Fatty acid ethyl esters are present in human serum after ethanol ingestion

Kathleen M. Doyle,[1] David A. Bird, Salih Al-Salihi, Youseff Hallaq, Joanne E. Cluette-Brown, Kendrick A. Goss, and Michael Laposata[2]

Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Abstract The aim of the study was to determine whether fatty acid ethyl esters, nonoxidative products of ethanol metabolism selectively present in organs damaged by ethanol abuse, are detectable in the serum after ethanol ingestion. Serum samples of hospital emergency room patients with positive (n = 32) and negative (n = 5) blood ethanol levels were assayed for fatty acid ethyl esters. In a separate study, five healthy subjects received an ethanol dose based on body weight mixed with fruit juice in a 1:2 ratio and administered by measured ingestion. Fatty acid ethyl esters were found in the serum of hospital emergency room patients with positive blood ethanol levels. The concentration of fatty acid ethyl esters in these patients correlated with the concentration of blood ethanol (r = 0.57; 95% confidence interval 0.28 to 0.77; P = 0.0002). In the controlled ethanol ingestion study with five healthy subjects, it was also determined that the serum fatty acid ethyl ester concentration began to decrease within 2 h of the time ethanol ingestion had been stopped. The fatty acid ethyl esters in the serum were bound to lipoprotein and albumin, and there was a higher percentage of saturated fatty acids in the FAEE pool than in the serum free fatty acid and triglyceride pools. These studies indicate that fatty acid ethyl esters, which have been implicated as mediators of ethanol-induced organ toxicity, are present in serum after ethanol ingestion.—**Doyle, K. M., D. A. Bird, S. Al-Salihi, Y. Hallaq, J. E. Cluette-Brown, K. A. Goss, and M. Laposata.** Fatty acid ethyl esters are present in human serum after ethanol ingestion. *J. Lipid Res.* 1994. **35:** 428–437.

Supplementary key words alcohol • lipoproteins • albumin

Fatty acid ethyl esters (FAEEs) are esterification products of fatty acids and ethanol (1). They are generated through the enzymatic action of FAEE synthase and, unlike acetaldehyde, represent a metabolic product of ethanol that is not generated by oxidation of ethanol. In a 1986 study (2) using postmortem organ samples from individuals who were acutely intoxicated at the time of death, FAEEs were found selectively in the organs most frequently damaged by ethanol abuse, with large amounts in pancreas and liver, and moderate amounts in heart and brain. There was also a substantial concentration of FAEEs in the adipose tissue of these subjects. Furthermore, it was reported that these five organs/tissues had the highest levels of FAEE synthase activity of all organs/tissues tested. This strong correlation between both FAEE accumulation and FAEE synthase activity and the well-recognized pattern of ethanol-induced organ damage led to the hypothesis that FAEEs are, at least in part, responsible for ethanol's pathological effect. In this report, there was no assessment of FAEE concentration or FAEE synthase activity in the blood. In support of the conclusion that FAEE may mediate ethanol-induced organ damage, we recently observed that FAEEs delivered to HepG2 cells via reconstituted LDL exert a direct toxic effect, decreasing [^{3}H]thymidine uptake by HepG2 cells by 45% when compared to cells incubated with LDL reconstituted with triglycerides (Szczepiorkowski, Z. M. and M. Laposata, unpublished observations).

Postmortem analysis of organs and tissues from known chronic alcoholics in the same study (2) who had undetectable levels of ethanol at the time of death showed FAEEs only in adipose tissue. The presence of FAEEs in adipose tissue, their absence in the other organs of chronic alcoholics, and the presence of FAEEs in highest concentration in liver and pancreas of acutely intoxicated individuals led to the hypothesis that FAEEs are synthesized in the the liver and pancreas, secreted from one or both of these organs, and transported through the blood to adipose tissue. It has been further suggested that in adipose tissue, FAEEs persist with a long half-life because the hydrophobic FAEEs are slowly degraded in this environment (2). If this hypothesis were true, there is reason to believe that FAEEs are present in the blood at some point

Abbreviations: FAEE, fatty acid ethyl esters; FAME, fatty acid methyl esters; TLC, thin-layer chromatography; GC, gas chromatography; GC–MS, gas chromatography–mass spectroscopy; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; PBS, phosphate-buffered saline; NEFA, nonesterified fatty acid; CI, confidence level.

[1]Present address: Department of Clinical Laboratory Sciences, University of Massachusetts Lowell, Lowell, MA 01854.

[2]To whom correspondence should be addressed at: Room 235 Gray Building, Massachusetts General Hospital, Boston, MA 02114.

after ethanol ingestion. As strong circumstantial evidence exists that FAEEs can be toxic mediators of ethanol-induced organ damage (2–4), serum FAEE measurement may have substantial clinical significance in the intoxicated patient, particularly if the FAEE can be eliminated before inducing organ damage. A separate report (5) indicated that white blood cells from outdated whole blood products had the capacity to synthesize FAEEs when incubated with very high concentrations of ethanol in vitro (0.1–1.0 mol/l), increasing the speculation that FAEEs are present in the blood. The use of serum for FAEE detection avoids the more difficult, if not impractical, alternative for clinical FAEE measurement using fat biopsy samples (6). Although obtaining fat or organ biopsies is not problematic in the postmortem state for forensic application, FAEEs must be detectable in the blood for routine clinical use.

In this study, we measured the serum FAEE concentration in two groups of subjects. The first group included patients who presented in the hospital emergency room with blood ethanol concentrations from 0.03 to 0.67 g%. The second group was comprised of five normal subjects who ingested a known amount of ethanol at a controlled rate. The data indicate that FAEEs are present in the serum after ethanol ingestion and that there is a correlation between the serum concentrations of ethanol and FAEEs. We also show that FAEEs are bound to lipoproteins and albumin in the serum, and that the serum concentration of FAEEs increases with ethanol ingestion and begins to decrease shortly after it is ended.

