ENZYMATIC FORMATION OF FATTY ACID AMIDES OF ETHANOLAMINE BY RAT LIVER MICROSOMES

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Ethanolamine is a major component of lipids, being present primarily as the basic portion of cephalin (phosphatidyl ethanolamine). During studies on the biosynthesis of this phospholipid it was found that rat liver microsomes readily incorporated ethanolamine-C^{14} into a lipid material, which, however, contained no phosphorous. Further studies showed that the microsomal system catalyzed a condensation reaction between fatty acids and ethanolamine to yield the corresponding amides. Some properties of this enzyme system and details of the identification of the enzymatically formed products are reported in this communication.

The reaction was first observed with microsomal preparations solubilized by treatment with snake venom (Crotalus adamantus, Ross Allen's Reptile Institute, Silver Springs, Florida). Such preparations incorporated ethanolamine-C^{14} into material which could be extracted into chloroform-methanol (2:1) or toluene. As shown in Table I no other additions were required for activity with a snake venom treated preparation. Using intact microsomes little ethanolamine incorporation was observed unless an acceptor fatty acid was added. Solvent extracts of snake venom treated microsomes also stimulated activity, making it apparent that one function of snake venom was to release a mixture of

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free fatty acids. In most of the studies reported here intact microsomes were used to permit experiments with individual fatty acids. With intact microsomes some stimulation was observed when ATP and CoA were added (Table I).

TABLE I

Incorporation of ethanolamine-C^{14} into lipid fraction

Additions	Relative incorporation of ethanolamine-C^{14} into lipid
Microsomes	20
Venom-treated microsomes	82
Microsomes + lipid extract of venom-treated microsomes	80
Microsomes + palmitic acid	100
" + " " + CoA + ATP	130
" + " " + Creatine P	100
" + " " + DPN + TPN	100

Reactions were conducted at 37° for 60 minutes in media consisting of 100 µmoles tris buffer pH 9.0, 60 µmoles ethanolamine HCl, and microsomes equivalent to 0.1 gm of rat liver. Additions were lipid extract from the equivalent of 0.1 gm rat liver microsomes, 1 µmole palmitic acid, 1 µmole ATP, 0.2 µmole CoA, 2 µmoles Creatine P, 2 µmoles DPN, and 0.2 µmole TPN. With microsomes and palmitic acid 0.3 µmole of ethanolamine was incorporated. This has been set at 100 for purposes of comparison.

The product which was formed on incubating palmitic acid and ethanolamine-C^{14} with microsomes was identified as palmityl ethanolamide [N-(2-hydroxyethyl) palmitamide] in the following way: Enzymatically formed material when subjected to chromatography on silicic acid with methanol-chloroform (1) was eluted with 2.5% methanol-97.5% chloroform. Synthetically prepared material (2) was eluted in the same manner. This is shown in Table II where comparisons of the elution patterns are made with known lipids. It can be seen that essentially all the incorporated ethanolamine-C^{14} was in the 2.5 to 5% methanol fraction. This clearly

TABLE II

Behavior of product on silicic acid column chromatography

Per Cent Methanol in Chloroform	Per Cent of Palmityl Ethanolamide Eluted			Known Lipid Compounds
	Synthetic	Enzymatic		
		A	B	
0 - 0.75	0.45	2.0	0	Neutral lipids, fatty acid
2.5 - 5.0	98.1	87	100	-
10	1.4	-	0	-
20	0	11	0	Phosphatidyl serine Phosphatidyl ethanolamine
40	0	0	0	Phosphoinositide; lecithin
80	0	0	0	Lysolecithins; sphingolipi

The lipids, in chloroform solution, were placed on silicic acid columns and eluted with chloroform containing increasing proportions of methanol as described by Hanahan et al.(1). The elution patterns of known lipid components (column 5) are according to Hanahan et al. A number of them were verified in our laboratory.

distinguishes it from the phospholipids and free fatty acids. The 2.5 to 5% methanol fraction was evaporated to dryness, and an aliquot dissolved in a small volume of chloroform and subjected directly to vapor phase chromatography. The remainder was taken up in pyridine and acetylated by treatment with acetic anhydride. Fig. 1 shows that the elution times of the enzymatically formed product and its acetylated derivative were identical with synthetic palmityl ethanolamide and O-acetyl palmityl ethanolamide respectively.

The products formed on incubation of ethanolamine-C^{14} with snake venom treated microsomes appeared to be a mixture of amides as shown by vapor phase chromatography. When subjected to hydrolytic conditions, the amides were stable in alkali, little or no hydrolysis occurring

in 1.0 N NaOH at 100° for 2 hours. On heating at 100° in 1.0 N HCl for 2 hours hydrolysis was almost complete so that little solvent extractable radioactivity remained and equivalent quantities of ethanolamine-C^{14} were released. The alkali stability and acid lability are evidence for the presence of an amide bond in the product.

