Studies of *trans*-3-methyl-2-hexenoic acid in normal and schizophrenic humans

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Abstract The report that sweat of certain schizophrenics contains the branched chain fatty acid, *trans*-3-methyl-2-hexenoic acid (TMHA), stimulated an investigation to evaluate the relationship between this fatty acid and schizophrenia. A sensitive and specific gas–liquid chromatography–mass spectroscopic procedure was developed for analyzing biological fluids for TMHA. Analysis of sweat samples from normal and schizophrenic subjects indicated that the sweat of both groups contains comparable quantities of this fatty acid. In addition, the fate of intravenously administered ^{14}C-labeled TMHA was shown to be similar in normal and schizophrenic subjects. It is concluded that there is no relationship between TMHA and schizophrenia.

Supplementary key words short-chain fatty acids · gas–liquid chromatography–mass spectroscopy

Investigations into the biochemical basis of schizophrenia have produced a vast number of abnormal findings, and many of these have been recently reviewed (1, 2). However, a specific biochemical defect has not been established for this disease. The identification of an abnormal metabolite in body fluids and tissues of a group of patients has often been the initial observation that stimulated investigations that eventually elucidated the biochemical basis of a disease (3, 4). Thus, the report that sweat of certain schizophrenic patients, selected because they had a characteristic odor (5), contains a short-chain fatty acid, *trans*-3-methyl-2-hexenoic acid (TMHA) (6), has stimulated further work to evaluate the relationship between this fatty acid and schizophrenia.

A quantitative gas–liquid chromatography–mass spectroscopy (GLC–MS) procedure was developed for the simultaneous quantitation and positive identification of TMHA in biological fluids. With this procedure, sweat samples of both schizophrenic patients and control subjects were found to contain comparable amounts of TMHA. In addition, the fate of intravenously administered ^{14}C-labeled TMHA was found to be similar in both groups. It is concluded, therefore, that there is no apparent correlation between TMHA and schizophrenia.

MATERIALS AND METHODS

TMHA was synthesized by the dehydration and hydrolysis of ethyl-3-hydroxy-3-methyl hexanoate, which was prepared by the Reformatsky condensation of ethyl bromoacetate and 2-pentanone (6). [1,2-^{14}C]TMHA was synthesized with ethyl [1,2-^{14}C]bromoacetate by the same reaction (7); it contained 81% *trans* and 19% *cis* isomers as measured by GLC. Hexanoic acid (Applied Science Laboratories, State College, Pa.) was chromatographically pure. 2-Hexenoic and 2-octenoic acids (L. Light and Co., Ltd., London, England) were 70% pure; however, the purity was adequate for use as mass spectroscopy standards. 2-Heptenoic acid was synthesized by the Pd–$BaSO_4$-catalyzed hydrogenation (8) of 2-heptynoic acid (Chem. Samples Co., Columbus, Ohio). Other standards for GLC–MS included valeric, hexanoic, heptanoic, and octanoic acids (Sigma Chemical Co., St. Louis, Mo.) and for TLC included monobutyrin, dibutyrin, and tributyrin (Applied Science).

Butyl esters of *cis*- and *trans*-3-methyl-2-hexenoic, valeric, hexanoic, *cis*- and *trans*-2-hexenoic, heptanoic, *cis*- and *trans*-2-heptenoic, octanoic, and *cis*- and *trans*-2-octenoic acids were prepared by the methods of Metcalfe and Schmitz (9). 1 ml of BF_3–butanol was added to 50 μg of each acid in a 10-cm screw-cap tube, and the tubes were placed in a block heater for 20 min at 100°C. The

Abbreviations: TMHA, *trans*-3-methyl-2-hexenoic acid; BTMH, butyl *trans*-3-methyl-2-hexenoate; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectroscopy; TLC, thin-layer chromatography; LCFA, long-chain fatty acid.

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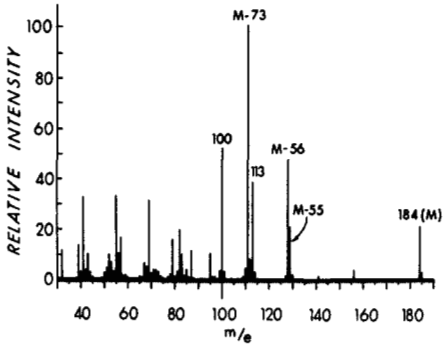


FIG. 1. Mass spectrum of butyl *trans*-3-methyl-2-hexenoate. The conditions used in the GLC–MS are described under Materials and Methods. The proposed structures for some of the major, higher molecular weight fragments are shown in Table 1.

BF_3 was neutralized by adding a small volume of water and solid $NaHCO_3$, and the bulk of the butanol was extracted into a 30% NaCl solution. The butyl esters were adjusted to an appropriate dilution in heptane for use as standards for GLC–MS.