METHODS

Patient specimens

Blood was obtained from 32 patients who presented in a hospital emergency room with elevated blood ethanol levels and from 5 control patients with undetectable blood ethanol levels. Serum was isolated by centrifugation of samples collected in vacuum tubes without anticoagulant at 1250 *g* for 10 min and was stored up to 3 months at −80°C. Serum ethanol levels were determined in the clinical laboratory by gas chromatography (GC) using standard methods (7). Briefly, an internal standard of 1-propanol was mixed with the serum sample, prior to injection of a 1-μl sample into a Hewlett-Packard 5890 gas chromatograph containing a 5% Carbowax 20 M/60/80 Carbopack B column (Supelco, Bellefonte, PA), with an oven temperature set isothermally at 100°C. The ethanol peak was identified by comparison to known standards.

FAEE isolation and quantitation

An internal standard of 50 nmol of ethyl heptadecanoate (ethyl 17:0) (Nu-Chek Prep, Elysian, MN) was added to each sample. Lipids were extracted by a modified Folch method (8). FAEEs were isolated from the organic phase by thin-layer chromatography (TLC) using a petroleum ether–diethyl ether–acetic acid 75:5:1 solvent system and silica gel 60 plates (E. Merck, Darmstadt, Germany) (9). The isolated FAEEs were scraped from the TLC plate in an atmosphere of nitrogen and eluted from the silica gel with 2.5 ml acetone. The FAEEs in the eluate were concentrated by drying the sample under nitrogen. A 1- to 2-μl sample of the concentrated eluate was then injected into a Perkin-Elmer 8500 GC with a WCOT Supelcowax capillary column (Supelco, Bellefonte, PA). The FAEEs were separated and quantitated using a temperature program from 150°C - 250°C increasing at 10°C/ min. Individual FAEEs were identified by comparison to known FAEE standards (Nu-Chek Prep, Elysian, MN). Using this temperature program, all FAEE tested (16:0, 16:1 n–7, 18:0, 18:1 n–9, 18:2 n–6, and 20:4 n–6) were clearly resolved from their corresponding fatty acid methyl esters. In addition, no fatty acid methyl esters were detected in any of the samples.

In a separate experiment, lipids were extracted from serum and separated by TLC as described above. FAEEs were then identified by gas chromatography–mass spectroscopy (GC–MS). GC–MS analysis was performed on a Hewlett-Packard 5890 gas chromatograph coupled to Hewlett-Packard 5970 mass spectrometer with a WCOT Supelcowax capillary column (Supelco, Bellefonte, PA). The injector was maintained at 260°C, the MS detector at 280°C, and the oven was heated from 150°C to 250°C increasing at a rate of 10°C/min and maintained at 250°C for 6 min. Total ion chromatograms were generated using an ionization energy of 70 eV.

Lipoprotein separation

Sera from 15 of the emergency room patients were pooled and subjected to density gradient ultracentrifugation according to Redgrave, Roberts, and West (10). Serum was adjusted to d 1.21 g/ml by addition of KBr. Subsequent solutions of d 1.063, d 1.019, and d 1.006 g/ml were carefully overlayered forming a discontinuous gradient. The samples were then centrifuged at 286,000 *g* at 20°C for 48 h. Serum lipoproteins (VLDL, LDL, HDL) and the serum fraction with a density > 1.21 g/ml were isolated. The above fractions were dialyzed, and from each the lipids were then extracted. FAEEs were isolated by TLC and quantitated by GC as described above.

LDL isolation and reconstitution with radiolabeled FAEE

LDL were isolated by density gradient centrifugation as described by Havel, Eder, and Bragdon (11) and reconstituted with [^{14}C]ethyl palmitate prepared from [^{14}C]tripalmitin (40 mCi/mmol) (DuPont-New England Nuclear, Boston, MA) using the method of Krieger et al. (12). The specific activity of the radiolabeled FAEEs was 29.3 dpm/pmol in a 4.0 ml volume of reconstituted LDL.

In the experiments to assess transfer of FAEEs from LDL to HDL and to albumin, the mixtures contained 4.0 ml of LDL (76.3 mg/dl apolipoprotein B, final concentration), 3.0 ml HDL (50 mg/dl apolipoprotein A-I, final concentration), and 1.7 ml delipidated serum with 1% thimerosol. After an incubation at 37°C for 4 h, the 8.7-ml volume was added as an overlay to 17.3 ml of a solution containing 0.269 mM Na_2 EDTA and 3.05 mM sodium azide with NaBr (2.47 mol/1000-g) at a density of 1.177 g/ml for ultracentrifugation at 115,250 *g* for 20 h to collect LDL. After the LDL was removed, the remaining infranatant was overlaid with a solution containing 0.269 mM Na_2 EDTA and 3.05 mM sodium azide with NaBr (8.15 mol/1000 g solution) at a density of 1.504 g/ml. The sample was centrifuged again at 115,250 *g* for 20 h and the HDL was removed. The albumin-bound FAEEs were in the infranatant from this centrifugation.

FAEE binding to albumin

To directly assess FAEE binding to albumin, 75 nmol of ethyl oleate in hexane was dried completely under nitrogen and then 40 μl human serum was added to the same tubes with vigorous mixing for 1 min. Human serum albumin was immunoprecipitated from the 40-μl serum samples which had been incubated with the ethyl oleate at 37°C for 0 or 2 h. After the addition of 480 μl of rabbit antiserum to human albumin (2.4 mg antibody protein) (Cappel Research Products, Durham, NC), the samples were diluted with distilled water to a total volume of 2 ml. The samples were agitated for 1 h at 8 inversions/min at room temperature and then incubated at 4°C overnight. The albumin-antibody complexes were then isolated by centrifugation at 2500 *g* for 20 min at 4°C. The precipitate was washed 3 times with cold phosphate-buffered saline (PBS) (8.1 mmol/l Na_2 HPO_4, 137 mmol/l NaCl, 1.5 mmol/l KH_2PO_4, 2.7 mmol/l KCl, pH 7.3), and then resuspended in 1 ml of PBS (13). The lipids were extracted from the precipitate using a modified Folch method (8) and the FAEEs were isolated by TLC and quantitated as described above.

