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REFERENCES

1. W. O. Godtfredsen, S. Johnson, H. Lorth, K. Proholt and L. Tybring, *Nature, Lond.* **193**, 987 (1962).
2. W. O. Godtfredsen and S. Vangedal, *Tetrahedron* **18**, 1029 (1962).
3. C. L. Harvey, C. J. Sih and S. G. Knight, *Bact. Proc.* (abstr. P3), 73 (1965).
4. H. J. Yamaki, *J. Antibiot., Tokyo* **18**, 228 (1965).
5. C. L. Harvey, C. J. Sih and S. G. Knight, *Biochemistry* **5**, 3320 (1966).
6. S. Tanka and A. Kaji, *Biochem. biophys. Res. Commun.* **46**, 136 (1972).
7. J. W. Bodley, F. J. Zieve, L. Lin and S. T. Zieve, *Biochem. biophys. Res. Commun.* **37**, 437 (1969).
8. J. W. Bodley, F. J. Zieve and L. Lin, *J. biol. Chem.* **245**, 5662 (1970).
9. A. Okura, T. Kinoshita and N. Tanaka, *J. Antibiot., Tokyo* **24**, 655 (1971).
10. G. R. Willie, N. Richman, W. O. Godtfredsen and J. W. Bodley, *Biochemistry* **14**, 1713 (1975).
11. H. Matsu, W. Nakagawa and M. Nakao, in *Organization of Energy Transducing Membranes* (Eds M. Nakao and L. Packer), pp. 63–9. University Press, Baltimore (1973).
12. W. O. Godtfredsen, *Fusidic Acid and Some Related Antibiotics*. Arrhus Stiftsbogtrykkerie, Copenhagen, Denmark (1967).
13. A. T. Florence, *Adv. Colloid Interface Sci.* **2**, 115 (1968).
14. M. Beaudoin, M. C. Carey and D. M. Small, *Gastroenterology* **65**, 527 (1973).
15. M. C. Carey, J.-C. Montet and D. M. Small, *Biochemistry* **14**, 4896 (1975).
16. C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*. Wiley, New York (1973).
17. T. W. Fuhremann, E. P. Lichtenstein, R. N. Zahlten, F. W. Stratman and H. K. Schnoes, *Pestic. Sci.* **5**, 31 (1974).
18. G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman), Vol. 10, pp. 448–63. Academic Press, New York (1967).
19. G. H. Sunshine, D. J. Williams and B. R. Rabin, *Nature New Biol.* **230**, 133 (1971).
20. J. R. Dice and R. T. Schimke, *J. biol. Chem.* **247**, 98 (1972).
21. A. A. Hochberg, R. N. Zahlten, F. W. Stratman and H. A. Lardy, *Biochemistry* **11**, 3143 (1972).
22. R. J. Mans and G. D. Novelli, *Biochem. biophys. Res. Commun.* **3**, 540 (1960).
23. W. O. Godtfredsen and S. Vangedal, *Acta chem. scand.* **20**, 1599 (1966).
24. H. N. Christensen, in *Proteins, Nutrition and Free Amino Acid Patterns* (Ed. J. H. Leathem), p. 40. Rutgers University Press, New Brunswick (1968).
25. R. Blasberg and A. Lajtha, *Archs Biochem. Biophys.* **112**, 361 (1965).
26. M. F. Holick and H. F. DeLuca, in *Advances in Steroid Biochemistry and Pharmacology* (Eds M. H. Briggs and G. A. Christie), pp. 111–55. Academic Press, New York (1974).
27. K. M. Botham, J. G. Chazarian, B. E. Kream and H. F. DeLuca, *Biochemistry* **15**, 2130 (1976).
28. E. Ziv and F. W. Stratman, *Fedn Eur. Biochem. Soc. Lett.* **68**, 86 (1976).
29. E. Ziv, M. J. Wagner and F. W. Stratman, *Fedn Eur. Biochem. Soc. Lett.* **86**, 219 (1978).

