. The mixture was centrifuged at 4°C and 1500 *g* for 15 min. The supernatant (1 ml) was treated as described in Section 2.4.

### 2.6. Gas chromatography

All samples were analyzed on a GC14A gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a $^{63}Ni$ electron-capture detector. A Hi-Cap CBP1-M25-025 capillary column (25 m×0.25 mm I.D.; film thickness, 0.25 μm; Shimadzu) was used. The electron-capture detector was maintained at 280°C. The column temperature was raised automatically from 100 to 200°C at a rate of 20°C/min, and the temperature was maintained at 200°C for 5 min, then the column temperature was raised to 220°C at a rate of 5°C/min, and then to 265°C at a rate of 30°C/min. The injector block temperature was adjusted to 270°C. The flow-rate of carrier gas

(nitrogen) was ca. 1.5 ml/min and its pressure was 1 $kg/cm^2$. The injection technique was splitless.

### 2.7. Liquid chromatography

In order to identify the peak of ADPH on GLC with ECD using mass spectrometry, a certain amount of ADPH had to be collected. ADPH derived from acetaldehyde in rat plasma after ethanol administration was separated on a 150×4.6 mm I.D. Inertsil ODS-80A column (GL Science, Tokyo, Japan), connected to a Shimadzu Model LC-3A liquid chromatograph (Shimadzu) to which a Shimadzu SPD-2A UV detector was attached. The hydrazone of acetaldehyde was prepared from rat blood as described in Section 2.8. The column was eluted with a mixture of water and acetonitrile (49:51, v/v) and monitored at 330 nm. The chromatograph was operated at 1 ml/min and 40°C. This fractionation procedure was repeated ten times.

### 2.8. Identification of peaks of ADPH on a gas chromatogram

Ethanol (20%) was given orally at a dose of 1 g/kg body weight to male Wistar albino rats weighing 220 g. Thirty min after the administration of ethanol, pentobarbital was injected intraperitoneally. Ten min after the pentobarbital injection, the neck was opened and 10 ml of blood were collected from the jugular vein. The syringe and centrifuge tube were wetted with 0.5 ml of 4.3% sodium citrate. The blood samples were centrifuged at 4°C and 1500 *g* for 10 min. To 6 ml of the plasma, 6 ml of 1.2 *M* $HClO_4$ were added and the mixture was centrifuged at 4°C and 1500 *g* for 10 min. The supernatant (10 ml) was placed in a 50-ml centrifuge tube with a tightly fitting cap. After adding 1 ml of a 20-m*M* 2,4-dinitrophenylhydrazine–6 *M* HCl solution, the mixture was reacted at 40°C for 1 h. ADPH was extracted with two 20 ml portions of *n*-hexane. The hexane layer was evaporated under reduced pressure using a rotary evaporator. The residue was dissolved in 0.5 ml of methanol. The entire sample was injected onto the HPLC column in ten runs. The peak corresponding to ADPH, which appeared at 10 min, was collected. The combined fractions were evaporated and the residue was dissolved in 0.5 ml of methanol. After the purity of the sample was confirmed by HPLC to show one peak at 10 min, it was again evaporated to dryness. The residue was dissolved in 1 ml of *n*-hexane and an aliquot of the hexane solution was diluted fifty times with *n*-hexane. The diluted solution was injected to the GLC–ECD system and identified to be one peak at 9.9 min. The purified ADPH thus obtained was applied to a VG-70SE (VG analytical, Manchester, UK) mass spectrometer in EI mode that was set at an ion source temperature of 250°C and an electron impact voltage of 40 eV.

### 2.9. Acetaldehyde concentration in rat tissues after ethanol administration

Male Wistar albino rats weighing 200–220 g were divided into two groups. One group was given intraperitoneally a disulfiram suspension (60 mg/ml) in 5% (w/w) gum arabicum at a dose of 150 mg/kg body weight. Pentobarbital (40 mg/kg body weight) was administered intraperitoneally 18 h after disulfiram injection, followed by oral administration of 20% ethanol (1 g/kg body weight). The other group was not given the disulfiram suspension, but was given 5% gum arabicum. The necks were opened and 500 μl blood samples were collected from the jugular vein at various times (20, 40, 60, 80 and 100 min) after ethanol administration. Acetaldehyde in blood samples was determined as described in Section 2.4.

To determine the acetaldehyde distribution in rat tissues, 3 male Wistar strain albino rats were orally administered 20% alcohol. At 10 min after administration of ethanol, pentobarbital was given, followed by excision of organs. Tissues from rats were homogenized and acetaldehyde was determined in a manner similar to that described in Section 2.4.

