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RECEIVED for review February 18, 1983. Resubmitted October 5, 1983. Accepted November 4, 1983. This research was supported by the Assistant Secretary for Fossil Energy and Division of Oil, Gas and Shale Technology, and the Bartlesville Energy Technology Center (Dexter Sutterfield, Project Manager) of the U.S. Department of Energy under Contract No. DEAC03-76SF00098.

Determination of Carbon-13 Labeled Lactate in Blood by Gas Chromatography/Mass Spectrometry

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A method is described for derivatizing lactate as *n*-propylamide heptafluorobutyrate. This derivative is an ideal choice for lactate metabolic studies with stable isotopes. It was stable to evaporation and had good gas chromatographic/mass spectrometric properties. Lactic *n*-propylamide heptafluorobutyrate gives prominent ions at *m/z* 327 and 241 upon 70-eV electron impact ionization. The former ion contains all three carbons of lactate, while the latter contains only carbons two and three. When [U-^{13}C]lactate was used as tracer, selected ion monitoring of the ion pairs *m/z* 327 vs. 330 and *m/z* 241 vs. 243 provided blood lactate enrichments, without and with lactate carbon recycling. The low isotopic background of these ion pairs (0.072 ± 0.004% and 0.80 ± 0.01%) means blood samples with low isotope enrichment can be determined with accuracy. The average coefficient of variation of blood samples was 4.8% with enrichments ranging from 0.2 to 1.0 mol % excess and a sample size of less than 50 μL of blood. This technique was applied to the study of lactate metabolism in newborn infants and pregnant women.

Lactate is a product of anaerobic metabolism in the glycolytic pathway in human (*1*, *2*). It is produced mainly from skeletal muscle and erythrocytes. The chief metabolic site is in the liver. Besides oxidation to carbon dioxide for energy purposes, lactate also serves as a major substrate for the synthesis of glucose (cori cycle). Because of the role of lactate in energy metabolism and acid–base balance, the determination of its production rate has been a subject of intensive investigation.

Estimates of lactate production can be obtained by measurement of regional blood flow and arterio-venous concentration differences of tissue and organs. The summation of the lactate production rates of all lactate producing tissues and organs is the total lactate production rate (*1*). Alternately, total lactate turnover rate can be determined by isotope dilution techniques (*3*–*7*). Various ^{3}H-labeled and U-^{14}C-labeled lactates were used by Okajima et al. (*5*) in the determination of lactate turnover rate in starved rats. Forbath et al. (*6*) used [U-^{14}C]lactate in normal and diabetic dogs. The same labeled lactate was used by Sparks et al. (*7*) in measurements in fetal lamb. In human studies, [U-^{14}C]lactate was employed by Kreisberg et al. (*3*) and Searle and Cavalieri (*4*).

Unfortunately, all the above tracers and techniques are not applicable in the study of lactate metabolism in infants and pregnant women due to the potential radiation hazards associated with radioactive tracers. Both of these populations happen to have appreciable numbers of metabolic disorders involving lactate production and disposal. A method capable of studying them without imposing any unnecessary risks is urgently needed.

The availability of stable isotope labeled compounds and the advancement in the stable isotope detecting gas chro-

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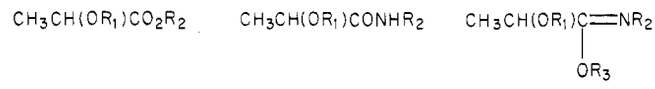
Table I. Derivatives of L-Lactate for GC/MS Analysis



I II III

derivative no.	skeleton	R_1	R_2	R_3	mol wt
1	I	$(CH_3)_3Si$-	$(CH_3)_3Si$-		234
2	I	CH_3CO-	n-C_4H_9-		188
3	I	CH_3CO-	$(CH_3)_3Si$-		204
4	II	H-	n-C_3H_7-		131
5	II	CH_3CO-	n-C_3H_7-		173
6	II	$(CH_3)_3Si$-	n-C_3H_7-		203
7	II	CF_3CO-	n-C_3H_7-		227
8	II	C_2F_5CO-	n-C_3H_7-		277
9	II	n-C_3F_7CO-	n-C_3H_7-		327
10	II	C_2H_5CO-	n-C_3H_7-		187
11	II	n-C_3F_7CO-	n-C_4H_9-		341
12	II	n-C_3F_7CO-	n-C_5H_{11}-		355
13	III	CH_3CO-	n-C_3H_7-	n-C_3F_7CO-	369
14	III	C_2H_5CO-	n-C_3H_7-	n-C_3F_7CO-	383
15	III	C_2H_5CO-	n-C_3H_7-	$(CH_3)_3Si$-	259
16	III	n-C_3F_7CO-	n-C_3H_7-	n-C_3F_7CO-	523
17	III	n-C_3F_7CO-	n-C_4H_9-	n-C_3F_7CO-	537
18	III	n-C_3F_7CO-	n-C_5H_{11}-	n-C_3F_7CO-	551

matograph/mass spectrometer (GC/MS) have made such studies feasible (*8–10*). However, unlike glucose and urea, lactate is usually present in smaller concentrations in blood. In addition, known chemical derivatives of lactate suitable for gas chromatographic/mass spectrometric analysis are usually too volatile to allow the concentration of sample for low-level analysis. In this laboratory, we have evaluated several relatively nonvolatile new derivatives of lactate for gas chromatographic/mass spectrometric properties and found that lactate *n*-propylamide *n*-heptafluorobutyrate is the best derivative for the study of lactate metabolism with stable isotope labeled lactate and GC/MS. The mass spectral fragmentation of several new lactic acid derivatives, the preparation of lactate *n*-propylamide *n*-heptafluorobutyrate, the gas chromatographic separation of D-(−)- and L-(+)-lactates, and the study of lactate metabolism using these techniques with [U-^{13}C]lactate as tracer are described in this report.