Sweat collection and preparation of samples

Normal subjects and schizophrenic patients were selected by one of us (K.S.). Their diets were not regulated. Before the test, the sweat from each individual was carefully evaluated for the characteristic odor; it was undetectable in the normal sweat and easily detectable in the schizophrenic sweat. After high levels of TMHA were found in the sweat of some of the normal subjects, their sweat was reevaluated and the absence of the odor was confirmed.

All subjects were bathed with Dial soap and then rinsed with water, a 5% solution of $NaHCO_3$, and finally with water. Sweat was collected by placing the subjects in large plastic bags containing powdered $NaHCO_3$ for 80 min as described previously (3).

The volumes of the sweat samples were measured, and the pH was adjusted to 11 by addition of about 0.5 ml of piperidine. Approximately 3×10^5 dpm of [1-^{14}C]hexanoic acid was added as internal standard, and the samples were lyophilized, redissolved in a small volume of water, and subjected to steam distillation after acidification (10). 3 vol of distillate was collected. Under these conditions, 100% of TMHA was recovered in the distillate. The distillate was extracted four times with diethyl ether. Two drops of piperidine were added to the ether extract, the volume was reduced under a stream of nitrogen, and butyl esters were prepared as described above. The recovery of TMHA as the butyl ester was about 70%, as determined by measuring the recovery of the internal standard, [1-^{14}C]hexanoic acid. In separate experiments, it was shown that [1-^{14}C]-hexanoic acid and TMHA, subjected to the entire procedure together, were recovered to the same extent.

The total long-chain fatty acids in the steam distillation residue were determined after saponifying an aliquot of the residue in 50% ethanol and 1.0 N NaOH for 30 min at 100°C. The fatty acids were extracted into petroleum ether; an aliquot was reduced to dryness, redissolved in 70% ethanol, and titrated with 0.001 N NaOH (11).

Gas–liquid chromatography–mass spectroscopy

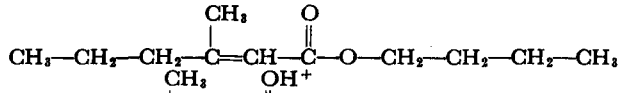
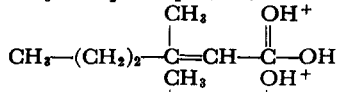
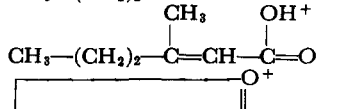
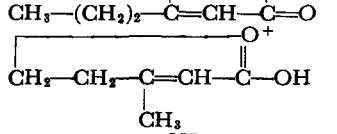
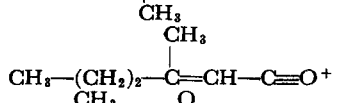
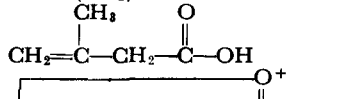
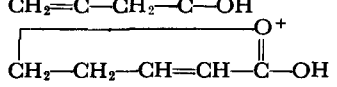
GLC–MS was performed on an LKB-9000 apparatus that was interfaced with a PDP-12 computer (12). An ionization voltage of 70 eV and a source of temperature of 270°C were used with an accelerating voltage of 3.5 kV. Perfluorokerosene was used for mass calibration. The scan speed was 7 (scan speed *m/e* 20 to *m/e* 500 in 4.2 sec), and the filter was 120. A 6-ft glass column packed with 10% diethylene glycol adipate (DEGA) and 2% H_3PO_4 on Gas-Chrom W with 6 inches of 1% SE-30 on Gas-Chrom W at the outlet was utilized. The carrier gas flow rate was 30 ml/min. The temperatures in the injector, the column oven, and the separator were 240, 100, and 240°C, respectively.

Prior to examination of unknown samples, a solution containing authentic ^{14}C-labeled butyl *trans*-3-methyl-2-hexenoate ([^{14}C]BTMH) was utilized as a standard to confirm the column and mass spectrometer conditions. Aliquots equivalent to 20–160 ng of TMHA in 0.5–5 μl were injected into the GLC–MS apparatus. About 9 min after sample injection, the mass spectrum of the effluent was scanned at 10 sec intervals until BTMH was eluted from the column (retention time of BTMH = 10 min ± 10 sec); then the temperature of the oven was increased to 190°C to elute other less volatile compounds. Other major peaks were also scanned for qualitative purposes. Each sample was analyzed in triplicate by GLC–MS.

Each scan across the BTMH peak was plotted (usually 11 scans), as illustrated in the mass spectrum shown in Fig. 1. The *m/e* 184 signal was multiplied five times (not shown in Fig. 1) to increase the accuracy and sensitivity of the quantitation at low concentrations of BTMH. The intensities of the *m/e* 100, *m/e* 111, and *m/e* 184 signals in each scan were measured. The sum of the *m/e* 100 signals was determined, and the background (the average of the signal from the first and last scans) was subtracted to determine the net signal of the *m/e* 100 fragment; the net signals of the *m/e* 111 and *m/e* 184 fragments were determined in the same way. The ratios of the net *m/e* 100 signal to the net *m/e* 111 signal and to the net *m/e* 184 signal were determined so that these could be compared in unknown samples. The mass-to-signal ratio (ng of [^{14}C]BTMH/net signals) was determined for each fragment.