## 3. Results

### 3.1. Gas chromatograms of ADPH

Fig. 1a shows the chromatograms of an authentic sample of ADPH, which was eluted as the two peaks corresponding to the *syn-* and *anti-* forms [5]. Fig. 1b is the chromatogram of ADPH derived from

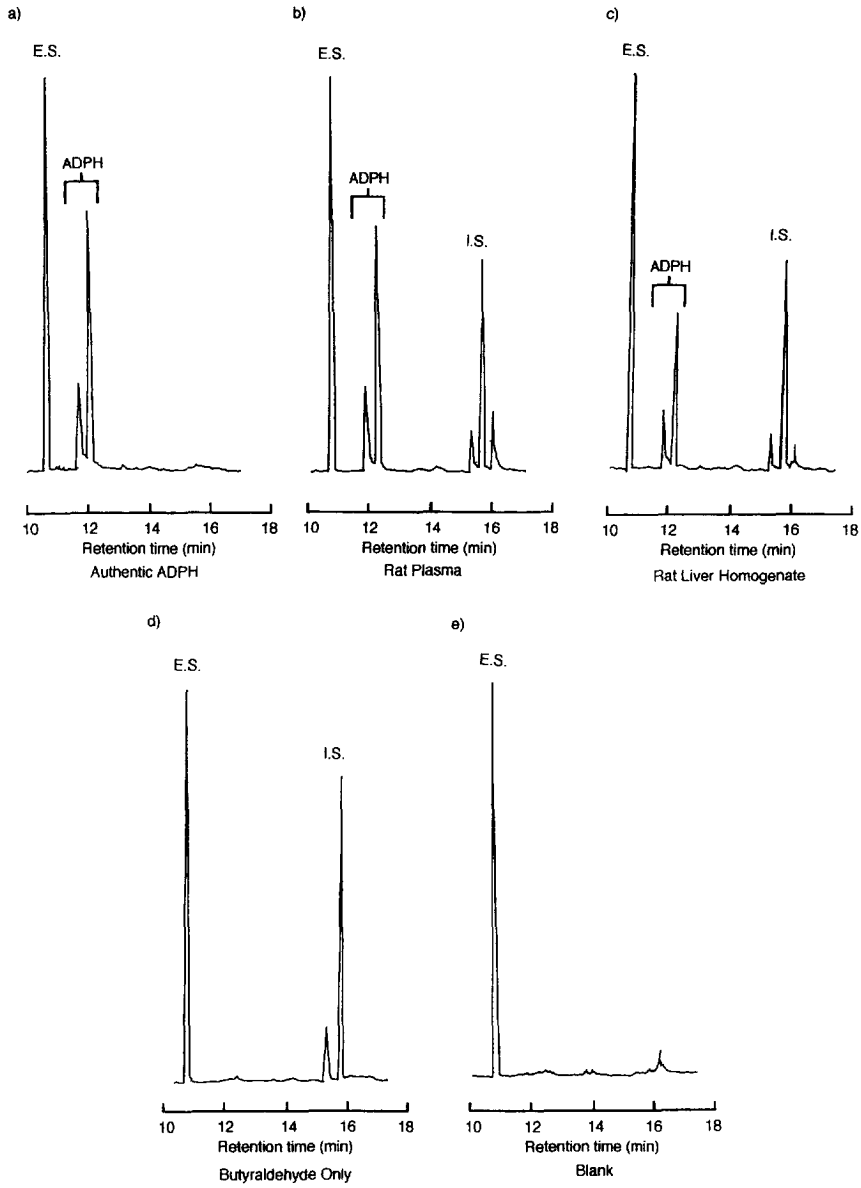


Fig. 1. Gas chromatograms of ADPH. (a) ADPH (authentic): 1 μl of a 1 nmol/ml standard solution of ADPH was injected. (b) ADPH (derived from acetaldehyde in the plasma of a rat that had been administered ethanol): deproteinized blood plasma of rat (1 ml) was treated as described in Section 2.4. (c) ADPH (derived from acetaldehyde in liver homogenate from a rat that had been administered ethanol): the liver (2 g) was treated as described in Section 2.5. (d) Butyraldehyde 2,4-dinitrophenylhydrazone: (derived from standard butyraldehyde solution): ice-cold 1 m*M* butyraldehyde (20 μl) was reacted with DNPH and analyzed as described in Section 2.3. (e) Solvent blank: $H_2O$ (1 ml) was placed in a 5-ml vial with a screw-cap and mixed with 2,4-dinitrophenylhydrazine (0.4 mg) dissolved in 0.1 ml of 6 *M* HCl. The mixture was reacted, extracted and analyzed by GLC as described in Section 2.3.