FAEE binding to lipid carriers in the serum

[^{14}C]FAEEs were prepared by incubating 0.2 mmol/l [^{14}C]oleate (56 mCi/mmol) (DuPont-New England Nuclear, Boston, MA) with 1 mol/l absolute ethanol and rat liver homogenate as a source of FAEE synthase for 4 h at 37°C (14). The [^{14}C]FAEEs generated were extracted and isolated by TLC as described above. The radiolabeled FAEEs (200 dpm/μl) in hexane were dried completely under nitrogen, after which 1 ml of human serum was added with mixing for 1 min. The mixture was incubated with normal human serum for 2 h at 37°C and then subjected to gel filtration using Sephacryl S-200 HR (Pharmacia/LKB Biotechnology, Piscataway, NJ), packed in a 1.5 × 100 cm column equilibrated with PBS. Fractions (0.35 ml/min) were collected and monitored for absorbance at 280 nm. Radioactivity in the fractions was measured by liquid scintillation counting.

Fatty acid composition

Blood was obtained from nine nonfasting healthy subjects. For the nonesterified fatty acid (NEFA) quantitations, lipids were extracted from the plasma as previously described. The NEFAs were isolated by TLC and scraped from the plate in an atmosphere of nitrogen, and then transesterified using acetyl chloride and methanol (15). Fatty acid methyl esters were separated and quantitated using a temperature program from 150°C to 250°C increasing at 10°C/min, and identified by comparison to known fatty acid methyl ester standards (Nu-Chek Prep, Elysian, MN).

For experiments involving fatty acid analysis of triglycerides from the sera of 15 of the emergency room patients with elevated blood ethanol and high serum FAEE concentrations (3.8–46.1 μM), the lipids were extracted from the samples using a modified Folch extraction (8) with a triheptadecanoin internal standard (20.9 nmol). The extract was dried to completion under nitrogen and the triglycerides were isolated by thin-layer chromatography using a solvent system of petroleum ether–diethyl ether 75/15 (v/v). The triglycerides were scraped from the plate using precautions to limit fatty acid oxidation (15) and then eluted from the silica gel with chloroform. The eluted triglycerides were then subjected to hydrolysis with fatty acid methylation in alkaline methanol (16). The fatty acid methyl esters were analyzed by GC–MS as described above for FAEE, and the data for each fatty acid were calculated as percent of total fatty acid.

Ethanol ingestion study

A controlled study to assess the relationship between ethanol intake and the production of FAEEs was conducted. The study was approved by the institutional review board, and written informed consent was obtained from each patient. The five healthy subjects ingested a low-fat breakfast 3–4 h prior to ethanol intake and they had ingested no ethanol for the previous 72 h. An initial serum sample for baseline ethanol and FAEE levels was taken. The subjects were then given measured amounts of vodka mixed with fruit juice in a 1:2 ratio based upon their weight to raise their blood ethanol levels to 0.07–0.10 g %. Four of the five subjects ingested 150 ml of vodka and the fifth (smaller) subject ingested 120 ml. The alcoholic drink was divided into 9 equal aliquots, and the volunteers drank each aliquot over a 10-min time period. Blood was drawn after cessation of drinking (90 min after the start of ethanol ingestion) and again 2 h later. Blood ethanol and total FAEEs were measured as described above.

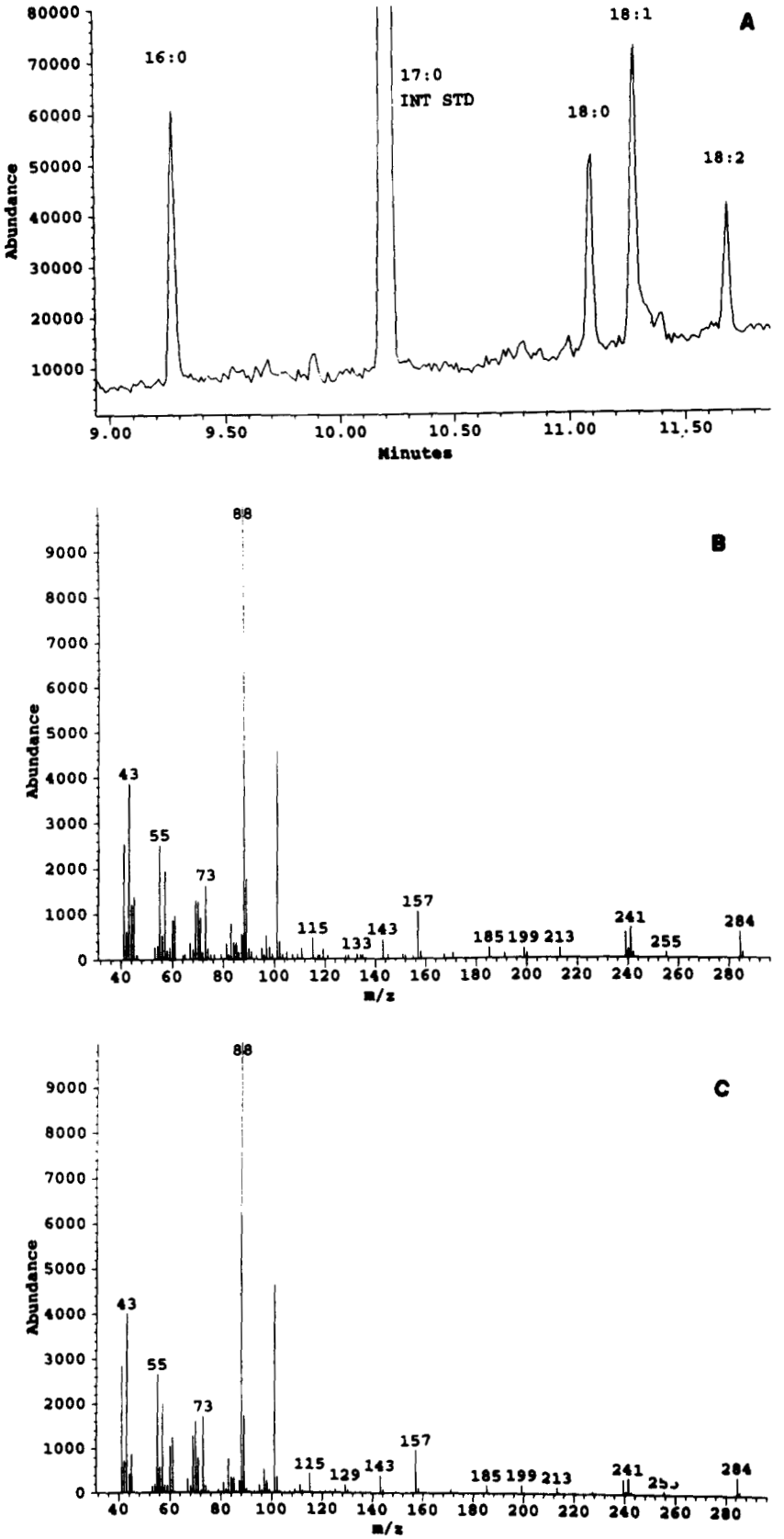

Fig. 1. A: GC-MS tracing of a serum sample containing fatty acid ethyl esters from a subject with a blood ethanol level of 0.1 g%. B: Electron-impact mass spectrum of ethyl 16:0 from Fig. 1A. C: Electron-impact mass spectrum of authentic ethyl 16:0.