Butyl esters of unknown fatty acids from the sweat samples were similarly subjected to GLC–MS, and the BTMH peak was scanned as above. The net signals of

TABLE 1. Probable structures of high intensity fragments

m/e		Structure
184	M	
129	M−55	
128	M−56	
113		
111	M−73	
100		
99		

The proposed structures of the high intensity, higher molecular weight fragments, *m/e* 184, 129, 128, 113, 111, and 100, in the mass spectrum of butyl *trans*-3-methyl-2-hexenoate (Fig. 1) and *m/e* 99 in the mass spectra of butyl *cis*- and *trans*-2-heptenoate (Fig. 2, *A* and *B*) are shown.

the *m/e* 100, 111, and 184 fragments were determined. The ratios of the net signals were calculated and shown to be the same as those of the standard BTMH. The quantity of BTMH in the samples was calculated from these three net signals and the mass/signal ratios of the standard.

In vivo studies with [^{14}C]TMHA

Four normal and three schizophrenic subjects received 10 μCi of [1,2-^{14}C]TMHA intravenously. Expired $^{14}CO_2$ was collected for 2 min in Hyamine at 5, 15, 30, 60, and 120 min after the injection. 10-ml blood samples were drawn at the same time intervals. 1 hr after the injection, the subjects underwent the sweating procedure. A urine sample was collected 140 min after the injection. All samples were processed immediately.

Expired $^{14}CO_2$ was assayed by counting an aliquot of the Hyamine in Bray's solution (13). 1 ml of each serum sample was also counted in Bray's solution. The lipids were extracted from the serum into chloroform–methanol (14) and separated into classes by TLC in a hexane–diethyl ether–glacial acetic acid 80:20:1 system (15). After visualizing the spots with iodine, the appropriate areas were scraped into counting vials containing Bray's solution and counted. The recovery of radioactivity from the plates was about 90%. Monobutyrin, dibutyrin, tributyrin, and TMHA were used as chromatographic standards. Approximately 90% of the ^{14}C activity migrated with TMHA on TLC. Analysis of an aliquot by gas–liquid radiochromatography (16) indicated that the radioactive compound in the serum was TMHA.

Aliquots of urine and sweat were assayed directly for radioactivity, and the remainder of each sample was lyophilized and steam distilled. The [^{14}C]TMHA recovered in the steam distillate was identified by both gas–liquid radiochromatography and by GLC–MS.

RESULTS

Identification of butyl ester of TMHA

One of the primary purposes of this study was to establish a procedure capable of positive identification of TMHA in biological samples. Attempts to accomplish this by GLC–MS of TMHA failed because of the low relative intensity of many of the fragments of the free acid. Efforts to improve sensitivity by making TMHA derivatives, such as 2,3-dihydroxy-3-methylhexanoate, via OsO_4 oxidation of the double bond (17) were unsuccessful because the derivatives could not be synthesized. However, butyl esters provided good GLC–MS sensitivity and several higher molecular weight fragments with high relative intensities (Fig. 1). The *m/e* 111 fragment is the strongest signal (base peak) in the spectrum; the relative intensity of *m/e* 100 is 52%, *m/e* 128 is 47%, *m/e* 113 is 39%, and both *m/e* 120 and 184 are 21%. The proposed structures of these higher mass fragments are shown in Table 1. The masses designated M (*m/e* 184), M − 55 (*m/e* 129), M − 56 (*m/e* 128), and M − 73 (*m/e* 111) are due to common fragments observed with fatty acid butyl esters (18). The *m/e* 113 fragment of BTMH is probably analogous to the *m/e* 99 fragment of butyl-2-heptenoate with a methyl,

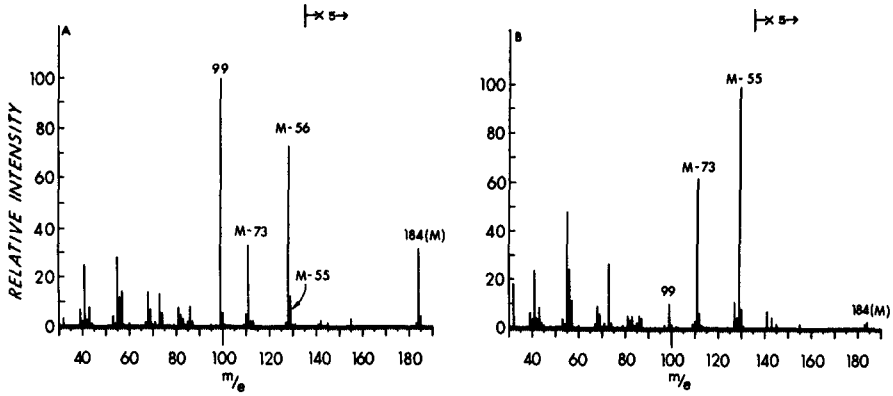


FIG. 2. Mass spectra of butyl *cis*-2-heptenoate (*A*) and butyl *trans*-2-heptenoate (*B*). The proposed stucture of *m/e* 99 is shown in Table 1.

instead of a hydrogen, on the third carbon (see below). The *m/e* 100 fragment represents a major fragment only in the spectrum of BTMH. Thus, in the mass spectrum of BTMH, there are six fragments of high relative intensity among the high molecular weight fragments.