acetaldehyde in plasma from a rat that was orally administered ethanol 60 min before being killed. Fig. 1c is the chromatogram of ADPH, which was converted from acetaldehyde in the liver of a rat given alcohol orally 60 min before being killed. As can be seen, ADPH appeared as two peaks on the

chromatogram and was well separated from the other peaks. Fig. 1d depicts the 2,4-dinitrophenylhydrazone derived from the standard butyraldehyde solution (0.4 nmol/ml). Fig. 1e is the chromatogram of the solvent blank.

### 3.2. Identification of the peak of ADPH

The peaks corresponding to ADPH on GLC were identified using the electron-impact ionization mass spectrometer. The parent peak ($M^+$) was at $m/z$ 224. The mass spectrum of ADPH derived from acetaldehyde in blood of rats that had been administered ethanol showed the same fragmentation pattern as previously reported [6].

### 3.3. Standard and calibration curves

The peak area ratio ($y$) of ADPH compared to the peak area of aldrin was exactly proportional to the ADPH concentration ($x$, $\mu M$): $y=0.1387x\pm0.0006$, $r^2=0.9997$. The determination limit was 100 fmol and the detection limit was 10 fmol.

The ratios of the peak area ($y$) of the hydrazone derived from various amounts of acetaldehyde to that from butyraldehyde were plotted as a function of the acetaldehyde concentration ($x$, $\mu M$) in the assay mixture and yielded a straight line, at least to 40 $\mu M$ (injection amount, 40 pmol): $y=0.0443x\pm0.002$, $r^2=0.9899$.

As for the calibration curve for relatively low acetaldehyde concentration, the ratios of the area ($y$) of ADPH to butyraldehyde hydrazone were directly proportional to the concentration of acetaldehyde ($x$, $\mu M$): $y=0.0451x\pm0$, $r^2=0.997$. The determination limit was 0.5 $\mu M$, corresponding to an injection amount of 500 fmol.

### 3.4. Reaction of glucose with DNPH

Aqueous glucose solution (0.5 ml; 1 to 10 m$M$), 0.5 ml of 1.2 $M$ $HClO_4$ and 0.1 ml of DNPH–6 $M$ HCl were reacted at 40°C for 60 min in a 5-ml vial. An aliquot (0.1 ml) of the reaction mixture was directly assayed by HPLC. The HPLC analysis procedure was the same as that described in Section 2.7. Glucose osazone was neither formed as crystals under these reaction conditions nor found in the chromatogram of the reaction mixture when compared to the authentic sample.

### 3.5. Reaction of acetaldehyde with DNPH in the presence of glucose

Since glucose is present in biological samples, it has to be tested whether glucose interferes with the formation of the acetaldehyde hydrazone. Ice-cold 1 m$M$ acetaldehyde (10 to 40 μl) and 100 μl of ice-cold 2,4-dinitrophenylhydrazine–6 $M$ HCl solution (4 mg/ml) containing 20 nmol of butyraldehyde were placed in a 5-ml vial. Two series of five vials were prepared. The reaction volumes were adjusted to 1 ml with ice-cold water for one set and with 4.5 m$M$ glucose for the second set. The mixtures were incubated and treated as described in Section 3.4. The resulting residue was dissolved in 1 ml of $n$-hexane containing 25 ng of aldrin. When acetaldehyde was reacted with DNPH alone and the ratios of the peak area ($y$) of the hydrazone derived from various amounts of acetaldehyde to that from butyraldehyde were plotted as a function of the acetaldehyde concentration ($x$, $\mu M$) in the assay mixture, a straight line was observed at least to 40 $\mu M$ (40 pmol as the injection amount): $y=0.0443x\pm0.002$, $r^2=0.9899$. In the presence of glucose, the ratios also yielded a straight line at least to 40 $\mu M$ (40 pmol as the injection amount): $y=0.0441x\pm0.001$, $r^2=0.9869$. These experimental results show that glucose did not interfere with the formation of acetaldehyde–hydrazone.