Statistical analyses

Data were analyzed for statistical differences using the unpaired Student's *t*-test (two-tailed). Correlation coefficients were determined by regression analysis.

RESULTS

To confirm the identity of FAEEs in serum isolated by TLC from serum lipid extracts, several FAEE-containing samples were analyzed by GC–MS. As shown in **Fig. 1A,** in a sample obtained from a subject who had ingested ethanol (blood ethanol 0.10 g %), the peak in the GC tracing thought to represent ethyl palmitate was positively identified by comparison of its electron impact MS pattern (Fig. 1B) with that of authentic ethyl palmitate (Fig. 1C) with a 98% match. Ethyl stearate was a 98% match, ethyl oleate (n–9) a 95% match, and ethyl linoleate (n–6) a 90% match. The major ions from each of the FAEE peaks (88 and 101) were those characteristic of ethyl esters, and the molecular ions from each of these peaks in the tracing reflected the exact molecular weight of that FAEE.

In the next series of studies, we obtained samples from emergency room patients with blood ethanol concentrations of 0.03–0.67 g%. The experimental advantage provided by this group of patients was that the blood ethanol concentrations in many cases were extremely high and therefore most likely to have FAEEs in the serum after ethanol ingestion. Higher FAEE concentrations in the serum were associated with higher blood ethanol levels (**Fig. 2**), and emergency room patients with no blood ethanol (control subjects) had no detectable serum FAEE. To provide an approximation of FAEE content relative to cholesteryl ester concentration, the total cholesterol concentration was determined in samples from 13 subjects with the highest FAEE levels. Assuming that 60% of the total cholesterol is esterified, the FAEE concentration was approximately 0.5% of the cholesteryl ester concentration. Because it was impossible in this group of patients to determine the amount of ethanol ingested, the time course of drinking, the time at which ingestion was stopped, and the specific alcoholic beverages consumed, a statistically significant correlation between patients' positive blood ethanol levels and serum FAEE concentrations was not anticipated. However, despite the many uncontrolled variables, there was a statistically significant correlation between blood ethanol levels and ethyl palmitate (r = 0.50; 95% confidence interval [CI], 0.19 to 0.73; P = 0.002), ethyl stearate (r = 0.60; 95% CI, 0.32 to 0.79; P = 0.00007), ethyl oleate (r = 0.58; 95% CI, 0.29 to 0.77; P = 0.0002), ethyl linoleate (r = 0.42; 95% CI, 0.08 to 0.66; P = 0.01) and total FAEEs (r = 0.57; 95% CI, 0.28 to 0.77; P = 0.0002). Thus, this study further documents that FAEEs are present in the serum after ethanol ingestion and that higher blood ethanol concentrations are associated with higher serum FAEE levels. Having identified FAEEs in the serum after ethanol ingestion, the possibility that FAEE synthesis occurs within the serum was assessed. FAEE synthase assays were performed using sera from 10 normal individuals as an enzyme source, and no enzyme activity was detected. Preliminary studies indicate that selected populations of white blood cells can synthesize FAEE from free fatty acids and ethanol (Gorski, N.P. and M. Laposata, unpublished observations). Thus, it remains to be determined whether the FAEEs detected in the sera are synthesized in organs and secreted into the blood or synthesized within the vascular compartment by white blood cells.

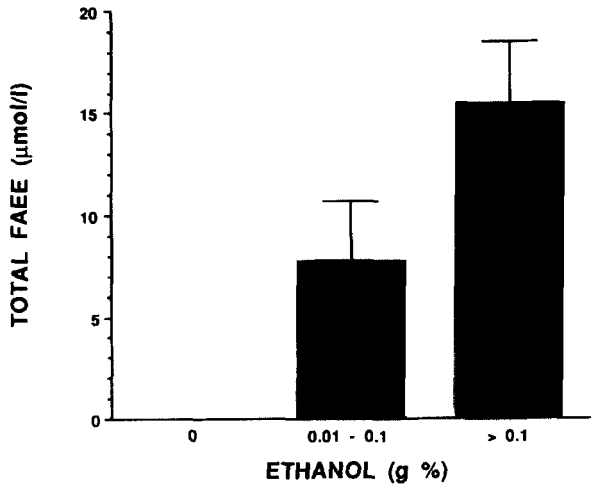

Fig. 2. Total serum FAEE versus serum ethanol concentration from 37 emergency room patients. Serum lipids were extracted, separated by TLC, and quantitated by GC. Controls, g % ethanol, n = 5; 0.01–0.1 g % ethanol, n = 7; > 0.1 g % ethanol, n = 25.

FAEEs have even greater hydrophobicity than triglycerides which must be enclosed in the core of lipoproteins for transport in the blood. The next series of experiments addressed the mode of transport of FAEEs in the serum. The sera from 15 emergency room subjects with elevated levels of FAEEs were pooled and subjected to density gradient ultracentrifugation to separate VLDL, LDL, and HDL from other serum proteins. As shown in **Fig. 3,** 68.3% of the FAEEs were found in the d > 1.21 g/ml fraction that contains albumin as well as other plasma proteins. In addition, 13.2% of serum FAEE were associated with HDL, 16.4% with LDL, and 2.1% with VLDL. Cumulatively, 31.7% of serum FAEEs were associated with lipoproteins.

