Fatty Acid Amides of Ethanolamine in Mammalian Tissues

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While investigating the metabolism of ethanolamine by rat liver microsomes, we observed an incorporation of this amine into microsomal lipid that was dependent on the presence of a fatty acid as cosubstrate (1). It was shown that the product of the enzymatic reaction was the fatty acid amide of ethanolamine and that the reaction was synthetic, since little hydrolysis of added fatty acid amide could be detected. The reaction was shown to involve the direct formation of the amide and not the rearrangement of an ester. Kuehl et al. (2) had previously reported the isolation of palmitoylethanolamide, as a naturally occurring anti-inflammatory agent, from egg yolks. Since palmitoylethanolamide was known to occur in nature and to have pharmacological activity, and since there existed an enzymatic mechanism in mammalian tissues for the synthesis of this amide, we looked for the occurrence of this new lipid in mammalian tissues. The isolation, characterization, and quantitative assay of fatty acid amides of ethanolamine from animal tissues are the basis of this report.

EXPERIMENTAL PROCEDURE

Materials

Palmitic and stearic acids for general work were obtained from the Fisher Scientific Company. For analytical studies, palmitic and stearic acids (99.9 + %) were obtained from Applied Science Laboratories, State College, Pennsylvania. Ethanolamine was obtained from Fisher Scientific Company. Chloroform (containing 0.75% ethanol) and methanol were analytical grade products of Merek and Company, Inc.

Palmitoylethanolamide, steareoylethanolamide, and other fatty acid ethanolamides were synthesized according to Roe, Miles, and Swern (3) and were recrystallized three times from hot ethanol before use. 1\(^{14}\)C-Palmitic acid was used to prepare 1\(^{14}\)C-palmitoylethanolamide in a similar manner, but the product was purified by silicic acid and thin layer chromatography (described below).

1\(^{14}\)C-Palmitic ethanolamine ester hydrochloride (\(\beta\)-aminoethylpalmitate hydrochloride) was synthesized from 1\(^{14}\)C-palmitoylethanolamide by mixing 100 mg of the amide with 80 ml of anhydrous ether. This mixture was gassed with dry hydrogen chloride for several minutes, during which time the amide completely dissolved. The flask was stoppered and allowed to stand at room temperature for 24 to 36 hours. During this time, delicate white crystals appeared in the solution. The solution was chilled to 0°, and the crystals were collected by filtration and washed with cold dry ether. The product was dried in a vacuum and was recrystallized twice from chloroform before use. The yield of white crystalline product, which had a melting point of 119-120°, was 60 to 70%. Elemental analysis was as follows.

\[\text{C}_{20}\text{H}_{39}\text{O}_2\text{NCl}\]

Calculated: C 64.35, H 11.40, N 4.17, Cl 10.56

Found: C 64.31, H 11.56, N 4.32, Cl 10.7

The infrared spectrum of this material was identical with palmitoylethanolamine ester hydrochloride, which was prepared and kindly supplied to us by Merck Sharp and Dohme Research Laboratories.

Silicic acid (100 mesh, for chromatography) was obtained from Mallinekrodt Chemical Works. To prepare this for columns, 5 kg were suspended in distilled water and allowed to settle for 10 minutes. The unsettled material and water were immediately decanted. When the decanting process was repeated 15 to 20 times, a uniform size of silicic acid resulted, representing about 20% of the starting material. Two additional washes with 5 liters of methanol and a final wash with 5 liters of diethyl ether were done before the material was allowed to dry in a fume hood overnight. Before use, the silicic acid was activated at 110° for 12 hours and stored in a tightly closed bottle.

Materials for thin layer chromatography were obtained from Brinkmann and Company. Silica gel G plates, 250 \(\mu\) thick, were used for lipid separations.

Tissues were obtained from stock male Sprague-Dawley rats or adult albino guinea pigs. After deprivation of food for 12 to 18 hours, the animals were killed by decapitation and the tissues were removed immediately for extraction.

Methods

Tissue Extraction—Isolated, pooled tissues, weighing from 5 to 50 g, were homogenized and extracted in a high speed blender with 20 volumes of chloroform-methanol (2:1) by the method of Folch, Lees, and Sloane Stanley (4). Upon addition of 0.2 volume of 0.73% NaCl solution to the filtered chloroform-methanol extract, the solution separated into two phases. The upper phase was discarded, and the lipid-containing lower phase was extracted four times with 0.5 to 1.0 volume of a wash solution consisting of 47 parts 0.53% NaCl, 45 parts methanol, and 3 parts chloroform. After extraction, the solvents of the lower phase were removed by evaporation under reduced pressure. In this and subsequent steps, all evaporation were carried out on a rotary evaporator under reduced pressure.