EXPERIMENTAL SECTION

Reagents. Analytical grade ion exchange resins, AG50W-X8 and AG 1-X8, were purchased from Bio-Rad Laboratories, Richmond, CA. 2,2-Dimethoxypropane (DMP) was obtained from Aldrich Chemical Co., Milwaukee, WI. *n*-Heptafluorobutyric anhydride, *n*-pentafluoropropionic anhydride, trifluoroacetic anhydride, *n*-heptafluorobutyrylimidazole, and bis(trimethylsilyl)trifluoroacetamide were purchased from Supelco (Bellefonte, PA), Pierce (Rockford, IL), or Regis (Morton Grove, IL). D,L-[U-^{13}C]Lactate (90 atom % excess isotope purity) was purchased from Merck/Isotopes, Quebec, Canada. The isotope purity of the product was verified by isotope cluster technique with GC/MS (*11*). The distribution of D and L isomers was determined by gas chromatography and found to be a 50:50 mixture. L-[U-^{13}C]-Lactate (90 atom % excess) was also obtained from the same source, it was synthesized biochemically from [U-^{13}C]glucose. Gas chromatographic/mass spectrometrical analysis showed that this product contained 3.86% of D isomer. L-[2,3-$^{13}C_2$]Alanine (90 atom % excess) was purchased from Merck.

Isolation of Lactate from Plasma or Blood. Whole blood, plasma, or red blood cells were deproteinized immediately with equal volumes of 10% perchloric acid solution. The supernatant was then neutralized with one-half volume of 14% potassium carbonate.

When only the isotope enrichment of lactate was to be analyzed, the neutralized protein-free supernatant (100 μL) was evaporated to dryness with a stream of air and directly derivatized for GC/MS.

When multiple tracers were infused or the isotope enrichments of substrates other than lactate were to be determined, the neutralized protein free supernatant (200 μL) was separated into glucose, urea, alanine, and lactate fractions through ion exchange resins, AG50W-X8 and AG 1-X8. A column of AG 1-X8 (formate form, 1.6 cm × 0.5 cm) was washed with 10 mL of 2 N formic acid, followed by 10 mL of distilled water. The washed AG 1-X8 column was then positioned on top of a column of AG 50W-X8 (H^+, 1.6 cm). The sample was added to the top column. The tandem columns were eluted with water. The first 5-mL fraction contained glucose. Urea was eluted in the second 5-mL fraction. After water elution, the columns were separated. The top column (AG 1-X8) was eluted with 5 mL of 2 N formic acid to obtain the lactate fraction. Amino acids (including alanine) were collected by eluting the bottom column (AG50W-X8) with 5 mL of 2 N ammonia. Each fraction was evaporated to dryness in a Vortex-Evaporator (Buchler, Fort Lee, NJ) at 40 °C.

Derivatization. The derivatives prepared for GC/MS evaluation are listed in Table I. Lactate *n*-butyl ester acetate (no. 2) was prepared by reacting sequentially with 1-butanol/HCl and acetic anhydride. Lactate trimethylsilyl ester acetate (no. 3) was obtained by silylating *O*-acetyllactic acid.

Lactic *n*-acylamide (no. 4–18) was prepared by the following method: Lactate (20 μg) was mixed with 200 μL of redistilled dimethoxypropane and 20 μL of dilute HCl in methanol (prepared by mixing 200 μL of concentrated HCl and 2 mL of methanol) in a 1-mL Reacti-vial capped with a Teflon faced laminated disk (Pierce). The mixture was allowed to react at room temperature for 1 h. *n*-Propylamine (or *n*-butylamine or *n*-pentylamine), 50 μL, was added to the reaction mixture and heated at 100 °C for 0.5 h. Reagents were then removed in a stream of dry nitrogen, and the residue was extracted with 200 μL of ethyl acetate. The ethyl acetate extract was transferred to another 1-mL Reacti-vial and evaporated to dryness. It was converted to monoperfluoroacylate ester (no. 7, 8, and 9) by reacting with 10 μL of *n*-heptafluorobutyric anhydride (trifluoroacetic anhydride or *n*-pentafluoropropionic anhydride) for 5 min at room temperature. The solution was evaporated to dryness and the residue was dissolved in ethyl acetate for GC/MS analysis. Similarly, lactic *n*-propylamide acyl ester (no. 5 and 10) and Me_3Si ester (no. 6) were prepared in this sequence. The diacyl esters (no. 13–18) were prepared by heating monoacylates in *n*-heptafluorobutyric anhydride or bis(trimethylsilyl)trifluoroacetamide for 20 min at 100 °C.

Gas Chromatography/Mass Spectrometry. A hewlett-Packard 5985A gas chromatograph/mass spectrometer (Palo Alto, CA) was used. The glass column ($^1/_8$ in. × 6 ft) was packed with 10% OV-17 on 100/200 mesh Gas Chrom Q (Applied Science, College Station, PA). The lactate derivatives were analyzed

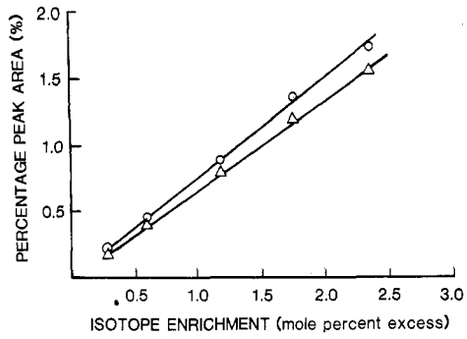


Figure 1. Representative standard curves for L-[U-^{13}C]lactate enrichment analysis with the GC/MS/SIM technique: (O) obtained from m/z 241 vs. 243 and (Δ) obtained from m/z 327 vs. 330. The percentage of peak area was obtained by dividing the area of m/z 243 peak with the total area of m/z 241 and 243 peaks. The same treatment was used for the m/z 327 vs. 330 pair.

isothermally at temperatures ranging from 90 °C to 140 °C. The injection port, jet separator, and ion source temperature were 200 °C.