As most of the serum FAEEs were found in the d > 1.21 g/ml fraction containing albumin, the next experiment determined whether FAEEs in serum were, like nonesterified fatty acids, bound to albumin. Serum samples from emergency room patients were pooled and serum albumin was immunoprecipitated. The lipids from the immunoprecipitate were extracted and the FAEEs

were isolated for quantitation. Although FAEEs were found in the immunoprecipitates, the recovery of FAEEs after immunoprecipitation, lipid extraction, and TLC was too low for reproducible quantitation. Therefore, to assess the capacity of albumin to bind FAEEs in serum samples, 75 nmol of FAEE (ethyl oleate) was incubated for 0 or 2 h with a sample of normal serum, from which the albumin was immunoprecipitated. FAEEs bound to serum albumin were detected in these studies (**Fig. 4**). This provided evidence to support the earlier density gradient studies that a significant fraction of serum FAEEs are bound to albumin.

To further investigate the question of FAEE binding to lipid carriers in the serum, radiolabeled FAEEs ([^{14}C]ethyl oleate) were incubated with normal serum for up to 3 h at 37°C. The serum was then gel-filtered using a Sephacryl-200 column, and the radioactivity was determined in each fraction. **Fig. 5** shows that the ethyl esters appeared in two major fractions in the plasma. One fraction eluted in the void volume of the Sephacryl-200 column and, on the basis of earlier evidence from density gradient ultracentrifugation, this likely represents lipoprotein-associated FAEEs. The other major fraction eluted with a molecular mass of approximately 66 kD and, consistent with both density gradient centrifugation and immunoprecipitation data, likely represents albumin-bound FAEEs. The results of multiple experiments, therefore, indicate that FAEEs in the serum are bound to both lipoproteins and albumin. In this in vitro study, the lipoproteins bound more of the FAEEs than albumin (Fig. 5), but in the sera of individuals ingesting ethanol, the density gradient fraction containing albumin

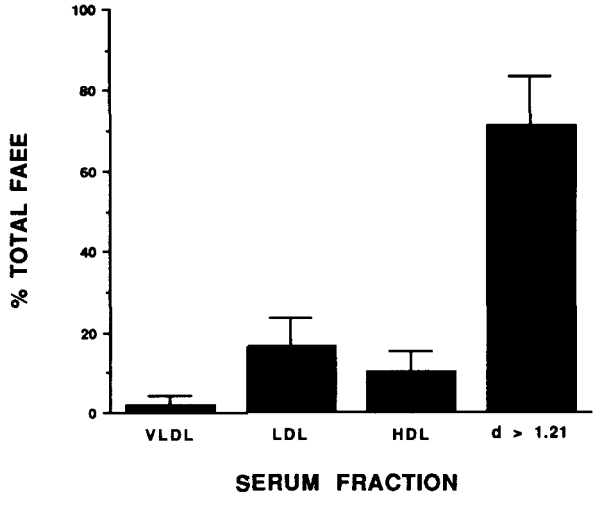

Fig. 3. Percent of total FAEEs in fractions obtained by density gradient ultracentrifugation. Pooled sera from 15 intoxicated emergency room patients were subjected to density gradient ultracentrifugation and serum fractions were isolated. Lipids were extracted and FAEEs were isolated by TLC and quantitated by GC.

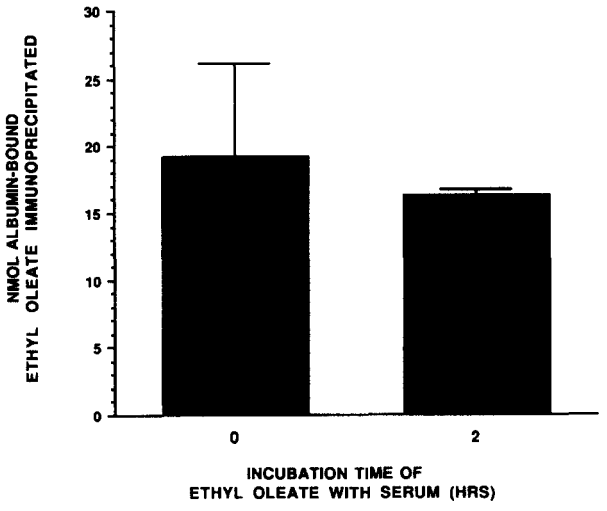

Fig. 4. Ethyl oleate binding to human serum albumin. Ethyl oleate was incubated for 0 or 2 h with normal serum. Albumin was immunoprecipitated by the addition of rabbit anti-human albumin antibody. Ethyl oleate was extracted, isolated by TLC, and quantitated by GC.

showed a higher amount of FAEEs than the lipoproteins (Fig. 3). To address the possibility that FAEEs may be transferred from newly synthesized lipoproteins to albumin, isolated human LDL was delipidated and reconstituted with radiolabeled ethyl palmitate. This LDL preparation was incubated with unlabeled HDL and delipidated serum containing albumin for 4 h to permit transfer of the radiolabeled FAEEs to HDL or to albumin. After this incubation, 10.9% of the FAEE mass remained associated with LDL, 31.3% was associated with HDL, and 57.8% was found in the density gradient fraction containing albumin. The results of this study indicate that FAEEs, like other lipids in the core of lipoproteins, can be transferred between lipid carriers. The mobility of FAEEs between lipid carriers provides a likely explanation for the predominant association of FAEEs with lipoproteins in the in vitro experiments but with albumin in the in vivo studies. A second possible explanation for the differences in FAEE distribution between lipid carriers in Figs. 3 and 5 may be provided by differences in experimental design. FAEEs may have preferentially bound to lipoproteins in the in vitro experiments (Fig. 5) because the FAEEs, dried on the wall of the test tube prior to addition of serum to assess lipoprotein and albumin binding of FAEEs, found the large lipoproteins to be a more available target for binding as they emerged from the wall of the test tube than the much smaller albumin molecules. The binding of FAEEs to lipid carriers in vivo is likely to occur through a variety of different mechanisms.