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Isolation. Lipid samples were dissolved in chloroform, and total phosphorus was determined on a small aliquot by the method of Bartlett (5). The silicic acid column was prepared to contain 1 g of silicic acid per 10 mg of phosphorus in the lipid sample. Columns containing less than 6 g of silicic acid were packed in columns 10 × 30 mm, while those larger than 6 g were packed in columns 20 × 45 mm. Silicic acid was packed as a chloroform slurry and washed with 10 volumes of chloroform before application of the sample. The lipid solution was then applied to the column, and the flow rate was adjusted to between 1 and 2 ml per minute. The volumes of solutions used for elution, per g of silicic acid, were as follows: lipid dissolved in chloroform, 3 ml; chloroform, 5 ml; 1% methanol in chloroform, 5 ml; 2% methanol in chloroform, 5 ml; 3% methanol in chloroform, 5 ml; 4% methanol in chloroform, 5 ml; 90% methanol in chloroform, 10 ml; 40% methanol in chloroform, 20 ml; 80% methanol in chloroform, 10 ml.

Since alcohols have an additive eluting effect on silicic acid, large volumes of wash chloroform (containing 0.75% ethanol) had a tendency to elute the ethanolamides in a nonreproducible manner. It was decided, therefore, to standardize the volume of wash chloroform at 5 ml per g of silicic acid and to standardize the eluting solutions in the manner given above. With this procedure, separations of the phospholipids were obtained similar to those reported by Hanahan, Dittmer, and Warashina (6). The 1% to 4% methanol fractions were evaporated individually and redisolved in 0.2 ml of chloroform, and aliquots of each were analyzed by gas-liquid chromatography. Those fractions containing fatty acid ethanolamides were pooled.

Thin Layer Chromatography—Each pooled sample was applied to a silica gel plate adjacent to synthetic palmitoylethanolamide standards and developed in an ascending manner with chloroform-methanol (9:1). After the plate had dried, it was stained in an iodine atmosphere; the silica corresponding to the area for standard palmitoylethanolamide was removed from the elution tract of each sample, mixed with 2 ml of absolute methanol, and centrifuged. The supernatant solution was transferred to another tube, and the methanol was evaporated to dryness. The residue was dissolved in 0.5 ml of chloroform and assayed by gas-liquid chromatography.

A second solvent system occasionally used to isolate the fatty acid amides was chloroform-acetic acid-water (90:10:0.5) (i). In the chloroform-methanol solvent, palmitoylethanolamide had an approximate R<sub>f</sub> of 0.75 while the palmitic ethanolamine ester had an approximate R<sub>f</sub> of 0.45. In the chloroform-acetic acid-water solvent, the approximate R<sub>f</sub> values of these two compounds were 0.40 and 0.11, respectively.

Gas-Liquid Chromatography—The instrument used was a Barber-Coleman model 10 gas chromatograph. For assay of fatty acid ethanolamides, a 12-foot 7% SE-30 column was used at a column temperature of 212°. The carrier gas was argon at 30 p.s.i. Under these conditions, standard palmitoylethanolamide had a retention time of 9 and 12 minutes, and stearoylethanolamide had a retention time of 18 to 24 minutes. All quantitative determinations were done by comparison of peak areas with those obtained with standard solutions of the corresponding amides. The detector response of the gas chromatograph was linear from 0.1 to at least 20 μg, which was the range utilized for these studies. A standard curve was included with each group of samples, since sensitivity of the detector varied slightly from day to day, and standard samples were assayed periodically each day to check the calibration of the detector response. Identical samples injected serially showed less than 10% variation in detector response. It was possible to detect as little as 0.025 μg of the amides and to determine with accuracy 0.1-μg quantities. Standard solutions of fatty acid ethanolamides were prepared fresh at least weekly, since noticeable breakdown of the amides occurred in both methanol and chloroform solutions. The procedure for assay of fatty acid esters utilized a 6-foot 15% ethylene glycol succinate column on 100-140 mesh Gas-Chrom P at 181° with argon carrier gas at 20 p.s.i.