It was possible that other seven-carbon monoenoic fatty acid butyl esters would have the same GLC retention times and similar mass spectra. The compound most likely to have properties similar to BTMH is butyl-2-heptenoate. *Cis*- and *trans*-2-heptenoic acid were synthesized by the reduction of heptynoic acid, and the butyl esters were prepared and analyzed by GLC–MS. The retention times of both compounds differed from that of BTMH. In addition, their mass spectra differed significantly (Fig. 2). Although both heptenoate isomers have the common M, M − 55, M − 56, and M − 73 fragments, the relative intensities of these fragments are different in the two isomers and different from those in the spectrum of BTMH. The base peak in the spectrum of butyl *cis*-2-heptenoate (Fig. 2*A*) is *m/e* 99; *m/e* 128 is 74%, *m/e* 111 is 34%, and the molecular ion *m/e* 184 is 6% of the base peak. The highest signal in the spectrum of butyl *trans*-2-heptenoate (Fig. 2*B*) is *m/e* 129, the relative intensity of *m/e* 111 is 62%, and both *m/e* 99 (10%) and the molecular ion are very small. It is clear that butyl-2-heptenoate could readily be differentiated from BTMH. Mass spectra and retention times of the butyl esters of valeric, hexanoic, *cis*- and *trans*-2-hexenoic, heptanoic, octanoic, and *cis*- and *trans*-2-octenoic acids were also examined and found to be different from those of BTMH. Thus, GLC–MS could provide positive identification of BTMH since it has a different retention time from most other similar fatty acid esters and it has a unique mass spectrum.

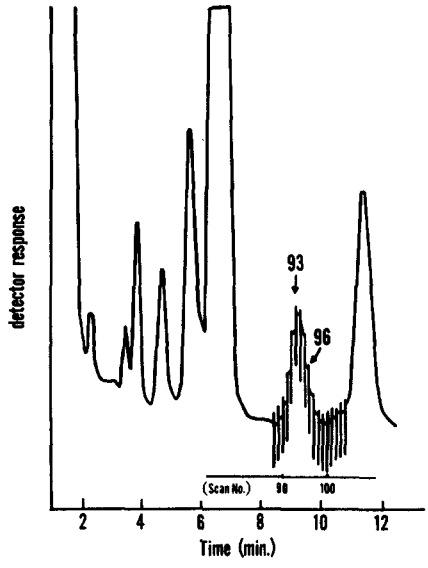


FIG. 3. Total ion current tracing of the column effluent during the GLC–MS analysis of sweat sample of normal subject G.J. Each vertical line represents a scan at 10-sec intervals. The retention time of BTMH (scan no. 96) is 30 sec after a major mass peak (scan no. 93).

Quantitation of BTMH

The other primary objective of this study was to measure the quantity of TMHA in biological samples. To accomplish this, it was first necessary to determine which fragments in the BTMH mass spectrum had no signal from other coeluting components when sweat and other biological samples were analyzed. Several sweat samples with and without added TMHA were processed and examined by GLC–MS to determine gas–liquid chromatographic and mass spectral resolution of BTMH from contaminants that chromatographed at the same retention time. Poor resolution was obtained on columns that contained either 1% SE-30 or 6% OV-225 stationary phases. Although gas–liquid chromatographic resolution of BTMH from contaminants was also poor on a column of 10% DEGA + 2% H_3PO_4, mass spectral resolution was adequate. As seen in Fig. 3, the total ion current tracing of butyl esters of the steam-volatile fatty acids of a sweat sample indicated components that

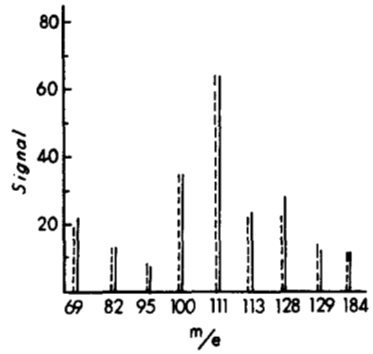


FIG. 4. A comparison of selected fragments from the mass spectrum of a BTMH standard (- - -) with those from scan no. 96 (Fig. 3) in a sweat sample (——). The indicated fragments were normalized to the *m/e* 111.