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2-Pyrrolidinone—A cyclization product of γ-aminobutyric acid detected in mouse brain

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The neurotransmitter candidate γ-aminobutyric acid (GABA) is structurally similar to glutamic acid. Cyclization of glutamic acid and GABA produces the lactams, 2 - pyrrolidinone - 5 - carboxylic acid (pyroglutamic acid, 5-oxoproline) and 2-pyrrolidinone respectively. While 2 - pyrrolidinone - 5 - carboxylic acid is known to occur in brain[1], studies on pyrrolidinone have been limited to exogenously administered compound. Pharmacological studies have indicated that pyrrolidinone in high doses exhibits anticonvulsant activity in animals, presumably by acting on the GABA system[2–4]. Other workers have been unable to verify these findings[5, 6]. Tower[7] has shown that cat cerebral cortex slices have the capacity to enzymatically convert [2-^{14}C]pyrrolidinone to [^{14}C]GABA. Thus, pyrrolidinone might potentially serve as a GABA precursor in the central nervous system. In this communication, mass spectral evidence is provided which suggests that pyrrolidinone is a natural constituent of mouse brain. Studies with labeled GABA are also presented which indicate that pyrrolidinone is not an artifact of the work-up process.

MATERIALS AND METHODS

Male ICR mice (25–30 g) were used for all the experiments. The animals were decapitated and the brains quickly removed, weighed and homogenized in cold

0.1 N formic acid (2 ml) containing internal standard. [5,5-$^2H_2$]pyrrolidinone (>97% deuterium incorporation) was prepared by the reduction of succinimide with lithium aluminum deuteride (Merck & Co., Inc./Isotopes, St. Louis, MO 63116) following the method of Duffield *et al.*[8]. [4,4-$^2H_2$]GABA [>97% deuterium incorporation, m.p. 202° dec. (unlabeled GABA lit.[9] m.p. 203° dec.)] was prepared by hydrolysis of [5,5-$^2H_2$]pyrrolidinone in refluxing 15% HCl for 2 hr and purified by ion exchange chromatography (AG 50W-X8). The brain homogenates containing standards were centrifuged at 12,000 *g* for 6 min and each supernatant fraction was placed on an ion exchange column (AG 50W-X8, 50–100 mesh, formate form, 1.0 *g* wet weight). Pyrrolidinone was contained in a water wash (10 ml) of the columns.

Identification of brain pyrrolidinone by thin-layer chromatography–mass spectrometry. The brains of three animals were homogenized in the presence of [$^2H_2$]GABA standard (0.60 μmole/brain) and the supernatant fractions applied to ion exchange columns as above. The combined water washes of the columns were extracted with an equal volume of Et_2O. The Et_2O layer which contained lipids was discarded. The aqueous layer was then extracted with 5 vol. chloroform–methanol (2:1, v/v). The combined organic layers were reduced in volume by distillation with a short path distillation apparatus to approximately 50 μl and the residue was streaked on a glass t.l.c. plate (silica gel G, 250 μm thick) and developed in benzene–methanol (9:1, v/v). The region co-chromatographic with authentic pyrrolidinone (R_f 0.2–0.3) was scraped and eluted with methanol (0.5 ml). The methanol layer was filtered and distilled to leave about 50 μl residue. The residue was transferred to a clean melting point tube and placed in a vacuum oven overnight (60°, house vacuum) to remove the remaining solvents. Pyrrolidinone was identified in the residue by direct probe insertion into a mass spectrometer (DuPont 21-490 mass spectrometer with 21-094 data system, electron impact mode, 70 eV).

Identification of brain pyrrolidinone as a pentafluoropropionyl derivative by gas chromatography–mass spectrometry. Supernatant fractions of the brains of three animals were applied to ion exchange columns and washed with water and the combined water washes extracted with Et_2O and $CHCl_3$–CH_3OH as described above. The combined organic layers were distilled to a volume of about 50 μl. Evaporation to dryness was carried out in a vacuum oven as above in a 10 ml pear shaped flask. To the residue was added 50 μl pentafluoropropionic anhydride and the mixture was heated with a heat gun until the residue dissolved. A portion of the sample (1–2 μl) was injected into a gas chromatograph (3% OV-17 on Chromosorb W.H.P., 2 m × 3 mm glass column, oven temp. 90–260° programmed at 15°/min) interfaced with a mass spectrometer. The molecular ion for *N*-pentafluoropropionylpyrrolidinone (M^+, m/e 231, relative intensity 6%) and its major fragment (M^+–C_2F_5, m/e 112, relative intensity 100%) were monitored in the g.c. effluent by the mass spectrometer-data system.