### 3.6. Recovery test

For the purpose of applying this procedure to biological samples, recovery tests were carried out. Various amounts of acetaldehyde were added to the 6000 $g$ rat liver homogenates, blood plasma from normal rats and to normal adult human blood plasma, and the amounts of acetaldehyde were measured. The recoveries were calculated from the calibration curve. The results are shown in Table 1, indicating the recoveries of 95.7±3.16, 94.1±2.18 and 96.5±3.73% of acetaldehyde added, for rat liver homogenate, rat plasma and human plasma, respectively.

Table 1
Recovery of acetaldehyde from rat liver homogenate, rat plasma and human plasma

| Acetaldehyde | Rat liver homogenate | | Rat plasma | | Human plasma | |
|---|---|---|---|---|---|---|
| added (nmol) | Found (nmol) | Recovery (%) | Found (%) | Recovery (%) | Found (nmol) | Recovery (%) |
| 0 | 2.5 | | 2.6 | | 3.0 | |
| 10 | 11.7 | 91.7±0.10 | 11.8 | 91.9±0.19 | 12.3 | 92.2±0.21 |
| 20 | 21.9 | 97.5±0.29 | 21.9 | 96.8±1.07 | 23.3 | 101.3±1.11 |
| 30 | 32.1 | 98.8±0.28 | 30.8 | 94.6±0.99 | 31.9 | 96.1±1.55 |
| 40 | 40.4 | 94.6±1.07 | 40.0 | 93.4±1.86 | 41.6 | 96.3±1.00 |
| Average | | 95.7±3.16 | | 94.1±2.18 | | 96.5±3.73 |

$n=3$.

### 3.7. Plasma level of acetaldehyde after ethanol administration

Fig. 2 shows the time course of plasma concentrations of acetaldehyde in rats treated with or without disulfiram treatment. The acetaldehyde concentration in the disulfiram-treated rats was about twice that of the untreated rats throughout the experiment. The plasma levels of acetaldehyde in both groups peaked at 40 min after ethanol administration and, thereafter, decreased slowly and remained at a higher level than before administration even 100 min after ethanol administration.

### 3.8. Acetaldehyde concentration in rat tissues after ethanol administration

Table 2 shows the acetaldehyde concentration in various rat tissues 40 min after ethanol administration. The highest concentration of acetaldehyde was found in the plasma and the second highest levels were found in liver and brain. Acetaldehyde was found at somewhat lower concentrations in the kidney and muscle. The total amount of acetaldehyde accumulated in these organs per animal 40 min after ethanol administration with disulfiram was calculated to be 22 μmol, which corresponded to 0.5% of the

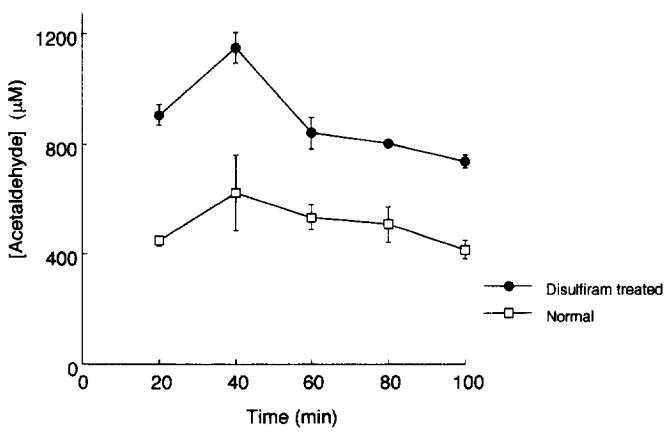


Fig. 2. Acetaldehyde concentration in the plasma of a rat after administration of ethanol. After rats were given disulfiram intraperitoneally, ethanol was orally administered (1 g/kg body weight) (●). The neck was opened and 500 μl blood samples were collected from the jugular vein 20, 40, 80 and 100 min after ethanol administration. Acetaldehyde in the blood samples was determined by GLC with ECD. Normal rat: (□).

Table 2
Concentration of acetaldehyde in rat tissues after ethanol administration

| Tissues | Normal rat (nmol/g tissue) | Rat administered ethanol (nmol/g tissue) |
|---|---|---|
| Liver | 2.50±0.50 | 170.8±13.6 |
| Muscle | N.D. | 52.7±7.1 |
| Kidney | 1.68±0.16 | 83.2±5.7 |
| Brain | 2.01±0.21 | 166.0±9.4 |
| Plasma | 2.53±0.47[a] | 573.1±25.5[a] |

Values are means±S.D. ($n$=3). N.D.: Not detectable.
[a] nmol/ml.

administered ethanol. If the acetaldehyde concentration in other tissues, such as lungs, intestines and adipose tissue, were determined and calculated, the total amount of acetaldehyde would be much higher. Considerable amounts of acetaldehyde were found in normal rat tissues, with the exception of muscle, even if alcohol was not administered, as shown in Table 2.