For electron impact ionization, helium was used as carrier gas at a flow rate of 30 mL/min. An electron energy of 70 eV was used for ionization. Spectra were obtained by repetitive scanning of gas chromatographic effluent from m/z 50 to m/z 550.

For chemical ionization study, methane was used as carrier and reagent gas at a flow rate of 10 mL/min. The gas chromatographic effluent was introduced directly into the mass spectrometric ion source without a separator. With the flow rate maintained, the pressure of the ion source was 0.5 torr. The ionization electron energy was about 150 eV. The spectra were scanned from m/z 80 to m/z 550.

Selected Ion Monitoring GC/MS. Lactate *n*-propylamide heptafluorobutyrate was the derivative used for isotope enrichment determination with selected ion monitoring GC/MS.

L-*Lactate.* A column of 10% OV-17 was used for this analysis. It was held at 100 °C isothermally with a helium flow rate of 26 mL/min. The ions, m/z 241, 243, 327, and 330 were monitored sequentially with dwelling times of 50 ms, 200 ms, 50 ms, and 200 ms, respectively. Standard solutions of known [U-^{13}C]lactate enrichments (prepared by mixing [U-^{13}C]lactate with unlabeled lactate) ranging from 0.1% to 3% were analyzed everyday to correct for the instrumental variation. The peak area percentages of the two ion pairs (m/z 241 vs. 243 and m/z 327 vs. 330) were used to construct standard curves (see Figure 1). The [U-^{13}C]lactate enrichments of the blood samples were determined by comparing the peak area percentage with the standard curves constructed. A Hewlett-Packard 9980 calculator with a linear regression program was used for this purpose.

D,L-*Lactate.* For studies with racemic D,L-[U-^{13}C]lactate as tracer, a capillary column had to be used for base line separation of D-lactate and L-lactate. A wall-coated glass capillary column with Chirasil-Val phase (Applied Science) and a dimension of 0.25 mm × 25 m was employed for the present investigation. The ends of the column were straightened with a glass column straightener (Supelco). It was coupled with the spectrometric ion source inlet with a low dead volume valve (*12*). A Hewlett-Packard 18835A capillary injection system was used for the GC/MS inlet. Splitless mode of injection was adopted with a helium flow rate of 1 mL/min and an injection port pressure of 6 psi. The solvent purging valve was programmed to open 0.7 min after injection. Column temperature was held at 60 °C during injection. It was programmed to rise at 30 °C/min to 110 °C immediately after injection. The column temperature was kept at 110 °C through the rest of the run. With this system, a base line separation of D-lactate propylamide heptafluorobutyrate (R_t = 3.9 min) and L-lactate propylamide heptafluorobutyrate (R_t = 4.2 min) was obtained. Standard curves were constructed from standards prepared from racemic D,L-[^{13}U]lactate and unlabeled L-lactate. The enrichments of D-lactate and L-lactate were both referred to L-lactate, e.g., the peak area of m/z 330 from D-[^{13}U]lactate was calculated with the peak area of m/z 327 from unlabeled L-lactate.

Derivation of Lactate from Blood as *n*-Propylamide Heptafluorobutyrate Derivative. The neutralized perchloric acid solution of blood, plasma, or red blood cells (100 μL) was evaporated to dryness in a stream of air. The residue was treated with 200 μL of dimethoxypropane and 20 μL of dilute HCl in methanol (3%) at room temperature for 1 h. The reaction mixture was decanted into another 1-mL Reacti-vial without transferring any of the solid deposit. Propylamine (50 μL) was added to this clear solution, and the procedure then proceeded as described under Derivatization.

The effluent from ion exchange column chromatography of protein-free blood was evaporated to dryness and derivatized as described above.

Lactate Turnover Rate Determined in Humans. Newborn infants were infused with L-[^{13}C]lactate or D,L-[^{13}C]lactate according to the prime-constant infusion technique for 4 h. The priming dose was 8 mg/kg and the infusion rate was 0.04 (mg/kg)/min. With this technique, a steady state of blood lactate enrichment was usually obtained 120 min after the start of infusion. The production rate was calculated by tracer dilution according to the equation, which is different from the one used conventionally (*13*)

$$P = [(1/E) - 1]\, I \qquad (1)$$

where P is the production rate, (mg/kg)/min, E is the [U-^{13}C]-lactate enrichment of blood, derived from the standard curve assuming [U-^{13}C]lactate tracer was 100% enriched, and I is the infusion rate of [U-^{13}C]lactate, (mg/kg)/min.

Quantitation of Contribution of Alanine Carbon to Lactate in Humans. Pregnant women were infused with L-[2,3-$^{13}C_2$]alanine with a loading dose of 4 mg/kg and an infusion rate of 0.07 (mg/kg)/min for 4 h. The blood enrichment of L-[2,3-$^{13}C_2$]lactate was then determined by the present technique. A steady-state enrichment of blood L-[2,3-$^{13}C_2$]alanine and L-[2,3-$^{13}C_2$]lactate was usually reached within 2 h after the start of infusion. The percentage of lactate carbons derived from alanine was calculated by the following equation

$$\%\ \text{lactate carbon from alanine} = (E_{\text{lactate}}/E_{\text{alanine}}) \times 100 \qquad (2)$$

where E is the ^{13}C enrichments of lactate and alanine at steady state.