Quantification of brain pyrrolidinone by gas chromatography–mass spectrometry. Brains from animals killed by exposing the skull to a beam of microwave radiation (2.0 kW, 3 sec, model BN-K2 General Medical Engineering Corp. Microwave Radiator) were homogenized as described above. The pyrrolidinone fraction of the homogenate, which contained added [$^2H_2$]pyrrolidinone (19.0 nmoles/brain), was extracted with an equal volume of Et_2O. To the aqueous layer was added an equal volume of conc. HCl and the mixture was held at reflux for 2 hr. The solution was then evaporated to dryness with a rotary evaporator and the residue dissolved in water (5 ml) and applied to a second ion exchange column. The column was washed with water (10 ml) followed by 1.0 N NH_4OH (15 ml). The NH_4OH wash was evaporated to dryness with a rotary evaporator and the residue treated with 50 μl of a solution of *N,N*-dimethylformamide dimethyl acetal in acetonitrile (2:1, v/v)[10, 11]. The derivatives, methyl 4 - (*N,N* - dimethyl-*N'* - formamidino)butanoate (I) where I equals $(CH_3)_2N$–CH=N–$[CH_2]_3$–CO_2CH_3 and [$^2H_2$]-I, were formed in 10 ml stoppered pear shaped flasks with gentle heating with a heat gun until the residue dissolved in the reagent. The gas chromatogram of the derivatives was continuously scanned during elution from mass range 51 to 219 a.m.u. at a scan rate of approximately 4 sec/scan which yielded a total of 6–7 scans for each compound eluted. Quantification of pyrrolidinone in the brain homogenate was accomplished from a ratio of the areas of computer constructed ion chromatograms of the ion current associated with the molecular ions of I (m/e 172) and standard [$^2H_2$]-I (m/e 174)[12].

RESULTS AND DISCUSSION

A comparison of the mass spectrum of authentic pyrrolidinone with the spectrum of the material isolated from mouse whole brain which exhibited the same chromatographic properties as pyrrolidinone is shown in Fig. 1. In this experiment, [4,4-$^2H_2$]GABA was added to the brain prior to homogenization to serve as a control for potential cyclization of GABA during the work-up procedure. The mass spectrum of pyrrolidinone from mouse brain to which [$^2H_2$]GABA had been added revealed that essentially all of the ionization resulted from unlabeled pyrrolidinone (Fig. 1). Since the brain homogenate contained approximately equal quantities of unlabeled and deuterium-labeled GABA during work-up, it is likely that pyrrolidinone produced as an artifact would exhibit the same unlabeled to labeled ratio. In this experiment, mass spectrometric analysis showed that

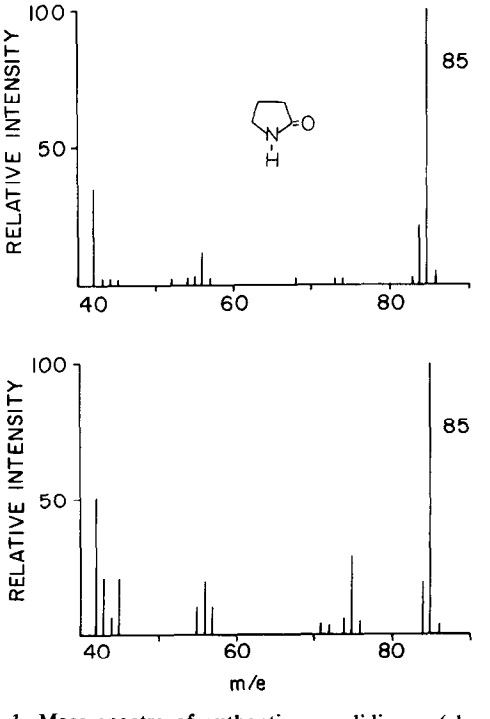

Fig. 1. Mass spectra of authentic pyrrolidinone (above) and of the pyrrolidinone fraction isolated from mouse brain (below). The spectra are not corrected for background. The brain pyrrolidinone spectrum (below) represents m/e values > 5 per cent of the relative intensity of m/e 85.

less than 2 per cent of the detected pyrrolidinone was accounted for as deuterated pyrrolidinone. This suggests that the brain pyrrolidinone was not derived from cyclization of GABA or [$^2H_2$]GABA during work-up of the tissue.

To further characterize the mouse brain pyrrolidinone, a derivative was formed by reaction of the pyrrolidinone fraction from the ion exchange column with pentafluoropropionic anhydride. Mass spectral analysis of the g.c. peak with the appropriate retention time showed the presence of the parent ion of *N*-pentafluoropropionylpyrrolidinone (M^+, m/e 231) and its major fragment ($M^+-C_2F_5$, m/e 112) in approximately the same ratio as found in authentic material.