## 4. Discussion

About 30 papers concerning methods for determining acetaldehyde have been published during the last 30 years. Half of these papers presented a determination method based on the formation of 2,4-dinitrophenylhydrazone, which was mainly analyzed by HPLC and, rarely, by GLC. A few of these determination methods were applied to biological samples [7–9] and the remainder were applied to industrial surfactants [10], automobile exhaust [11], polluted air [12], cigarette smoke [13] and aqueous acetaldehyde solutions [14,15]. From another point of view, when we searched the papers that were published over the last ten years that dealt with the concentration of acetaldehyde in biological samples, we found that acetaldehyde was determined mainly using the head-space technique of GLC, followed by the HPLC method. The former, however, was less sensitive, with a sensitivity in the range of 0.4 to 5.7 μ$M$ [16,17].

The literature survey revealed that there was no simple and sensitive determination method for acetaldehyde in biological samples. Accordingly, we had to develop a new determination method for acetaldehyde to study the cause of hangovers.

This paper describes a GLC determination method for acetaldehyde using ECD and a capillary column, since nitro groups can be detected by ECD. The use of ECD as the detector is the important device to be emphasized in the method and resulted in high sensitivity. The detection and determination limits of acetaldehyde in aqueous solution were 0.1 and 0.5 μ$M$ using this method. The sensitivity was comparable to that obtained using the method of Nakashima et al. [18], in which HPLC was combined with fluorescence detection. The GLC–ECD method has another advantage of high specificity because the detector is responsive to nitro groups.

We also tried to use pentafluorophenyl-, 2-chloro- and 2,4-dichlorophenylhydrazine as labeling compounds for the GLC–ECD method, however, they were not satisfactory; pentafluorophenylhydrazone (m.p. 54°C) was easily lost during the extraction procedure, due to its tendency to sublimate. The monochlorophenylhydrazone (oil) showed a broad peak and the dichlorophenylhydrazone (oil) was eluted too slowly.

As far as we know, this is the first report on the precise measurement of the acetaldehyde content in tissues from rats, with or without alcohol administration. As can be seen in Table 2, small amounts of acetaldehyde were found in normal rat tissues. There are hitherto two reports that deal with the measurement of acetaldehyde in normal rats. Eriksson et al. [19] reported that endogenous acetaldehyde could not be detected in normal rat blood when perchloric acid was used as a precipitant; however, it could be measured when the blood was hemolyzed and directly heated to 65°C for 15 min prior to head-space analysis. Eriksson reported later [20] that it was because of artefactual acetaldehyde formed during the preparative procedures for the direct head-space gas chromatography and hemolysation. Yourick and Faiman [21] reported that no acetaldehyde could be detected even in the blood of rats receiving ethanol. They used the head-space chromatographic method for the determination of blood acetaldehyde and the sensitivity was at the m$M$ level.

Acetaldehyde concentrations in human blood from

normal subjects were reported to be 0.89±0.41, 0.4±0.2 and 0.60±0.08 μ*M* by Nakashima et al. [18], Fukunaga et al. [22] and by Takayanagi et al. [23], respectively. The acetaldehyde level in normal rat blood in this report was lower than that of humans.

Here, we will discuss the problems of whether acetaldehyde binds to proteins and, if so, whether the bound acetaldehyde is quantitatively recovered. Acetaldehyde (60 nmol) was mixed vigorously with 2 ml of aqueous bovine serum albumin (BSA) (50 mg/ml) and allowed to stand at 37°C for 60 min. The mixture was filtered using a Centricon YM-30 (Amicon, Beverly, MA, USA) at 4°C and 4500 *g* for 50 min. Acetaldehyde in the filtrate was determined in a similar manner to that described in Section 2.4. This experiment showed that 89% of the acetaldehyde added was found in the filtrate. When the reaction mixture of BSA and acetaldehyde was deprotenized by perchloric acid, a major portion (90%) of the acetaldehyde added was recovered in the supernatant. These values are calculated as 100% when acetaldehyde was added to water instead of to an aqueous albumin solution.

Since glucose is present in the blood of rats and human subjects at a concentration of 4 m*M*, it is thought that glucose interferes with the determination of acetaldehyde using this method. However, as shown in Section 3, there was no interference from glucose using our method.