We next determined the serum concentration of the different NEFAs relative to the concentrations of the

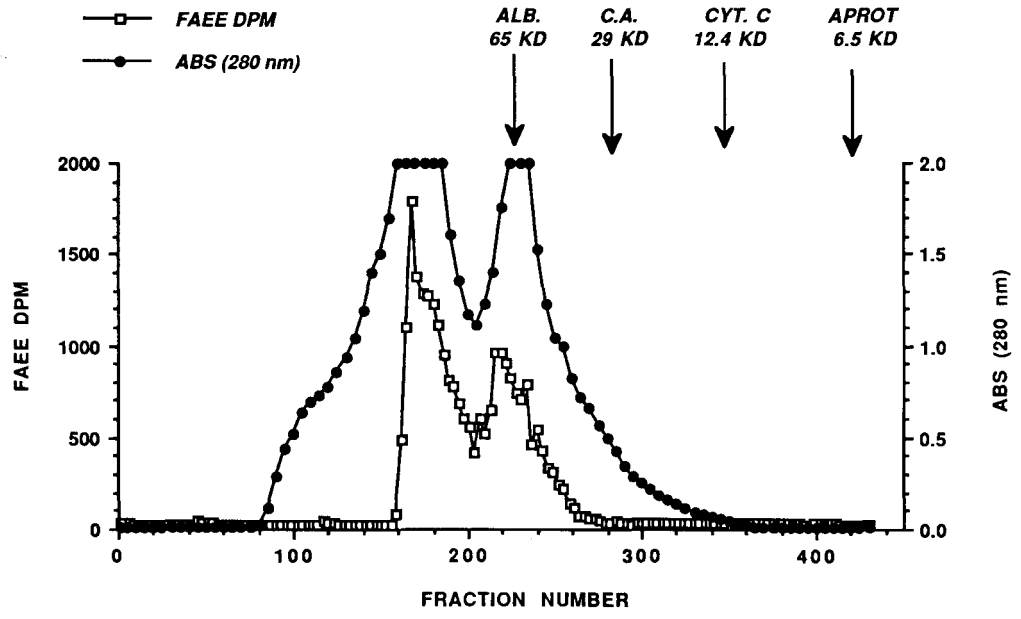

Fig. 5. [^{14}C]ethyl oleate binding to serum lipid carriers. Normal human serum was incubated with [^{14}C]ethyl oleate (200 dpm/μl) and subjected to gel filtration. Fractions were collected and monitored for absorbance at 280 nm. Radioactivity was measured by liquid scintillation counting. Alb, albumin; C.A., carbonic anhydrase; Cyt. C, cytochrome C; Aprot, aprotinin.

different FAEEs. In these studies, individual NEFA concentration was determined after isolation from plasma lipid extracts by TLC and quantitation by GC using multiple internal standards for precise measurement as previously described (15). The data in **Fig. 6** show that the fatty acid composition of NEFAs and FAEEs are similar but not identical. Relative to the fatty acid composition of NEFAs, there was a preference for the saturated fatty acids in FAEEs, with significantly higher concentrations of ethyl 16:0 (ethyl palmitate) in the FAEE pool (P = 0.00489 for FAEE vs. NEFA and P = 0.313 (NS) for FAEE vs. triglyceride). The polyunsaturated fatty acid ethyl esters, ethyl 18:2 n-6 (ethyl linoleate) and ethyl 20:4 n-6 (ethyl arachidonate), represented a significantly lower percentage of total fatty acid than corresponding compounds in the serum NEFA pool (the P values for linoleate and arachidonate percentages in the NEFA vs. the FAEE pools were 0.0037 and < 0.00001, respectively). On the other hand, the linoleate in serum FAEE and serum triglyceride pools was present in very similar percentage concentrations. The lower values for polyunsaturated FAEE relative to polyunsaturated NEFA could have been due to loss by oxidation during FAEE isolation and identification, preference for saturated fatty acids by FAEE synthase, or accelerated hydrolysis or utilization of polyunsaturated FAEE.

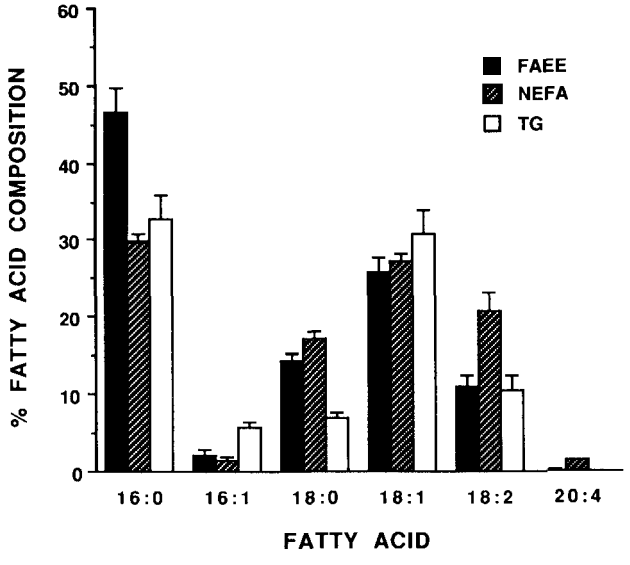

Fig. 6. The fatty acid composition of serum FAEE and serum triglycerides from hospital emergency room patients and plasma NEFA of healthy subjects. The values represent the percent of each fatty acid within either FAEE, triglyceride, or NEFA pools. Fatty acid composition was determined as described in Methods.

In an effort to eliminate many of the variables associated with serum samples obtained from emergency room patients, a study was performed involving five volunteers ingesting known amounts of ethanol at a controlled rate and providing blood samples for measurements of blood ethanol and serum FAEE concentrations. The mean FAEE concentration in these subjects (**Fig. 7A**) increased from 0 at baseline to 0.51 μmol/l (0.51 μM) after ethanol ingestion was completed. Two hours later, the FAEE concentration decreased to a mean of 0.30 μmol/l