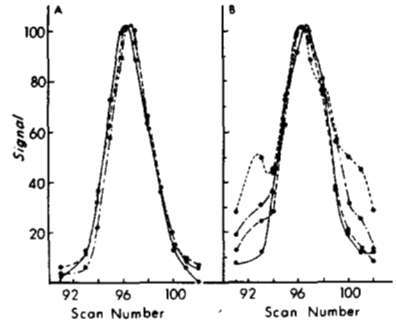


FIG. 5. The normalized *m/e* signals, obtained during the analysis of a sweat sample (Fig. 3), are plotted against scan number. The maximum intensity of each signal (scan 96 or 97) was made equal to 100% signal, with the same correction made to the relative intensity of the other scans. The *m/e* 100 (— - —), *m/e* 111 (- - -), and *m/e* 184 (——) fragments have symmetrical peaks in *A*, while the fragments shown in *B*, *m/e* 128 (——), *m/e* 129 (— - —), *m/e* 113 (– –), and *m/e* 82 (- - -), gave asymmetric peaks.

eluted at 9.5 min (scan no. 93), whereas the retention time of BTMH in this sample was 30 sec later (scan no. 96). Although BTMH was one of several compounds under the mass peak, there were several fragments in the BTMH spectrum that were uncontaminated.

Two methods of analyzing mass spectrometry data from a sweat sample are demonstrated in Figs. 4 and 5. In the experiment of Fig. 4, the signal intensities of selected fragments from scan no. 96 (Fig. 3), the mass spectrum of BTMH in the sweat sample, were normalized to the *m/e* 111 signal and compared with the same fragments from a mass spectrum of a BTMH standard. Fragments in the sweat sample of *m/e* 184, *m/e* 100, and *m/e* 82 were the same intensity as the standard, while contaminants contributed to the intensity of *m/e* 69, *m/e* 113, and *m/e* 128 signals. The results of this experiment suggested that the fragments of *m/e* 184, *m/e* 111, *m/e* 100, and *m/e* 82 could be used in quantitation of BTMH in a sweat sample. Another means of evaluating the data (Fig. 5) was to measure the relative intensities of selected *m/e* signals of the BTMH spectrum from each of the continuous scans of a sample (scans 91–102, Fig. 3) in the region of the BTMH retention time, normalize the most intense signal from each fragment to 100%, with an appropriate correction of the same fragment in the other scans, and plot the normalized relative intensity against scan number. Symmetrical and superimposed mass chromatography peaks with the same half-width were obtained from a sweat sample with *m/e* 100, *m/e* 111, and *m/e* 184 (Fig. 5*A*), demonstrating that these fragments were free from contaminants, whereas *m/e* 128, *m/e* 129, *m/e* 113, and *m/e* 82 gave asymmetric patterns, having leading or trailing shoulders (Fig. 5*B*). This procedure most clearly identified BTMH in an unknown sample, since all the characteristic fragments cochromatographed. Two other less critical methods (not shown) used to evaluate the uncontaminated fragments and the spectral properties of the contaminants were also applied: *1*) a normalized BTMH mass spectrum was substracted from a mass spectrum of a sweat sample within the BTMH retention time, the remaining signals were from the contaminants, while the clean BTMH fragments were eliminated; *2*) all the major *m/e* signals obtained from continuous scans across the total ion current mass peak were measured and plotted against scan number to determine the mass chromatographic properties of contaminant signals.

Although some of the fragments were obviously contaminated in the sweat samples, *m/e* 100, *m/e* 111, and *m/e* 184 were clean by all four analyses. These *m/e* signals were used to quantitate the BTMH in sweat samples. The net signal, which is comparable to the integral value of the mass chromatography peak, was determined (as described under Materials and Methods) for *m/e* 100, *m/e* 111, and *m/e* 184. A standard curve for *m/e* 111 and *m/e* 184 obtained with authentic BTMH is shown in Fig. 6. A similar curve was obtained for *m/e* 100. The mass of BTMH in the sweat samples was calculated from such standard curves.

Analysis of TMHA in sweat samples

After establishing that the GLC–MS technique could be utilized for both quantitation and positive identifica-

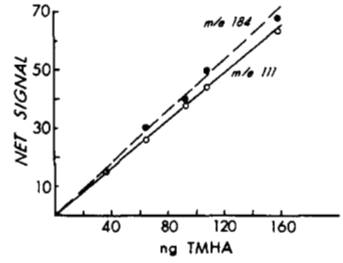


FIG. 6. A standard curve of the net signal of the *m/e* 111 and *m/e* 184 fragments from BTMH plotted against nanograms of TMHA. The *m/e* 184 signal was multiplied by 5.