It is not common knowledge that acetaldehyde is found in normal rat tissues and in blood samples from normal subjects that did not receive alcohol. The origin of acetaldehyde in normal rat tissues is being investigated by us. As a preliminary report, when diacetyl, acetoin and 2,3-butanediol ($C_4$ compounds) were orally administered to rats, the acetaldehyde levels in blood were about ten times higher than those found in normal rat blood 1 h after the administration of $C_4$ compounds. One source of acetaldehyde in the tissues of normal animals is $C_4$ compounds, which are always present in the range of 1 to 8 μ*M* in rat tissues [1,2].

Disulfiram is an inhibitor of liver mitochondrial low $K_m$ acetaldehyde dehydrogenase in vivo. Blood acetaldehyde in rats given disulfiram before receiving ethanol was measured by Yourick and Faiman. [21]. In their report, its level peaked 30 min after ethanol ingestion and the peak level was 450 μ*M*, corresponding to a 3% blood ethanol concentration. Eriksson [20] observed in rats given disulfiram that blood levels of acetaldehyde were about 200 μ*M*, which corresponded to a 0.9% blood ethanol concentration. However, they analyzed acetaldehyde by the head-space chromatographic method, which has lower sensitivity. In this paper, when the rats that had been pretreated with disulfiram were injected with alcohol, their blood was found to contain 1 m*M* acetaldehyde (Fig. 2). This concentration was two- or five-fold higher than that determined by Yourick and Faiman [21] and by Eriksson [20].

## Acknowledgments

We are grateful to Dr. J. Shiozawa of the Max Planck Institute of Biochemistry (Munich) for improving our English. We thank the Ryoubi Teien Memorial Foundation for supporting this work.

## References

[1] M. Otsuka and S. Ohmori, J. Chromatogr., 577 (1992) 215.
[2] M. Otsuka and S. Ohmori, J. Chromatogr. B, 654 (1994) 1.
[3] M. Otsuka, T. Mine, K. Ohuchi and S. Ohmori, J. Biochem., 119 (1996) 246.
[4] R.L. Shriner, R.C. Fuson and D.Y. Curtin, The Systematic Identification of Organic Compounds. A Laboratory Manual, Wiley, New York, 6th ed., 1956, p. 537.
[5] Y. Hoshika and Y. Tanaka, J. Chromatogr., 120 (1976) 379.
[6] D. Lucas, J.F. Menez, F. Berthou, Y. Pennec and H.H. Floch, J. Chromatogr., 382 (1986) 57.
[7] C. Lynch, C.K. Lim, M. Thomas and T.J. Peters, Clin. Chim. Acta, 130 (1983) 117.
[8] E. Mentasti, J. Chromatogr., 417 (1987) 253.
[9] G.A. Cordis, D. Bagchi, N. Maulik and D.K. Das, J. Chromatogr. B, 661 (1994) 181.
[10] J.R. Dahlgran and M.N. Jameson, J. Assoc. Off. Anal. Chem., 71 (1988) 560.
[11] F. Lipari and S.J. Swarin, J. Chromatogr., 247 (1982) 297.
[12] K. Kuwata, M. Uebori and Y. Yamasaki, J. Chromatogr. Sci., 17 (1979) 264.
[13] Y. Hosuhika, J. Chromatogr., 120 (1976) 379.
[14] V.P. Uralets, J.A. Rijks and P.A. Leclercq, J. Chromatogr., 194 (1980) 135.
[15] K. Fung and D. Grosjean, Anal. Chem., 53 (1981) 168.
[16] J.M. Christensen, H. Angelo and J. Knop, Clin. Chim. Acta, 166 (1981) 389.

[17] J.F. Brien and C.W. Loomis, Clin. Chim. Acta, 87 (1978) 175.
[18] K. Nakashima, Y. Hidaka, T. Yoshida, N. Kuroda and S. Akiyama, J. Chromatogr. B, 661 (1994) 205.
[19] C.J.P. Eriksson, Y. Mizoi and T. Fukunaga, Anal. Biochem., 125 (1982) 259.
[20] C.J.P. Eriksson, Biochem. Pharmacol., 34 (1985) 3979.
[21] J.J. Yourick and M.D. Faiman, Biochem. Pharmacol., 38 (1989) 413.
[22] T. Fukunaga, P. Sillanaukee and C.J.P. Eriksson, Alcoholism: Clin. Exp. Res., 17 (1993) 1198.
[23] M. Takayanagi, S. Goto, Y. Kokubo, M. Suzuki and T. Yashiro, Chem. Pharm. Bull., 37 (1989) 200.