As a means of quantifying pyrrolidinone in single whole brain, a second derivative and gas chromatography–mass spectrometry were utilized[10]. In this method, pyrrolidinone, which had been separated from GABA by ion exchange chromatography, was hydrolyzed in acid. The resulting GABA was treated with *N,N*-dimethylformamide dimethyl acetal[11] to form methyl 4-(*N,N*-dimethyl-*N'*-formamidino)butanoate (I). Compound I has suitable properties for gas chromatography–mass spectrometry quantitative analysis. [$^2H_2$]Pyrrolidinone served as internal standard. The molecular ions of the hydrolyzed and derivatized pyrrolidinone products I (m/e 172) and [$^2H_2$]-I (m/e 174) were monitored in the g.c. effluent from reconstructed ion current profiles derived from successive scanning[12]. The ratio of the peak area of m/e 172 and of the known amount of standard m/e 174 yielded a quantitative estimate of the quantity of pyrrolidinone in the sample. By this method the concentration of pyrrolidinone in single whole mouse grain was found to be 42 ± 9 nmoles/g wet tissue (mean of six mice ±S.E.M.).

While the biosynthetic source of the brain pyrrolidinone and a relationship between pyrrolidinone and the functioning GABA system have not been established, the possibility exists that a physiologically significant GABA–pyrrolidinone equilibrium may occur in mouse brain.

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REFERENCES

1. P. van der Werf, R. A. Stephani and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1026 (1974).
2. J. E. Hawkins, Jr. and L. H. Sarett, *Clinica chim. Acta* **2**, 481 (1957).
3. J. E. Lightowler and J. A. R. MacLean, *Archs int. Pharmacodyn. Thér.* **145**, 233 (1963).
4. J. Sieroslawska, *Archwm Immun. Terap. Exp.* **13**, 70 (1965).
5. P. Pinelli, *Il Farmaco (Sci.)* **25**, 187 (1970).
6. E. W. Maynert and H. K. Kaji, *J. Pharmac. exp. Ther.* **137**, 114 (1962).
7. D. B. Tower, in *Inhibition in the Nervous System and Gamma-Aminobutyric Acid* (Ed. E. Roberts), p. 562. Pergamon Press, New York (1960).
8. A. M. Duffield, H. Budzikiewicz and C. Djerassi, *J. Am. chem. Soc.* **86**, 5536 (1974).
9. J. G. Grasselli (Ed.), *Atlas of Spectral Data and Physical Constants for Organic Compounds*, p. B367. CRC Press, Cleveland (1973).
10. P. S. Callery, L. A. Minor, S. M. Remmers and M. Stogniew, *Abstracts, Twenty-fifth Annual Conference on Mass Spectrometry and Allied Topics*, p. 553. American Society for Mass Spectrometry, Washington, D.C. (1977).
11. J. P. Thenot and E. C. Horning, *Analyt. Lett.* **5**, 519 (1972).
12. M. F. Schulman and F. P. Abramson, *Biomed. Mass Spectrom.* **2**, 9 (1975).

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Maternal transport of chlorophenoxyisobutyrate at the foetal and neonatal stages of development

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Administration of the widely used anti-hypercholesterolemic drug 'clofibrate' (ethyl -α-*p*-chlorophenoxyisobutyrate) to the rat has been shown to produce many changes in the liver. Mention, in this regard, may be made of hepatomegaly[1], proliferation of mitochondria[2,3] and increase in the activity of the mitochondrial enzyme α-glycerolphosphate dehydrogenase* (L-glycerophosphate (acceptor) oxidoreductase, EC 1.1.99.5)[3, 4]. Both mitochondria and GPD are known to increase post-natally in animals[5]. It was therefore, of interest, to know the effect of administration of clofibrate to the mother during pregnancy and lactation on the above-mentioned developmental changes. The results of such a study are presented and discussed in this paper.

Female albino rats weighing 120–150 g from the Central Animal Facility of this Institute were maintained on a stock diet obtained commercially. Experimental animals were administered with clofibrate orally (50 mg per rat per day) as an emulsion in water (0.2 ml) during the entire period of mating, gestation and lactation. Animals not receiving the drug served as controls. The offspring were weaned from their mothers after 21 days of lactation.

Offspring of age groups as indicated were killed and the serum and liver collected. The livers of 12–15 new born pups were pooled for subcellular fractionation which was done essentially as described earlier[3]. Mitochondrial GPD was assayed manometrically[3]. The level of chlorophenoxyisobutyrate in serum and liver was estimated spectrophotometrically[6] as well as by using methyl [^{14}C] CPIB. Protein was estimated using Folin's reagent[7].

Administration of clofibrate to the adult animal causes cessation of weight gain as well as enlargement of the

* Abbreviation used: GPD, α-glycerolphosphate dehydrogenase; CPIB, *p*-chlorophenoxyisobutyrate (free acid).