TABLE 2. Quantitative data from the 12 normal and 7 schizophrenic subjects

	Sweat Volume	TMHA Concn	Total TMHA	Total LCFA	μmoles TMHA/ μmoles LCFA
	ml	*ng/ml*	*μg*	*μmoles*	*× 10⁴*
Normals					
CJ	98	8	0.75	23.7	2.5
NM	210	6	1.18	18.4	5.0
KS	98	5	0.50	43.6	0.9
PK	66	198	13.10	26.2	38.9
CP	168	4	0.71	15.2	3.7
MT	172	5	0.86	17.3	3.9
JC[a]	145	13	1.78	33.6	4.1
GJ[a]	196	104	19.70	25.6	62.0
JS	105	102	10.70	7.2	120.0
JJ	139	4	0.55	22.1	2.0
AB	56	<5	<0.20	16.3	<0.1
HT	74	<2	<0.10	13.0	<0.8
Median	122	5.5	0.85	20.3	3.8
Schizophrenics					
EH[a]	37	72	2.4	25.0	10.2
PO[a]	161	11	1.7	35.0	3.7
RO	174	90	12.5	106.0	9.3
RB	182	45	8.1	89.0	7.1
JC	158	9	1.5	52.5	2.2
PH	152	8	1.2	25.2	3.8
JF	209	10	2.1	79.2	2.1
Median	161	11	2.1	52.5	3.8

The volume and the TMHA and long-chain fatty acid (LCFA) contents of sweat from the subjects were determined as described under Materials and Methods. In two subjects, AB and HT, BTMH was not detected; the lowest measurable level of BTMH was estimated, and the amount of TMHA in these samples was expressed as less than that level. The μmoles TMHA/μmoles LCFA ratios were multiplied by 10^4 for tabulation.

[a] These subjects were subjected to the study more than once; the data are means of individual determinations shown in Table 3.

tion of nanogram quantities of TMHA in sweat, a group of 12 normal and 7 schizophrenic subjects was investigated (Table 2). Both groups included males and females ranging in age from 24 to 50 years. Since the distribution of much of the data is skewed to the left, the medians, rather than the means, were determined and statistically compared by the nonparametric χ^2 test (19).

The volume of sweat obtained from the normal group ranged from 56 to 210 ml, whereas that from the schizophrenic group ranged from 37 to 209 ml. The median values for the two groups (normal, 122 ml; schizophrenic, 161 ml) were not statistically different ($P > 0.2$). The concentration of TMHA in the sweat ranged from < 2 to 198 ng/ml (median, 5.5 ng/ml) in normals and from 8 to 90 ng/ml (median, 11 ng/ml) in schizophrenics. The concentration was the same ($P > 0.2$) in both subject groups. The total amount of TMHA in the sweat samples of normal subjects ranged from < 0.1 to 19.7 μg (median, 0.85 μg), and this was not statistically different ($P > 0.2$) from the amount in the sweat of the schizophrenics, which ranged from 1.2 to 12.5 μg (median, 2.1 μg). In the sweat of two normal subjects (A.B. and H.T.) in whom BTMH was not detected, the lowest level of sensitivity of the GLC–MS procedure (≤10 ng of BTMH), the volume of the sample applied to the GLC–MS analysis, and the recovery of the internal standard were used to estimate the lowest measurable level of BTMH; the amount of TMHA in these samples was expressed as less than that level.

It was desirable to measure another substance in the sweat samples of both groups in order to establish that any possible difference in TMHA content was specific. For this purpose the total long-chain fatty acid (LCFA) content was determined on all samples. As noted in Table 2, the content of LCFA in the sweat of normal subjects ranged from 7.2 to 43.6 μmoles, whereas the content in the sweat of the schizophrenic subjects ranged from 25.0 to 106.0 μmoles. The median values for the normal group (20.3 μmoles) and schizophrenic group (52.5 μmoles) were the same ($P > 0.2$). Comparison of the ratios of total TMHA to total LCFA (μmoles TMHA/μmoles LCFA × 10^4) in the sweat samples indicated that the normals ranged from < 0.1 to 120, whereas the schizophrenics ranged from 2.1 to 10.2. The median of the ratios from both groups was 3.8 ($P > 0.8$).

Two normal (J.C. and G.J.) and two schizophrenic (E.H. and P.O.) subjects were tested more than once, at intervals of several weeks, to evaluate the reproducibility of the data (Table 3). The volumes of sweat and the total amount of TMHA in the sweat had approximately the same average variations (± 42%) about their means, and the concentration of TMHA and the ratio of TMHA to LCFA had average variations of about ± 23% about their mean values. In spite of these variations, it is apparent that the amount of TMHA in the sweat of an individual subject was quite constant over a period of several weeks. The sweat of G.J. collected during the first test contained the greatest amount of TMHA (16.8 μg) in both groups of subjects; the results of the second test (22.6 μg) confirmed this high value.

At various times throughout the study, 10 plastic bags were rinsed with 100 ml of 5% $NaHCO_3$ at either 25 or 50°C for 30 min, and the resulting solutions were processed by the same procedure as the sweat samples to demonstrate that TMHA was not a contaminant of the bags, the glassware, or the reagents. No TMHA was detected in these experiments.