In some cases, particularly with brain extracts, other substances persisted through the purification method to the point of gas chromatography. However, it was possible to remove these impurities by the following procedure. The sample was dissolved in 1 ml of 1 N potassium hydroxide in methanol and allowed to stand for 60 minutes at room temperature. The solution was then evaporated to near dryness, taken up in 3 ml of water, and extracted three times with 2-ml volumes of diethyl ether. The ether extracts were pooled, evaporated, and taken up in 1 ml of chloroform for gas-liquid chromatography. Since the amides were resistant to alkaline hydrolysis, they remained unaffected while the contaminating materials were apparently destroyed by the alkali.

Hydrolysis of Isolated Ethanolamide Samples of isolated palmitoylethanolamide were hydrolyzed by heating them to 100° for 60 minutes in 0.5 ml of 6 N methanolic HCl. The resultant solution was diluted to 2 ml with water, and then was extracted three times with 2-ml portions of petroleum ether. The petroleum ether extracts were used for fatty acid analysis, while the aqueous phase was used for ethanolamine analysis.

Standard Procedure Used for Tissue Assay—The lipid was extracted from fresh tissue samples by the method of Polich et al. (4). The chloroform-methanol extract was then taken to dryness, redissolved in chloroform, and applied to a silicic acid column. The samples eluted with 1 to 4% methanol in chloroform were assayed for palmitoylethanolamine, and those samples which contained the appropriate peaks were combined and evaporated to dryness. The lipid residue was taken up in 0.5 ml of chloroform or methanol and assayed for fatty acid ethanolamides either directly or after alkaline hydrolysis.

RESULTS

Recovery of Palmitoylethanolamide Added to Tissues

Synthetic 1-<sup>14</sup>C-palmitoylethanolamide was added to liver homogenates, and the radioactivity was followed through the isolation procedure. As shown in Table I, recoveries were...
essentially quantitative at each step except for small losses on the silicic acid columns. These were found to be due to a small amount of tailing of the amides into later fractions. Concomitant assay of the total palmitoylethanolamide (radioactive plus nonradioactive) at various stages in the isolation procedure produced similar recovery data.

**Amounts of Fatty Acid Amides Present in Tissues**

Palmitoylethanolamide was found in several tissues of the rat and guinea pig (Table II). The amounts found in liver were quite variable, but the ethanolamide was consistently found in brain, liver, and muscle and was not detected in the other tissues examined. Since the method was sensitive to 0.025 μg levels, 0.005 μg per g, wet weight, could have been detected. Stearoyl-

**TABLE I**

Recovery of palmitoylethanolamide from rat liver lipids

The recovery data of four experiments were averaged, and the percentage recovery from each step was calculated by comparing the starting material at each step with the material recovered at that step. Over-all recovery was calculated by comparing the initially added 1-14C-palmitoylethanolamide with the amount obtained after the entire isolation procedure. In three experiments, 40 g of rat liver were homogenized with 300 μg of palmitoylethanolamide, and the radioactivity was followed through the isolation technique described. In the fourth experiment, 13.3 g of rat liver were homogenized with 2 μg of 1-14C-palmitoylethanolamide, and the radioactivity was similarly followed. Both methods produced similar recoveries at each level of the isolation.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>99.3</td>
</tr>
<tr>
<td>Wash procedure</td>
<td>98.0</td>
</tr>
<tr>
<td>Silicic acid column</td>
<td>93.1</td>
</tr>
<tr>
<td>Thin layer chromatography</td>
<td>97.2</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>100.0</td>
</tr>
<tr>
<td>Over-all recovery</td>
<td>90.7</td>
</tr>
</tbody>
</table>

**TABLE II**

Tissue levels of palmitoylethanolamide

The values are calculated as micrograms per g, wet weight. Numbers in square brackets show range of values obtained. Numbers in parentheses show number of experiments. Since the smallest quantity of tissue used was 5 g and the method is sensitive to 0.025 μg, 0.005 μg of palmitoylethanolamide per g of tissue could have been detected.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Palmitoylethanolamide</th>
<th>Rat</th>
<th>Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>35.7 [33-36] (3)</td>
<td>45.0 (1)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;0.005 (1)</td>
<td>&lt;0.005 (1)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;0.005 (1)</td>
<td>&lt;0.005 (1)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5.75 [1.0 10.0] (13)</td>
<td>17.0 (1)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;0.005 (1)</td>
<td>&lt;0.005 (1)</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>&lt;0.005 (1)</td>
<td>&lt;0.005 (1)</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;0.005 (2)</td>
<td>&lt;0.005 (2)</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>1.2 (1)</td>
<td>0.88 (2)</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>&lt;0.005 (2)</td>
<td>&lt;0.005 (1)</td>
<td></td>
</tr>
</tbody>
</table>

**Identification of Ethanolamides Found in Tissues**

**Properties on Gas-Liquid Chromatography**—When isolated palmitoylethanolamide was cochromatographed on gas-liquid chromatography with synthetic palmitoylethanolamide, a single peak appeared which was indistinguishable from the synthetic standard (Fig. 1). The same was true when isolated stearoyl-ethanolamide was chromatographed with synthetic material.