RESULTS AND DISCUSSION

For a successful study of lactate metabolism in humans using stable isotopes and gas chromatography/mass spectrometry, suitably labeled lactate and a volatile derivative are required. The tracers used in the metabolic study have to be metabolically stable (in regard to label) and capable of being determined at low enrichment. Besides, more information is obtained if this tracer is capable of being used for recycling and nonrecycling production rate determinations. For these reasons, we chose [U-^{13}C]lactate as the tracer. The determination of the blood enrichments of L-[1,2,3-$^{13}C_3$]lactate will yield a "true" lactate production rate that is devoid of recycled lactate. At the same time the determination of blood enrichment of L-[2,3-$^{13}C_2$]lactate will yield a "recycled" lactate production rate. The difference between the two production rates indicates the degree of recycling. To achieve these goals, the GC/MS derivative of lactate has to have the following characteristics: abundant mass fragment containing all three lactate carbons, abundant mass fragment containing carbon two and three only, low isotopic background at (M + 3) and (M + 2) to permit an accurate low enrichment determination, and a simple derivatization procedure to permit handling of large numbers of samples generated from metabolic studies.

Derivatives for Gas Chromatography/Mass Spectrometry (Table II). The simplest of lactate derivatives is the bis(trimethylsilyl) derivative. Its mass spectrum (EI) has a low abundance of three carbon fragments at m/z 219 ($M^{+\cdot}$ − 15). The fragment at m/z 117, resulting from cleavage between carbons one and two, contains lactate carbons two and three. However, the M + 2 ion (m/z 119) has a high background due to the natural abundance of silicon isotope

Table II. Mass Spectral Fragmentation of L-Lactate Derivatives

derivative no.	EI/CI[a]	mass spectra
1	EI	*m/z*, 219 (6),[b] 191 (12), 190 (11), 147 (73), 117 (100), 75 (16), 73 (100)
2	EI	*m/z*, 143 (6), 133 (18), 115 (30), 105 (20), 87 (100), 57 (10), 43 (10)
	CI	*m/z*, 189 (6), 147 (12), 133 (16), 115 (100), 105 (26), 91 (8), 89 (18), 87 (26)
3	EI	*m/z*, 189 (8), 147 (46), 129 (20), 119 (10), 118 (11), 117 (100), 116 (20), 101 (11), 75 (36), 74 (10), 73 (70)
4	EI	*m/z*, 131 (33), 116 (15), 102 (12), 88 (40), 87 (100), 86 (66), 74 (22), 72 (30), 70 (8), 59 (52), 58 (33)
	CI	*m/z*, 172 (10), 160 (13), 132 (100)
5	EI	*m/z*, 173 (12), 132 (13), 130 (9), 115 (33), 88 (100), 87 (50), 86 (60), 70 (23), 61 (21)
	CI	*m/z*, 214 (12), 202 (30), 174 (100), 132 (78), 114 (17)
6	EI	*m/z*, 203 (1), 188 (10), 159 (25), 144 (8), 117 (100), 73 (92)
7	EI	*m/z*, 227 (22), 198 (10), 169 (8), 141 (50), 126 (8), 113 (11), 86 (100), 69 (61), 58 (8), 56 (14)
8	EI	*m/z*, 277 (20), 248 (9), 191 (40), 163 (8), 147 (9), 119 (54), 114 (9), 86 (100), 69 (18), 58 (6), 56 (12)
9	EI	*m/z*, 327 (20), 298 (8), 241 (50), 214 (8), 197 (6), 169 (34), 119 (6), 114 (6), 100 (8), 86 (100), 69 (39), 58 (6), 56 (12)
10	EI	*m/z*, 187 (8), 143 (5), 129 (15), 102 (50), 101 (13), 86 (37), 74 (22), 57 (100)
11	EI	*m/z*, 341 (9), 312 (13), 298 (11), 241 (62), 213 (10), 197 (9), 169 (42), 128 (10), 119 (8), 100 (90), 85 (22), 72 (12), 69 (35), 58 (8), 57 (100), 56 (20), 55 (10)
12	EI	*m/z*, 355 (3), 326 (11), 312 (15), 298 (10), 241 (72), 213 (11), 197 (9), 169 (46), 142 (9), 114 (100), 100 (6), 86 (10), 85 (40), 72 (16), 71 (95), 69 (38), 56 (18), 55 (13)
13	CI	*m/z*, 310 (100), 256 (10), 131 (14), 129 (58), 101 (18), 75 (18)
14	EI	*m/z*, 129 (10), 101 (24), 57 (100)
15	EI	*m/z*, 169 (8), 129 (10), 101 (35), 69 (10), 57 (100)
16	EI	*m/z*, 310 (7), 268 (19), 254 (11), 241 (100), 213 (28), 197 (19), 169 (52), 100 (8), 69 (52), 56 (14)
17	EI	*m/z*, 324 (6), 296 (10), 268 (30), 241 (100), 213 (25), 197 (14), 169 (39), 154 (9), 100 (8), 69 (24), 57 (30), 56 (18), 55 (8)
18	EI	*m/z*, 338 (8), 310 (12), 282 (26), 268 (23), 241 (100), 213 (23), 197 (18), 169 (50), 114 (6), 71 (20), 70 (18), 69 (23), 56 (8), 55 (10)

[a] EI spectra were obtained with an electron energy of 70 eV. CI spectra were obtained with methane as reagent gas. [b] The numbers in parentheses indicate the percentage of that fragment to base peak (100%). Only fragments with abundance above 5% of base peak are tabulated here. The spectra were recorded from *m/z* 50 to *m/z* 550 using a gas chromatograph as inlet and with an ion source temperature of 200 °C.