In vivo studies with [^{14}C]TMHA

The second part of the present study was designed to evaluate the fate of intravenously administered [^{14}C]-TMHA in four normal and three schizophrenic subjects. The quantity of expired $^{14}CO_2$ and the rate of $^{14}CO_2$ output were similar in the normal and schizophrenic subjects after the injection of [^{14}C]TMHA. The rate of disappearance of radioactivity from the serum after the injection

TABLE 3. Reproducibility of the data

	Sweat Volume	TMHA Concn	Total TMHA	Total LCFA	μmoles TMHA/ μmoles LCFA
	ml	*ng/ml*	*μg*	*μmoles*	× 10^4
Normals					
JC	202	8	1.57	26.8	4.5
	162 (145)	16 (13)	2.60 (1.78)	47.0 (33.6)	4.3 (4.1)
	71	17	1.16	26.9	3.4
GJ	220	76	16.8	28.2	47
	(196)	(104)	(19.7)	(25.6)	(62)
	172	131	22.6	22.9	77
Schizophrenics					
EH	64	63	4.0	45.1	6.8
	(37)	(72)	(2.4)	(25.0)	(10.2)
	10	80	0.8	4.8	13.5
	187[a]	14	2.6	ND	ND
PO	161	11	1.7	35.0	3.7
	212[a]	8	1.6	ND	ND

The quantitative data from two normals and two schizophrenics who were subjected to the study more than once. The mean of each group of determinations is shown in parentheses; these means were also utilized in Table 2.

[a] The sweat volume and TMHA content are presented for purposes of comparison but were not included in the calculated means because the LCFA content was not determined (ND).

was similar in both groups of subjects, and the radioactive material in the serum was shown to be the free fatty acid, TMHA, by gas–liquid radiochromatography.

The urine samples collected 140 min after the intravenous injection of [^{14}C]TMHA contained 1–9% of the administered radioactivity in both the normal and schizophrenic subjects. Approximately 40% of the radioactivity in the urine samples was identified as [^{14}C]-TMHA. The remaining radioactive material was not identified. Urine of a schizophrenic subject who had not been injected with TMHA contained no detectable TMHA.

The sweat samples collected after [^{14}C]TMHA injection contained only 0.1–1% of the administered radioactivity in both groups of subjects, and only about 5% of the radioactivity in the sweat of both groups was identified as [^{14}C]TMHA. The remaining radioactive material in the sweat was not identified.

DISCUSSION

Quantitative GLC–MS, or mass chromatography, was adapted for use in this study because it provides a sensitive assay with positive identification of TMHA by the quantitation of unique fragments of BTMH. Mass chromatography has been employed previously to study the metabolism of pharmacological agents (20). Before mass chromatography could be utilized in this study, it was necessary to demonstrate that the retention time and the mass spectrum of BTMH are different from those of other fatty acid butyl esters of similar molecular weight. The fatty acid most likely to simulate TMHA in this procedure is another seven-carbon monoenoic acid. Thus, the butyl esters of both *trans*- and *cis*-2-heptenoic acid were examined. Both compounds were shown to have retention times and mass spectra different from BTMH, and therefore they posed no problem. It was similarly shown that butyl esters of *cis*- and *trans*-2-hexenoate, *cis*- and *trans*-2-octenoate, and their saturated analogs could be readily differentiated from BTMH by GLC–MS.

The position and the stereochemistry of the double bond in most unsaturated fatty acids are difficult to determine by mass spectroscopy without derivatization of the double bond (17, 18, 21); α,β unsaturated fatty acids are an exception to that rule. A fragment with the mass of 113 is a characteristic of α,β unsaturated fatty acid methyl esters of five or more carbon atoms and is depicted as a sixmembered ring (22–24); its structure is similar to that proposed for mass 99 in Table 1, except the hydrogen of the acid is replaced with a $-CH_3$ of the methyl ester. In addition, *cis* isomers of these methyl esters have a more intense 113 signal than their *trans* isomers because they form the cyclic fragment without isomerization of the double bond; all of the *cis* isomers of the 2-hexenoic, 2-heptenoic, and 2-octenoic acid butyl esters have a more intense *m/e* 99 signal than the corresponding *trans* isomers. BTMH has no *m/e* 99 fragment, but *m/e* 113 has the same *cis-trans* isomer relative intensity characteristics described above; therefore, its structure is proposed to be the same as *m/e* 99 except that the third carbon contains a methyl group in place of the hydrogen.

A major *m/e* 113 fragment is also present in the mass spectrum of butyl heptanoate; however, its structure, a seven carbon straight chain acylium ion without a branched methyl group, is analogous to the M − 73 fragment shown in Table 1. All of the other straight chain saturated and α,β unsaturated fatty acid butyl esters have no *m/e* 113 fragment.

The structure of *m/e* 100 fragment was proposed by Holmstedt[2] for TMHA and is probably the same for its butyl ester (Table 1). In spite of the uncertainty of its structure, it is clear that *m/e* 100, as major fragment, is unique to BTMH. Butyl *cis*- and *trans*-2-hexenoate, butyl *cis*- and *trans*-2-heptenoate, and the butyl esters of their saturated counterparts have less than 5% signal at *m/e* 100, while butyl *cis*- and *trans*-2-octenoate have about 15% *m/e* 100 signal.