**Behavior on Hydrolysis**—Hydrolysis of the samples in 6 N methanolic HCl at 100° for 60 minutes completely destroyed the natural ethanolamides, while hydrolysis in 1 N methanolic potassium hydroxide for 60 minutes at room temperature left the ethanolamides unchanged. Synthetic ethanolamides behaved in the same manner.

**Analysis of Hydrolysis Products**—Several samples of palmitoylethanolamide which had been isolated from rat tissues and purified by the previous procedures were hydrolyzed as described under “Methods,” and the hydrolysis products were assayed for ethanolamine and palmitic acid.

Freshly prepared diazomethane was added to the pooled petroleum ether extracts until the yellow color persisted. The solution was then evaporated to dryness, the residue was dis-
solved in about 0.1 ml of isoctane, and the fatty acid esters were determined by gas chromatography. The predominant fatty acid present in the tissue extract was palmitic acid, but stearic acid was also present in the same proportion noted in the original fatty acid liberated by acid hydrolysis and the parent amide is identical with that of dinitrophenylethanolamine.

The stoichiometric relationship between ethanolamine and fatty acid liberated by acid hydrolysis and the parent amide is shown in Table III. It can be seen that for each mole of amide hydrolyzed, 1 mole of ethanolamine and 1 mole of fatty acid appeared.

**Characteristics of Acetyl Derivatives**—Microgram quantities of the amides were acetylated by evaporating solutions of amides to dryness and adding 1 ml of acetic anhydride and 0.05 ml of dry pyridine to the residue. After heating in a steam bath for 30 minutes, the solution was evaporated to dryness, and the residue was taken up in 0.5 ml of methanol. Gas-liquid chromatography of the acetylated palmitoylethanolamide derived from the tissues yielded a peak identical with that obtained with authentic acetylpalmitoylethanolamide (Fig. 1). Natural and synthetic acetylated stearoyl derivatives were also found to be identical.

**Infrared Spectroscopy**—Several samples of palmitoylethanolamide derived from rat tissues were combined (2.08 mg, total) and rechromatographed on a 4-g silica acid column. The amide was collected in the 2% methanol fraction, taken to dryness, and redissolved in 1 ml of chloroform. The infrared spectrum of this and of a comparable standard of palmitoylethanolamide are shown in Fig. 2. Except for the small area of 5.8 \( \mu \), which represents water contamination, the traces are identical.

**Procedures Used to Rule out Possibility That Fatty Acid Ethanolamides Represent Artifacts**

The effects of variations in the isolation procedure, particularly alkaline conditions, on the amounts of palmitoylethanolamide isolated were investigated in the following manner. The lipids extracted from 10- to 40-g quantities of rat liver or brain were divided into equal portions, and each portion was treated in the manner described in Table IV and assayed for palmitoylethanolamide. The amounts of palmitoylethanolamide obtained from the procedures with alkaline hydrolysis were found to be the same as from routine column chromatography and thin layer chromatography (Procedure A). Even though hydrolytic conditions were intensified by increasing time, temperature, or both (Procedures C, D, and E), as shown in Experiments 3 and 4, the amounts of palmitoylethanolamide isolated from rat liver and rat brain remained unchanged.

In Experiment 5, the lipid from 67 g of rat liver was fractionated by silicic acid chromatography according to the standard procedure; when the resultant fractions were analyzed for palmitoylethanolamide by gas chromatography, 498 \( \mu g \) of palmitoylethanolamide (7.45 \( \mu g \) per g of liver) were found in the 3% methanol in chloroform fraction. Each fraction was then subjected to alkaline hydrolysis in 1 \( \pi \) methanolic KOH for 12 hours at room temperature, followed by evaporation and extraction of the lipids into diethyl ether. The ether extracts were analyzed for fatty acid ethanolamides. The 3% methanol fraction contained 482 \( \mu g \) of palmitoylethanolamide (7.2 \( \mu g \) per g of liver), and 3.22 \( \mu g \) (0.048 \( \mu g \) per g of liver) appeared in the 2% methanol fraction. None of the other fractions contained detectable quantities of palmitoylethanolamide.