from the trimethylsilyl group. Apparently, it is not an ideal derivative for our purpose. The next logical derivative for lactate would be the lactate diesters. Kamerling et al. (*14*) used O-acetylated methynyl esters for capillary gas chromatographic separation of lactate isomers. The preparation of this derivative is cumbersome. We prepared O-acetylated *n*-butyl ester. The EI mass spectrum of this derivative shows extensive fragmentation, with moderate abundant peaks at *m/z* 115, 133, and 143 containing three lactate carbons. The base peak, *m/z* 87, contains lactate carbons two and three. Methane chemical ionization of this derivative increased the three-carbon fragment (*m/z* 115) and decreased the two-carbon fragment (*m/z* 87). The CI mass pair *m/z* 115 vs. 118 can be used to determine the enrichment of [U-^{13}C]lactate. A major disadvantage of this derivative is the difficulty of preparing it in small quantity, such as those which would be encountered in blood samples. *n*-Butyllactate is extremely volatile. Any attempts to concentrate this derivative resulted in loss of most or all of the lactate. Increasing the carbon numbers of the alcohol used to make esters of higher series is infeasible and the volatility problem remains. Alcohols higher than 1-butanol are more difficult to remove in the derivatization mixture. In addition, alcohols as high boiling as menthol still resulted in a lactate ester that was still too volatile to concentrate (*14*). To avoid this problem of lactate loss due to evaporation, we attempted derivatizing the hydroxyl function first. The O-acetylated lactic acid was completely nonvolatile. It was then derivatized as the trimethylsilyl ester (Table I, derivative 3). The mass spectrum of this derivative did not have suitable fragments for quantitative determination of lactate enrichment. Apparently, the approach from ester preparation failed to produce any useful derivative that is not too volatile and also have the desired fragmentation pattern. Amide derivatives of an organic acid are generally much less volatile than the corresponding ester derivatives. The amide is seldomly used for gas chromatographic analysis because it is more difficult to prepare and has poorer gas chromatographic properties than esters. Having an acidic hydrogen, amide usually results in tailing in a gas chromatographic analysis. We have synthesized a number of lactate amide derivatives by using a convenient method developed in our laboratory. These derivatives were stable to evaporation. They can be concentrated, even evaporated to dryness. Besides, they all have good chromatographic properties, especially in high-load nonpolar phases, such as 10% OV-17. Their EI mass spectral fragmentation (Table II, derivatives 4–12) all showed relatively high abundance of fragments containing all three lactate carbons and fragments containing lactate carbons two and three. Amides were prepared by using *n*-propylamine, *n*-butylamine, and *n*-pentylamine. Alkylamines lower than *n*-propylamine (bp 48 °C) were inconvenient for amide synthesis due to their low boiling point, whereas those higher than *n*-pentylamine (bp 104 °C) were also infeasible for amide preparation due to their high boiling point; this prevented the removal of starting alkylamine after reaction. Lactic *n*-butylamide and *n*-pentylamide derivatives have more extensive mass fragmentation than *n*-propylamide as expected from the longer alkyl chains. The

extensive fragmentation resulted in lower abundance of useful fragments. Thus, as far as the amide of lactic acid is concerned, *n*-propylamide is the best derivative in terms of mass spectral properties. Lactic *n*-propylamide with an underivatized hydroxyl group (derivative 4) was volatile enough to be analyzed with a gas chromatograph. However, this derivative resulted in more tailing when compared with O-acylated *n*-propylamide. In addition, its mass spectral fragments were all in low mass range ($<m/z$ 131), inadequate to permit an interference free low background analysis. So, lactic *n*-propylamide was converted to a number of O-acylated derivatives (trimethylsilyl, acetyl, propionyl, trifluoroacetyl, pentafluoropropionyl, and heptafluorobutyryl). Perfluoroacyl derivatives have an advantage over other derivatives: they shifted the mass fragments to a higher mass range without increasing natural isotope background due to the single isotope nature of the fluorine atom. Trifluoroacetyl, pentafluoropropionyl, and heptafluorobutyryl derivatives of lactic *n*-propylamide all had appreciable abundance of useful fragments for isotope enrichment determination. Out of this, heptafluorobutyryl derivative was preferred over two other perfluoroacyl derivatives. First it was stable in evaporation. Second, it shifted lactate fragments to a higher mass region with less interference with minimal loss of their abundance due to longer alkyl chains (chance for more extensive fragmentation).

A number of lactic acylamide O-acylated derivatives with the other amide hydrogen also acylated were also prepared. The so-called lactic acylamide *O,O*-bis(acylate) or lactic acylamide *O,N*-bis(acrylate) were prepared by heating lactic acylamide or lactic acylamide *O*-acylate in silylating reagent or perfluoroacyl anhydride. It is not sure whether the second acyl group (R_2) on the amide group existed as $-CON(R_1,R_2)$ or $-C(OR_2)=NR_1$. From mass spectral data, it is probably existing in the latter form (lactic acylamide *O,O*-bis(acylate)). These derivatives have the advantage over lactic acylamide *O*-acylates in producing more symmetrical gas chromatographic peaks due to the removal of acidic amide hydrogen. They have shorter chromatographic retention times than the corresponding monoacylates. Their mass spectral fragmentations (EI) are similar to the corresponding monoacylate. The molecular ions were absent and the highest mass ions were usually produced through losing a molecule of acid. For example, the mass spectrum of derivative 16 (lactic *n*-propylamide *O,O*-bis(heptafluorobutylate)) showed a low abundant ion at m/z 310, which resulted from losing one molecule of heptafluorobutyric acid from the molecular ion (m/z 523, absent from the spectrum). The structure of this ion is probably as shown, $[CH_3C(OCOC_3F_7)=C=NC_3H_7]^{+\cdot}$. This ion is more likely to form if the original structure of the derivative is $[CH_3CH(OCOC_3F_7)C(OCOC_3F_7)=NC_3H_7]$ instead of $[CH_3CH(OCOC_3F_7)CON(COC_3F_7)(C_3H_7)]$. Unfortunately, the low abundance of ions containing all three lactate carbons excluded the use of these derivatives for the present investigation.