Although the proposed structures of the other fragments shown in Table 1, designated M, M − 55, M − 56, and M − 73, are those of BTMH, analogous fragments are common to all saturated and unsaturated fatty acid butyl esters (18). The relative intensity of the molecular ion signal (M) depends on the stability of the free radical ion that is generated by the loss of an electron. The butyl group is lost from these esters relatively easily, and the acid (M−56) or the protonated acid (M−55) is often an abundant fragment. Perhaps the most characteristic fragment in the mass spectrum of butyl esters is the acylium ion (M−73), which is generated by a complex rearrangement after the loss of a butoxyl group (25).

GLC alone did not permit quantitation of BTMH due to the poor resolution of the BTMH from contaminants that had almost the same retention times. Four tests were used to demonstrate that *m/e* 100, *m/e* 111, and *m/e* 184 were unique fragments at the retention time of BTMH with every sample analyzed. Since the net *m/e* signal is an expression of the area under a fragment's mass chromatography peak, the fact that the ratios of the net *m/e* signals in the sweat samples were always the same as those in the BTMH standard confirmed the uncontaminated quality of these fragments. These fragments were therefore used in the quantitation of the BTMH.

The studies reported above indicate that there is no significant difference between the amount of TMHA in the sweat of normal and of schizophrenic subjects. Although the range of concentrations of TMHA in the sweat of both groups was from < 2 to 198 ng/ml, the median concentration was 9 ng/ml. The broad range of concentrations among control subjects is interesting but unexplained. It may be related to dietary intake, but this aspect was not investigated since diets were not regulated. The use of GLC–MS permitted accurate quantitation of TMHA at concentrations as low as 2 ng/ml. Such sensitivity has not been previously achieved. Previous studies led to a report (6) that the sweat of schizophrenic subjects contained about 0.1 μg of TMHA/ml of sweat, while TMHA was not detected in most normal control subjects. Although the higher values reported in the present study are in the range reported for the schizophrenics, sweat from both normal and schizophrenic subjects contained equally high concentrations, and, in fact, the highest concentration found was in a normal control. The failure to detect TMHA in normal subjects studied earlier (6) cannot be explained. Perry et al. (26) reported that TMHA was not detected in the sweat of schizophrenic or of normal subjects. However, concentrations of 20 ng/ml of sweat were required for detection in the analytical procedure used in that study.

A group of normal and schizophrenic subjects received 10 μCi of [^{14}C]TMHA intravenously and its fate was monitored to evaluate its disappearance from blood, its appearance in sweat and urine, and its oxidation to CO_2. Since these studies were carried out at the same time that the sweat samples were being analyzed by GLC–MS, it was thought that investigations with the [^{14}C]TMHA might allow further insight into possible differences between normals and schizophrenics, provide information about body fluids other than sweat, and especially help to delineate differences in the metabolism of TMHA between the two groups. As was the case in the sweat analyses, there were no significant differences in the fate of [^{14}C]TMHA in normal and in schizophrenic subjects. The rate of clearance of [^{14}C]TMHA from the blood and the rate of $^{14}CO_2$ output from the lungs were similar in both groups. A large and similar amount of the injected radioactivity was excreted in the urine in both groups. However, TMHA was not detected in urine of a subject who had not been injected with the compound. A significant, though smaller, amount of the injected radioactivity was found in the sweat of both groups. Thus it is concluded that exogenous TMHA is metabolized similarly by normal and schizophrenic subjects.

The major stimulus for the present investigation was the report that TMHA is found only in the sweat of schizophrenics. TMHA is a fatty acid that contains a branched methyl group on C-3. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), which accumulates in patients who have Refsum's disease (27), also contains a branched methyl group on C-3, and the enzyme defect in this inherited disorder has been shown to be a defect in the α-hydroxylation of the fatty acid (28). Thus, the possibility that a defect in α-hydroxylation might lead to the accumulation of TMHA was considered at the start of this investigation. Now that it is established that TMHA is present in the sweat of most subjects, the source of this fatty acid might be considered. Its structure does not suggest an obvious metabolic precursor; thus, it is not possible to guess whether this fatty acid is derived from

[2] Holmstedt, B. Personal communication.

an exogenous precursor or is synthesized in the skin, which is known to synthesize a large number of fatty acids (29, 30).

The sweat of both normals and schizophrenics contains TMHA; therefore it is difficult to explain how this acid could lead to a characteristic odor in schizophrenics (6). One possibility is that there might be a difference in skin pH in the two groups. The pK_a of TMHA was measured and found to be about 5.0. If certain schizophrenics have skin pH below this value, the TMHA in those individuals would be a volatile fatty acid that might be smelled. Another possibility is that the odor is due to another compound.

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