To examine further the possibility that free ethanolamine contributed to the formation of palmitoylethanolamide during the isolation, either by condensation with fatty acid or by aminolysis of an ester, 1.0 \( \mu e \) of ethanolamine-1,2-\( ^{14}C \) was homogenized with 30 g of rat liver; palmitoylethanolamide was isolated as described. After thin layer chromatography, the band corresponding to palmitoylethanolamide contained only 39 c.p.m. per \( \mu g \) above background, or a specific activity of 51 c.p.m.

![Fig. 2. Infrared spectra of palmitoylethanolamide obtained from rat tissues and by synthesis. The solvent was chloroform.](http://www.jbc.org/)
per μmole. The specific activity of free, reisolated ethanolamine was 6380 c.p.m. per μmole. Since less than 1% of the palmitoyl-ethanolamide could have been derived from free ethanolamine, no further purification of the palmitoyl-ethanolamide to remove the few detectable counts was attempted.

It remained possible that the material present in tissues was the palmitic ethanolamine ester which rearranged to the amide during the routine extraction and isolation procedure. This was investigated in the following manner. 1-14C-Palmitoyl-ethanolamide ester hydrochloride (2 mg; 1 μc/0.1 mmole) was homogenized with 20 g of rat liver in 20 volumes of chloroform-methanol (2:1), and this was carried through the routine isolation technique. Another identical sample of ester and rat liver homogenate was allowed to stand for 30 minutes before extraction with chloroform methanol. On passage through the silicic acid column, palmitoyl-ethanolamide was eluted with 1 to 4% methanol in chloroform while the ester was eluted with 20% methanol in chloroform. After thin layer chromatography, the amide fractions were quantified by gas chromatography. The total amount of palmitoyl-ethanolamide recovered from the zero time sample was 30 μg or 1.5 μg per g, while the 30-minute sample contained 22 μg or 1.6 μg per g. The measured radioactivities of these samples were 23 and 102 c.p.m., giving respective specific activities of 280 and 956 c.p.m. per pmole. The specific activity of free, reisolated ethanolamine was calculated to contain the fatty acid ethanolamides. It remained possible that the material present in tissues was the fatty acid ethanolamides. In whole mice, the incorporation of 1-14C-palmitic acid into palmitoyl-ethanolamide indicates an active synthesis of the ethanolamide in vivo.5 The enzymatic activity of both liver and brain is sufficient to account for the amount of fatty acid ethanolamides detected in these tissues. Since these fatty acid amides also may be accumulated from the diet of the animals, the levels obtained in liver may reflect both the enzymatic synthesis of the ethanolamide and a dietary contribution. Fasted animals were used to minimize the latter. However, it is unlikely that the diet can contribute to the levels of the fatty acid amides found in brain, since little if any palmitoyl-ethanolamide passes the liver after intestinal absorption. It is noteworthy that the level of the amide in brain is higher than in other tissues, representing almost 0.1% of the total brain lipid. In the liver, palmitoyl-ethanolamide accounts for 0.015% of the total lipid; in the skeletal muscle, it accounts for 0.002% of the total lipid. Brain also maintains a rather constant level of the amide, which in rats has been found to vary in the range from 30 to 37 μg per g of tissue.

The role of the fatty acid amides in metabolism is unexplained as yet. However, pharmacological studies by Ganley, Graebe, and Robinson (10, 11) have shown palmitoyl-ethanolamide to have potent anti-inflammatory and antianaphylactic properties. Whether this is related to the function of the amide as found in nature remains to be shown.

The problem of production of artifacts in the isolation technique was seriously considered since it is known that at alkaline pH, amide formation can occur through condensation of fatty acids and amines, by rearrangement from ester to amide linkage, or by aminolysis of esters. To minimize such artifacts, the pH of all the solutions employed in the extraction and isolation procedure was checked and noted to be at or below neutrality. Only in the case of brain lipids were alkaline conditions used to remove impurities at the final stage of the isolation. At this stage, the amides had already been detected on gas chromatography, and any increase in amide levels would have been quite apparent. Such increases were never observed. It should be pointed out that palmitoyl-ethanolamide was not detected in all tissues. Were it an artifact, one would expect to find it in all tissues that contain fatty acids, fatty acid esters, and lipid-bound ethanolamine at concentrations similar to those shown to contain the fatty acid ethanolamides.