Mass Spectrum of Lactic *n*-Propylamide *n*-Heptafluorobutyrate (Derivative 9). As mentioned earlier, the best derivative for the lactic acid metabolic study using stable isotope and gas chromatography/mass spectrometry is lactic *n*-propylamide *n*-heptafluorobutyrate. The EI mass spectrum of this derivative is displayed in Figure 2. The D-lactic acid derivative had an almost identical EI mass spectrum as its L isomer. The molecular ion (m/z 327) is relatively abundant. The loss of ethyl group from *N*-propyl side chain produced an ion at m/z 298. A split between carbon 1 and 2 of lactate produced two prominent ions. One of the ions (m/z 241) contains carbon two and three of lactate, while the other ion (m/z 86) contains carbon one of lactate. The latter ion is also

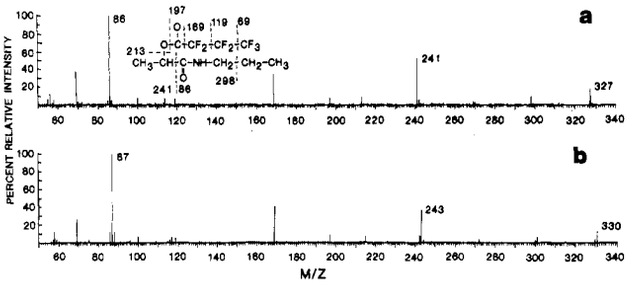


Figure 2. Electron impact ionization (70 eV) mass spectra of (a) L-lactic *n*-propylamide heptafluorobutyrate and (b) L-[U-^{13}C]lactic *n*-propylamide heptafluorobutyrate. The spectra were obtained through a gas chromatographic inlet. Ion source temperature was 200 °C.

$$CH_3CH(OH)CO_2H \xrightarrow[DMP]{H^+} CH_3CH(OH)CO_2CH_3 \xrightarrow{CH_3CH_2CH_2NH_2} CH_3CH(OH)CONHC_3H_7 \xrightarrow[HFBIM]{HFBA\ or} CH_3CH(OCOC_3F_7)CONHC_3H_7$$

Figure 3. Reaction sequence for the preparation of lactic *n*-propylamide heptafluorobutyrate: DMP, 2,2-dimethoxypropane; HFBA, heptafluorobutyric anhydride; HFBIM, heptafluorobutyrylimidazole.

the base peak. It is obvious that this derivative possesses the advantage of determining separately the enrichments of lactate C1–C2–C3, C2–C3, and C1. The ions at m/z 213, 197, 169, 119, and 69 were produced from the *n*-heptafluorobutylate group ($C_3F_7CO_2$) by successive cleavage of oxygen, CO, and CF_2. An EI mass spectrum of [U-^{13}C]lactate derivative is also shown in Figure 2.

Preparation of Lactic *n*-Propylamide Heptafluorobutyrate (Derivative 9). One of the reasons that an amide derivative of an organic acid is seldom used in gas chromatography is the difficulty in preparation, especially in small scale. Unlike ester formation, which has many convenient and high yield methods (*15*), the derivatization of an acid to amide for gas chromatographic analysis is almost nonexistent. In organic synthesis, an amide can be prepared by activating the carboxylic acid group to acyl halide, anhydride, or ester. The activated carboxylic acid derivative is then reacted with an amine to produce the amide. In the present investigation, the ester formation resulted in the only successful procedure for lactic amide preparation. 1,2-Dimethoxypropane (DMP) was used as the esterification agent. This reagents is not only convenient to use but also forms methyl ester in high yield at room temperature (*16*). The synthetic sequence is as depicted in Figure 3. The methyl lactate formed is very volatile, so the reaction mixture cannot be concentrated. Amide formation was accomplished by heating with excess *n*-propylamine. *n*-Propylamine served two functions; it neutralized the acid used in the DMP esterification procedure in addition to participating in the reaction. The residue, after evaporation, contained lactic *n*-propylamide, *n*-propylammonium chloride, and possibly some imine produced through reaction of *n*-propylamine and acetone (end product of DMP). Most of the *n*-propylammonium chloride was left behind after extraction with ethyl acetate; the reaction (amide formation) was relatively complete. When the reaction mixture was concentrated and silylated, it showed only 5% of the unreacted lactic acid on gas chromatographic analysis. The percentage of unreacted lactic acid did not change when the reaction time was prolonged or the amount of *n*-propylamine was increased.

The conversion of lactic *n*-propylamide to heptafluorobutyrate derivative can be accomplished with either heptafluorobutyric anhydride or heptafluorobutyrylimidazole.