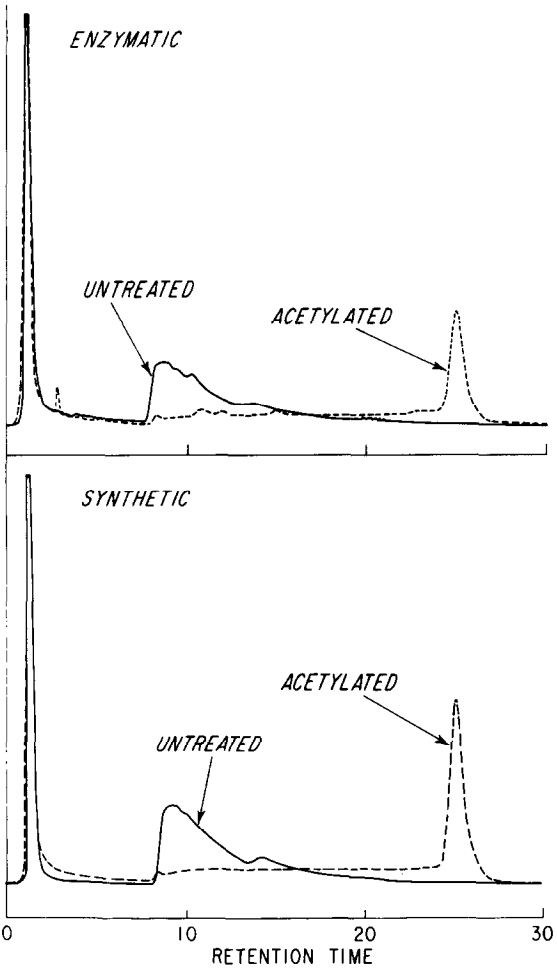

Fig. 1. Comparison of enzymatically formed palmityl-ethanolamide and its acetylated product with synthetic materials. The product was isolated and acetylated as described in the text and subjected to gas-liquid chromatography. The following system was used: Column 9 ft x 3 mm (I.D.) glass U tube packed with 7% SE-30 polymer on 100 mesh Gas-Chrom. P.; argon pressure 34 p.s.i.; temperature 233°.

With intact microsomes it was found that even numbered fatty acids from C_{12} to C_{24}, both saturated and unsaturated, could substitute for

palmitate in the reaction. The requirements were found to be somewhat more exacting for the amine acceptor. Thus, choline, serine, glycine, alanine, phenylalanine and threonine were found to be inactive. However, other amines such as phenethylamine, β-hydroxyphenethylamine and 1-amino-2-propanol were found to be about as active as ethanolamine.

With ethanolamine and related hydroxyamines the possibility existed that the primary product could have been the corresponding ester, as reported by Kennedy (2), which then rearranged to the amide. However, the incorporation of phenethylamine to the corresponding amide ruled this out. Even with ethanolamine as substrate the amide was obtained under conditions which made ester-acyl migration highly unlikely; enzymatic formation at or near neutrality and extraction from an acidified incubation mixture into organic solvent. Finally, if this were the ester forming system of Kennedy (2) then choline should have been an excellent substrate.

Although amino acids were not converted to fatty acid amides by this system the reaction may be compared with the reversible formation of amino acid-fatty acid amides reported by Fukui and Axelrod (3). In the reaction described by these authors the hydrolysis of the fatty acid amide proceeded at a faster rate than its synthesis. To investigate the possibility that in the present system amide formation occurred due to the reversal of a hydrolytic reaction synthetic C^{14} palmityl-ethanolamide was incubated with microsomes. It was found that less than 0.01 micromole of the amide was hydrolyzed in a preparation which formed 0.15 micromole from palmitate and ethanolamine. Furthermore, addition of micromole quantities of the amide to the reaction did not inhibit amide formation. It would appear then that the microsomal activity described here represents a synthetic mechanism for forming an amide bond. Such a mechanism should require energy. The failure to show marked stimulation with added cofactors may indicate that the proper energy sources were not tried or that intact

microsomes already contain or can produce adequate amounts of these substances.

Although enzymatic formation of fatty acid amides of ethanolamine has not been reported before this, such amides have been isolated from natural sources. Coburn et al. (4) and Long and Martin (5) isolated material from egg yolk which was later shown by Kuehl et al. (6) to contain palmityl ethanolamide. Similar materials were found in several natural oils. It is of further interest that these amides of ethanolamine have been reported to have anti-inflammatory and anti-serotonin activity (7). The physiologic significance of the fatty acid amides of ethanolamine and of the enzyme involved in their biosynthesis remain to be elucidated.

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REFERENCES

1. HANAHAN, D. J., DITTMER, J. C., and WARASHINA, E., J. Biol. Chem., 228, 685 (1957).
2. KENNEDY, E. P., Canadian J. Biochem. and Physiol., 34, 334 (1956).
3. FUKUI, T., and AXELROD, B., J. Biol. Chem., 236, 811 (1961).
4. COBURN, A. F., GRAHAM, C. E., and HANINGER, J., J. Exp. Med., 100, 425 (1954).
5. LONG, D. A., and MARTIN, A. J. P., Lancet, 464 (1956).
6. KUEHL, F. A., JR., JACOB, T. A., GANLEY, O. H., ORMOND, R. E., and MEISINGER, M. A. P., J. Am. Chem. Soc., 79, 5577 (1957).
7. GANLEY, O. H., and ROBINSON, H. J., The Journal of Allergy, 30, 415 (1959).