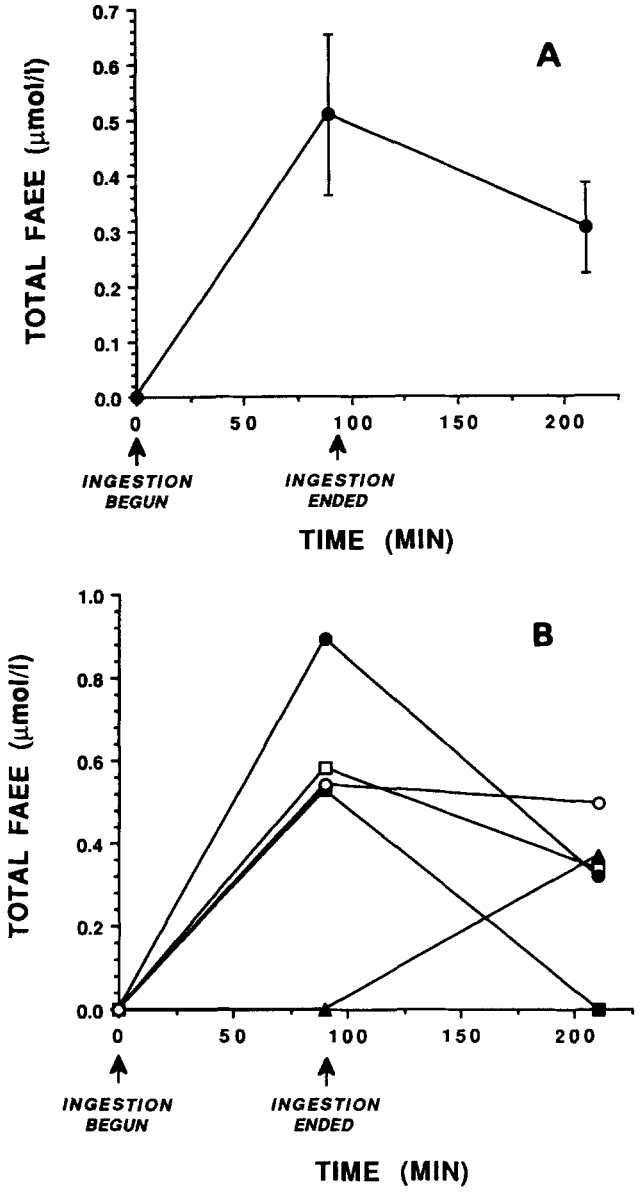

Fig. 7. A: Mean total serum FAEE versus time in five volunteer subjects ingesting measured amounts of ethanol at a controlled rate. Values represent mean ± SEM for five subjects. Lipids were extracted, and FAEEs were isolated and quantitated by GC. B: Total FAEE versus time in five volunteers ingesting ethanol at a controlled rate.

(0.30 μM). The data for the five individual subjects (Fig. 7B) show that four of the five subjects experienced a decrease in FAEE concentration 2 h after ethanol ingestion was stopped.

DISCUSSION

The observation that FAEEs are present in serum after ethanol ingestion allows for determination of FAEE concentrations in living patients. Because evidence currently exists that FAEEs may be toxic metabolites of ethanol (3, 4), this observation of FAEE presence in the serum could lead to the development of a valuable diagnostic test in patients ingesting an overdose of ethanol.

Earlier observations that FAEEs are present in organs after ethanol ingestion, while they are highly significant, do not have great diagnostic implications in living patients. The collection of a fat biopsy sample with the use of a vacutainer tube is the only reasonable option for samples other than blood. However, this approach still represents an invasive and painful procedure for FAEE quantitation. The measurement of serum FAEEs may not only be valuable as an assessment of alcohol ingestion, but it may have another important diagnostic advantage over ethanol measurement. There have been reports (6, 17) indicating that the half-life of FAEEs in adipose tissue is prolonged, on the order of 16–24 h. This has led to the hypothesis that adipose FAEEs may be analogous to red blood cell hemoglobin A_{1C}, in that they may represent a long term marker of ethanol ingestion. Because of the difficulties associated with collection of fat samples from living patients, the current study raises the possibility that serum FAEEs rather than adipose FAEEs might be useful as a convenient long-term marker of ethanol ingestion. Studies are ongoing to determine the time of appearance and disappearance of FAEEs in the serum after ethanol ingestion in a highly controlled clinical study with 19 time points over 24 h for FAEE and ethanol quantitation. The value of serum FAEEs as a diagnostic marker for ethanol ingestion is independent of its potential role as a toxic metabolite of ethanol-induced organ damage.

It is not stated in the 1986 autopsy study in which FAEE were found in tissues and organs (2) whether serum was tested for the presence of FAEEs or whether an examination of the blood in these subjects revealed FAEEs. We speculate that FAEEs would not have been detected in the earlier study because a packed column GC methodology was used. The use of capillary GC in the current study increased the sensitivity of FAEE detection at least 100-fold over packed column methods and, therefore, may explain why it was possible to detect and quantitate serum FAEEs. It is also possible that FAEEs are degraded after death and that postmortem blood would be negative for FAEEs as a result of esterase action upon the FAEE. This may not occur as quickly in organs and tissues, particularly adipose, where the FAEEs are in a more hydrophobic environment than in the aqueous-based environment of the blood.

The origin of the FAEEs in the serum has not yet been determined. It has been reported (5), and we have recently demonstrated in our laboratory, that FAEEs can be synthesized in vitro by white blood cells. It is possible, therefore, given the presence of FAEE synthase in white blood cells, that NEFAs in the plasma or NEFAs in white blood cells serve as substrates for white blood cell FAEE

synthase and that FAEEs are made within the vascular compartment. Upon ethanol ingestion, FAEEs could be synthesized by white blood cells and released into the plasma with subsequent binding to albumin or lipoproteins. It is also possible that the major organs for FAEE production, pancreas and liver, synthesize FAEEs and secrete them into the blood. In the case of the liver, FAEEs could be packaged into the core of lipoproteins at the time of synthesis. At present, the location of the FAEE synthase responsible for generation of FAEEs detectable in the serum after ethanol ingestion remains to be identified.