Heptafluorobutyric anhydride converted lactic *n*-propylamide to lactic *n*-propylamide heptafluorobutyrate in 5 min at room temperature with the coproduction of about 5% of lactic *n*-propylamide *O,O*-bis(heptafluorobutyrate) (derivative 16) as side product. The amount of this side product increased as the reaction was prolonged or the reaction temperature was increased. At elevated temperature (such as 90 °C) bis(heptafluorobutyrate) derivative was the exclusive reaction product. Unreacted heptafluorobutyric anhydride has to be removed completely under a gentle stream of nitrogen before GC or GC/MS analysis. In contrast, derivatization with heptafluorobutyrylimidazole did not produce any bis(heptafluorobutyrate) side product, even under prolonged reaction time. Besides, unreacted heptafluorobutyrylimidazole did not have to be removed before analysis on nonpolar phases, such as 10% OV-17. The disadvantage was that the column had a shorter life due to the accumulation of imidazole, which eventually raised the base line. Overall, we prefer to use heptafluorobutyric anhydride as derivatizing agent for the –OH group on lactic *n*-propylamide.

Isolation of Lactate from Whole Blood, Plasma, or Red Blood Cells for Derivatization. The biological samples wre deproteinized first with perchloric acid and then neutralized. The dried neutralized sample can be derivatized directly, which is the preferred method, or the neutralized sample can be separated into glucose, urea, amino acid, and lactate fraction through ion exchange columns if multiple tracers are used or the isotope enrichment of other compounds is to be determined. However, in the latter procedure, extreme caution has to be taken to avoid the dilution of lactate enrichment by unlabeled lactate produced in the column and to avoid the interfering compounds eluted from the column.

The most used procedure for the separation of biological samples into fractions of glucose, alanine, and lactate has been that of Kreisberg (*17*) or the modification of that procedure (*8*). The former procedure used separate columns of anion and cation exchange resins while the latter used one single column packed with both anion and cation resins. We observed that dilution of lactate enrichment occurred through ion exchange columns using both methods. Apparently, the ion exchange resins produced lactic acid. After numerous determinations, it was found that the anion exchange resin, AG 1X8 (formate form), was the one that produced lactic acid. This contaminated resin can be cleaned by washing with 2 N formic acid. However, after the washed resin was stored at 5 °C for a couple of days, the resin was again contaminated with lactic acid. Since lactic acid is produced by many microorganism, it is possible that this lactate was produced by a microorganism contaminant in the resin using formic acid as nutrient. The only possible way of avoiding lactate enrichment dilution was washing the column with 2 N formic acid immediately before the application of sample.

Gas Chromatography/Mass Spectrometry. Upon electron impact ionization, lactic *n*-propylamide heptafluorobutyrate gave prominent ions at m/z 327 and 241, which contain carbons one, two, and three and carbons two and three of lactate, respectively. In the selected ion monitoring mode, the ions m/z 241, 243, 327, and 330 were focused alternatively. Standard curves were constructed by using standards with known [U-^{13}C]lactate enrichments. These are shown in Figure 1. The background for natural lactate in M + 2 (m/z 241 vs. 243) was 0.80% ± 0.01% (mean ± standard deviation, n = 5, coefficient of variation = 1.25%), and that of M + 3 (m/z 327 vs. 330) was 0.072% ± 0.004% (mean ± standard deviation, n = 5, coefficient of variation = 5.6%). Theoretically, this method is capable of determining with accuracy the isotope enrichment down to at least 0.05%. In actual determination, a standard with 0.14% of [U-^{13}C]lactate enrichment gave isotope abundance value of 0.11 ± 0.01 mol % excess (n = 3) for (M + 2) and 0.11 ± 0.01 (n = 3) for (M + 3). Samples from human studies gave similar precision. For example, samples from a lactate production study gave the following typical results: 0.45 ± 0.02 (n = 3); 0.23 ± 0.02 (n = 3); 0.62 ± 0.02 (n = 3); 1.00 ± 0.03 (n = 3); the data are expressed as mole percent excess, mean ± standard deviation. Overall, the coefficient of variation (CV) of determining blood isotope enrichment in a typical study of 0.2–1.0 mol % excess was only 4.8%. The samples used for these determinations were 50–100 μL of neutralized perchloric acid precipitated protein free sample, which is equivalent to only 17–33 μL of whole blood or plasma sample. The amount of lactate injected in each determination was about 0.1–0.3 μg.

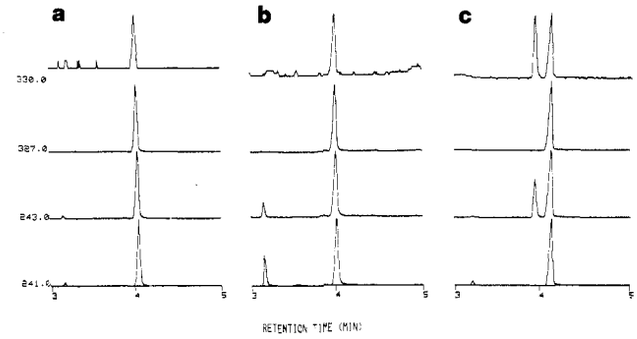


Figure 4. Selected ion chromatograms of lactate in blood. Lactate was derivatized as lactic *n*-propylamide heptafluorobutyrate. Ions m/z 241, 243, 327, and 330 were monitored. The peak intensities were normalized to maximum, e.g., the ion intensities of 243 and 330 were magnified more than 100-fold: (a) blood lactate before isotope infusion; (b) blood lactate after L-[U-^{13}C]lactate infusion (An enrichment of 0.48% was indicated.); (c) blood lactate after D,L-[U-^{13}C]lactate infusion. Blood L-[U-^{13}C]lactate enrichment was 0.47% in this case. The peak with lower retention time (3.9 min) was D-[U-^{13}C]lactic acid.