### TABLE IV

**Procedures used to rule out artifact**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tissue</th>
<th>Procedure</th>
<th>Palmitoyl-ethanolamide found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rat liver</td>
<td>A</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>Rat liver</td>
<td>A + B</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>Rat liver</td>
<td>A</td>
<td>6.41</td>
</tr>
<tr>
<td>4</td>
<td>Rat brain</td>
<td>A</td>
<td>36.5</td>
</tr>
<tr>
<td>5</td>
<td>Rat liver</td>
<td>Silicic acid column</td>
<td>7.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silicic acid column + B</td>
<td>7.20</td>
</tr>
</tbody>
</table>

1. N. R. Bachur and S. Udenfriend, unpublished observations.
Separate experiments were conducted to show that there was no incorporation of free ethanolamine into the fatty acid ethanolamides and that there was no rearrangement of fatty acid ethanolamine ester to the ethanolamide during the isolation of the amides. In both cases only a small fraction of the isolated ethanolamine could have been formed by the artifactual mechanisms of direct condensation, aminolysis of an ester, or rearrangement of ester to amide linkage. Palmitic ethanolamine ester clearly rearranges to the palmitoylethanolamide under the conditions of alkaline hydrolysis employed to remove impurities at the final stage of the isolation. By this stage, however, any ethanolamine ester would have been removed, since the elution characteristics would separate ethanolamide from ethanolamine ester in both column and thin layer chromatography.

The evidence accumulated so far indicates that the fatty acid ethanolamides are probably formed directly by the enzymatic condensation of fatty acid with the ethanolamine. Since the microsomal system that we described previously (12) could utilize ethanolamine as well as amine compounds that had no adjacent hydroxyl group (e.g., phenethylamine, tyramine, and histamine), it appears that the amide bond is formed directly rather than being the product of ester amide rearrangement. It is conceivable, but not likely, that the amides of ethanolamine and phenethylamine are formed by different mechanisms.

The possibility was also considered that the amides were breakdown products of some hypothetical compound,

\[
O \quad R-\overset{\text{N}}{\bigg\|}\overset{\text{CH}_{3}}{\text{CH}_{2}}-\overset{\text{O}}{\text{R'}}
\]

In the experiments in which the lipid extracts were subjected to alkaline hydrolysis before and after silicic acid fractionation, no changes in the levels of fatty acid ethanolamides were detected. A very small amount of palmitoylethanolamide which appeared in the alkaline-hydrolyzed 2% methanol fraction from silicic acid column chromatography was most likely free palmitoylethanolamide that was masked by contaminating materials on gas-liquid chromatography. Since a highly diluted sample must be analyzed on gas-liquid chromatography in the early stages of purification, very small amounts of palmitoylethanolamide can escape detection. After column and thin layer chromatography, more concentrated samples can be analyzed, and smaller quantities of the fatty acid amides detected. A preliminary report has appeared (13) of variations in the levels of fatty acid ethanolamides, obtained from plant materials, dependent on hydrolytic conditions. This does not seem to be the case for palmitoylethanolamide in rat or guinea pig tissues.

While the fatty acid ethanolamides are pharmacologically active compounds, they also represent an important interaction between the hydrophobic acids and the charged amino compound. Similar interactions are known to occur between fatty acids and other amines. For example, rat liver microsomes catalyze the synthesis of fatty acid amides of histamine, tyramine, phenethylamine, β-hydroxyphenethylamine, and tryptamine (12). Whether these amides exist in vivo is unknown. This type of interaction may occur more generally than has been realized previously, and may preface unforeseen biochemical and physiological activities of such compounds.

**SUMMARY**

A method for the isolation, purification, and quantitative assay of fatty acid ethanolamides from natural sources is described. In rats and guinea pigs from which food has been withheld, the amide is found in brain, liver, and skeletal muscle, the level being highest and most constant in brain. Verification of the structure of the isolated palmitoylethanolamide was obtained through gas-liquid chromatography, infrared spectroscopy, and hydrolysis and analysis of the component fatty acid and amine. Evidence was presented to rule out the artifactual production of palmitoylethanolamide.

**Acknowledgments—**The authors gratefully acknowledge the aid of Dr. J. Bieri and Miss E. I. Andrews in the determination of the fatty acids by gas-liquid chromatography, and Dr. H. Fales and Mrs. K. Warren in preparing and interpreting infra-red spectograms.

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