Recently, encapsulated n-3 fatty acid dietary supplements in the form of FAEEs instead of triglycerides have become available. The use of FAEEs rather than triglycerides as n-3 fatty acid carriers permits delivery of a higher concentration of n-3 fatty acids relative to other fatty acids. Studies on the efficiency of absorption of FAEEs from the gut and the extent of FAEE hydrolysis within the gut have yielded a variety of different results probably due to many differences in experimental design. In rats given FAEEs, FAMES, and triglycerides by stomach tube, Yang, Kuksis, and Myher (18) demonstrated during peak absorption that the appearance of fatty acids in lymph chylomicrons and in intestinal mucosal cells from the digestion and absorption of FAEE and FAME was nearly 50% lower than corresponding triglycerides. They also demonstrated uptake by enterocytes of intact FAMEs and FAEEs (4–22% of administered amounts). Other studies in humans by Nordoy et al. (19) suggest that intestinal absorption of fatty acids as FAEEs is equivalent to the absorption of fatty acids within triglycerides. In addition, the data in this report (19) suggest that there is little or no absorption of intact FAEEs with complete or near complete hydrolysis of FAEEs in the gut. Interestingly, Newsome and Rattray (20) demonstrated that pancreatic extracts were able to enzymatically esterify fatty acids with long chain alcohols in vitro, raising the possibility that ethyl ester formation may also occur within the gastrointestinal tract from ingested ethanol and fatty acids. In preliminary studies in our laboratory, after ingestion of FAEE-containing capules, FAEEs do appear in both blood and adipose tissue. If FAEEs are shown to be toxic mediators of ethanol abuse, the long-term use of these newly available dietary supplements may have pathological implications. In conclusion, the present observation that FAEEs are detectable in serum after ethanol ingestion could lead to a major improvement in the monitoring of ethanol ingestion and the treatment of ethanol-induced organ damage.

This research was supported by the National Institutes of Health grants R01 DK37454 and R01 DK43159. The authors would like to acknowledge Dr. Ann Thor for performing the fat biopsies in pilot studies involving ethanol ingestion and FAEE quantitation in adipose tissue, Ms. Patricia Puopolo and Dr. James Flood for the determination of the blood ethanol levels, Dr. Jerome Hojnacki for assistance in the density gradient ultracentrifugation studies, and Dr. David Schoenfeld for the statistical analyses.

Manuscript received 13 May 1993 and in revised form 23 August 1993.

REFERENCES

1. Lange, L. G., S. R. Bergmann, and B. E. Sobel. 1981. Identification of fatty acid ethyl esters as products of rabbit myocardial ethanol metabolism. *J. Biol. Chem.* **256:** 12968–12973.
2. Laposata, E. A., and L. G. Lange. 1986. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. *Science.* **231:** 497–499.
3. Lange, L. G., and B. E. Sobel. 1983. Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol. *J. Clin. Invest.* **72:** 724–731.
4. Mair, D. C., M. E. Donnell, and E. A. Laposata. 1990. Fatty acid ethyl esters, a family of ethanol metabolites, mediate changes in protein secretion in HepG2 cells. *Clin. Res.* **38:** 264A.
5. Wright, M., K. J. Bieser, P. M. Kinnunen, and L. G. Lange. 1987. Nonoxidative ethanol metabolism in human leukocytes: detection of fatty acid ethyl ester synthase activity. *Biochem. Biophys. Res. Commun.* **142:** 979–985.
6. Laposata, E. A., D. E. Scherrer, and L. G. Lange. 1989. Fatty acid ethyl esters in adipose tissue: a laboratory marker for alcohol-related death. *Arch. Pathol. Lab. Med.* **113:** 762–766.
7. Schroeder, T. J. 1984. Alcohol screen. *In* Clinical Chemistry Theory, Analysis and Correlation. L. A. Kaplan and A. J. Pesce editors. C. V. Mosby Company, St. Louis, MO. 1335–1336.
8. Cohen, P., M. J. Broekman, A. Verkley, J. W. W. Lisman, and A. Derksen. 1971. Quantification of human platelet inositides and the influence of ionic environment on their incorporation of orthophosphate-^{32}P. *J. Clin. Invest.* **50:** 762–772.
9. Kinnunen, P. M., and L. G. Lange. 1984. Identification and quantitation of fatty acid ethyl esters in biological specimens. *Anal. Biochem.* **140:** 567–576.
10. Redgrave, T. G., D. C. K. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
11. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoprotein in human serum. *J. Clin. Invest.* **34:** 1345–1353.
12. Krieger, M., M. S. Brown, J. R. Faust, and J. L. Goldstein. 1978. Replacement of endogenous cholesteryl esters of low density lipoprotein with endogenous cholesteryl linoleate: reconstitution of a biologically active lipoprotein particle. *J. Biol. Chem.* **253:** 4093–4101.
13. Marsh, J., and A. F. Whereat. 1959. The synthesis of plasma lipoprotein by rat liver. *J. Biol. Chem.* **234:** 3196–3200.
14. Mogelson, S., and L. G. Lange. 1984. Nonoxidative ethanol metabolism in rabbit myocardium: purification to homogeneity of fatty acid ethyl ester synthase. *Biochemistry.* **23:** 4075–4081.
15. Hallaq, Y., T. C. Becker, C. S. Manno, and M. Laposata.

1993. Use of acetyl chloride/methanol for assumed selective methylation of plasma nonesterified fatty acids results in significant methylation of esterified fatty acids. *Lipids.* **28:** 355–360.
16. Turk, J., B. A. Wolf, J. B. Lefkowith, W. T. Stump, and M. L. McDaniel. 1986. Glucose-induced phospholipid hydrolysis in isolated pancreatic islets: quantitative effects on the phospholipid content of arachidonate and other fatty acids. *Biochim. Biophys. Acta.* **879:** 399–409.
17. DePergola, G., C. Kjellström, C. Holm, N. Conradi, P. Pettersson, and P. Björntorp. 1991. The metabolism of ethyl esters of fatty acids in adipose tissue of rats chronically exposed to ethanol. *Alcohol Clin. Exp. Res.* **15:** 184–189.
18. Yang, L-Y., A. Kuksis, and J. J. Myher. 1990. Intestinal absorption of menhaden and rapeseed oils and their fatty acid methyl and ethyl esters in the rat. *Biochem. Cell Biol.* **68:** 480–491.
19. Nordoy, A., L. Barstad, W. E. Connor, and L. Hatcher. 1991. Absorption of the n–3 eicosapentaenoic and docosahexaenoic acids as ethyl esters and triglycerides by humans. *Am. J. Clin. Nutr.* **53:** 1185–1190.
20. Newsome, W. H., and J. B. M. Rattray. 1965. The enzymatic esterification of ethanol with fatty acids. *Can. J. Biochem.* **43:** 1223–1233.