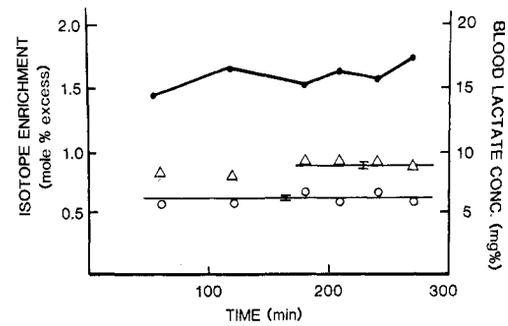


Figure 5. Blood lactate concentration and L-[U-^{13}C]lactate enrichment of a normal newborn infants after primed constant infusion of D,L-[U-^{13}C]lactate: (●) blood lactate concentration; (△) blood ^{13}C lactate enrichment measured from m/z 241 vs. 243; (○) blood ^{13}C lactate enrichment measured from m/z 327 vs. 330; (I) mean ± 1 standard deviation.

In some of the studies, racemic D,L-[^{13}C]lactate was used as tracer. This necessitated the development of a method capable of separating the optical isomers of lactate. Kamerling et al. (*14*) used an optically active derivatizing agent, (–)-menthol, to prepare diasteric (–)-methynyl esters. They were then separated on an OV-101 capillary column. We found that base line separation was obtained when lactic propylamide heptafluorobutyrates were chromatographed on a capillary column wall coated with an optically active stationary phase such as Chirasil-Val from Applied Science. Typical selected ion chromatograms from samples containing D- and L-[U-^{13}C]lactate are exemplified in Figure 4.

Lactate Production Rate Measurement in Humans. By use of the prime-constant infusion technique, the lactate production rate was measured in newborn infants. A repre-

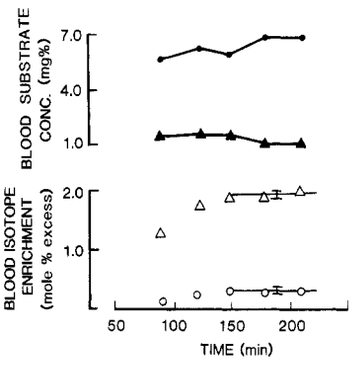


Figure 6. Blood substrate concentrations and enrichments after infusing L-[2,3-^{13}C]alanine in nonpregnant women: (●) blood lactate concentration; (▲) blood alanine concentration; (○) blood ^{13}C lactate enrichment measured from m/z 327 vs. 329; (△) blood ^{13}C alanine enrichment measured from m/z 141 vs. 143 of its n-butyl ester trifluoroacetamide derivative; (I) mean ± 1 standard deviation.

sentative study is shown in Figure 5. A steady state of lactate enrichment is usually reached between 60 and 120 min after the start of infusion. This particular example showed a "true" lactate production rate of 3.75 (mg/kg)/min, as measured from the enrichment of M + 3 (m/z 330 vs. 327).

If lactate carbon recycling occurred, the enrichment obtained from M + 2 (m/z 243 vs. 241) would be higher then that obtained from M + 3. Hence an "apparent" lactate production rate, which contained the recycled lactate, would be obtained from (M + 2) enrichment. The difference between the two rates is the extent of lactate recycling. In this example, the recycling of lactate carbons was 31%.

Production of L-[2,3-$^{13}C_2$]Lactate from L-[2,3-$^{13}C_2$]Alanine in Humans. The conversion of L-[2,3-$^{13}C_2$]alanine into L-[2,3-$^{13}C_2$]lactate was studied by the prime-constant infusion of L-[2,3-$^{13}C_2$]alanine into man, and the L-[2,3-$^{13}C_2$]lactate enrichment of the lactate pool was determined by the present method. A representative experiment is shown in Figure 6. In a steady state, the percentage of lactate derived from alanine was calculated to be 16% in this particular example. The details of these investigations will be published separately.

Other Applications. By use of optically active stationary phase capillary columns, this method can also be used to determine the concentration of D-lactate in blood, plasma, and red blood cells using less than 50 μL of blood. In contrast, the D-lactate dehydrogenase method of determing D-lactate required 2 mL of plasma (*18*).

Gas chromatographic analysis of organic acids using alkylamide derivatives is a novel approach. It has the advantage of rendering a derivative that is relatively nonvolatile for subsequent workup. Besides, alkylamide derivatives of an acid are usually less volatile than the acid itself. With some modification, this method may also be used for the gas chromatographic and GC/MS determination of volatile acids, such as acetic, propionic, isovaleric, and β-hydroxybutyric acids. Currently, volatile fatty acids are usually analyzed as free acids after isolation by solvent extraction or vacuum distillation (*19*). The derivatization of these acids as their alkylamide derivative would simplify the handling of these acids.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Kathe L. Lubke for her able technical assistance and patience. The secretarial assistance of Sandee Riedrich is also gratefully acknowledged.

Registry No. 1, 80372-11-8; 2, 88392-18-1; 3, 88392-19-2; 4, 88392-20-5; 5, 88392-21-6; 6, 88392-22-7; 7, 88392-23-8; 8, 88392-24-9; 9, 88392-25-0; 10, 88392-26-1; 11, 88392-27-2; 12, 88392-28-3; 13, 88392-29-4; 14, 88392-30-7; 15, 88392-31-8; 16, 88392-32-9; 17, 88392-33-0; 18, 88392-34-1; $CH_3CH(OH)CO_2H$, 50-21-5; $CH_3CH(OH)CO_2CH_3$, 547-64-8; $CH_3CH_2CH_2NH_2$, 107-10-8; $CH_3CH(OH)CONHC_3H_7$, 74421-70-8; L-[U-^{13}C]lactic acid, 88392-35-2; L-alanine, 56-41-7.

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RECEIVED for review June 23, 1983. Accepted December 12, 1983. This study was supported by Grants HD 11089 and AM 25596 from the National Institutes of Health. S.C.K. is recipient of a Research Career Development Award (1K04 AM 00801) from the National Institutes of Health.