Gas chromatographic-mass spectrometric identification of epinine in rat superior cervical ganglia and formation in vivo

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Summary. Epinine was identified in rat superior cervical ganglia by a gas chromatographic-mass spectrometric method. The deuterated methyl group of i.v. administered labeled methionine was incorporated into epinine at a slow rate, although epinephrine-CD_3 was rapidly formed. These results indicate that epinine found in the ganglia is not a precursor of epinephrine.

In 1906, Halle[3] proposed that epinephrine might be formed by β-hydroxylation of epinine, but the latter compound was not reported in any animal tissues until 1962, when it was found in the parotid gland of Bufo marinus[4]. In 1973, Laduron[5] demonstrated the presence of epinine in bovine adrenal medulla and showed that it could be synthetized in vitro from dopamine by the action of an N-methyltransferase isolated from the adrenal medulla[6] as well as in perfused adrenals[7]. Although norepinephrine is generally considered to be the precursor[8–10] of epinephrine, on the basis of these observations, Laduron proposed that epinephrine could also be formed by a pathway involving the N-methylation of dopamine to epinine and subsequent β-hydroxylation to epinephrine. Epinephrine is present in the superior cervical ganglion[11], perhaps in chromaffin cells[12] which are similar to the principal cell type of adrenal medulla[13]. Since chromaffin or small intensely fluorescent (SIF) cells in the superior cervical ganglia disappear during the development[14], it was of interest to determine if epinine was present in ganglia of neonatal rats and if its levels would decline during development in the same manner as do chromaffin cells. The relative synthesis rates of epinephrine and epinine in newborn rats was examined by determining the relative rate of incorporation of CD_3 from administered methionine-CD_3 into the N-methyl group of the 2 compounds.

Materials and methods. Pregnant rats were obtained from Zivic Miller (Allison Park, Pa., USA) on the 18th day of gestation and were housed individually until birth of the litters. Pups of different ages were killed by decapitation and superior cervical ganglia were removed, cleaned and

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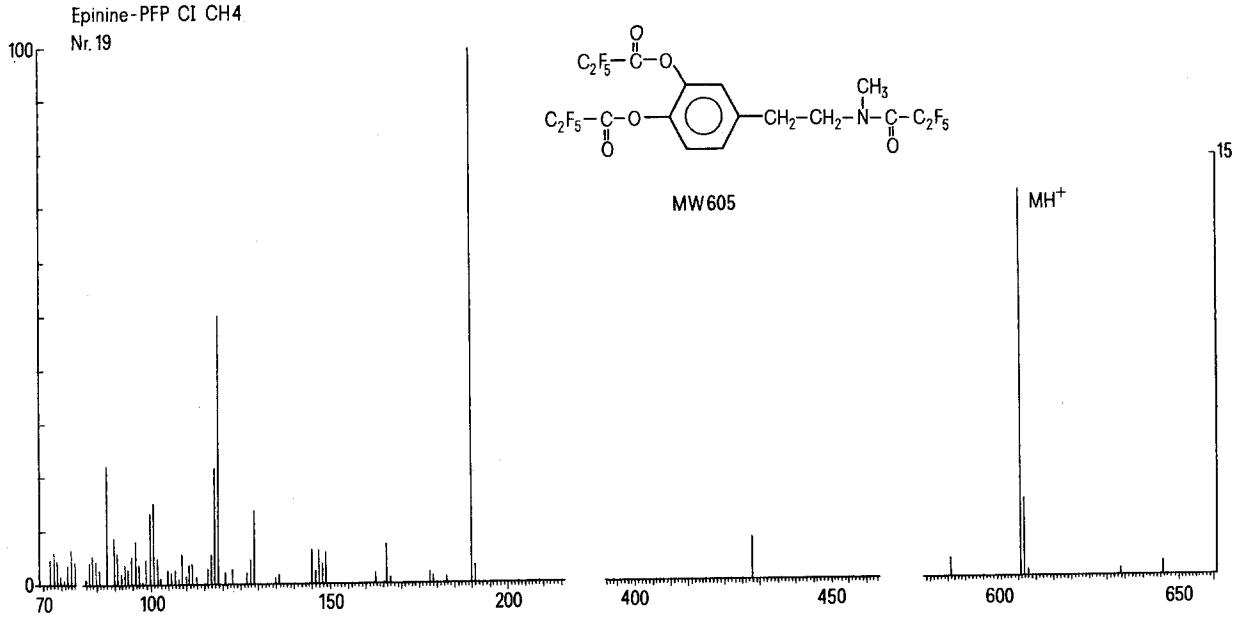

Fig. 1. Chemical ionization mass spectra of epinine-PFP on a Finnigan Model 1015C Mass Spectrometer using methane as reagent gas.

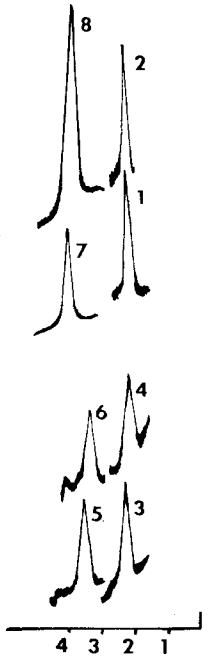

Fig. 2. Typical GC-MS tracing, under chemical ionization conditions with methane as reagent gas, of pentafluoropropionic anhydride derivatives of catecholamines in rat superior cervical ganglia and of added authenic deuterated catecholamines. *1* Epinephrine-PFP, m/e 604; *2* E-D_3-PFP, m/e 607; *3* norepinephrine-PFP, m/e 590; *4* NE-D_3-PFP, m/e 593; *5* dopamine-PFP, m/e 592; *6* DA-D_2-PFP, m/e 594; *7* epinine-PFP, m/e 606; *8* Ep-PFP, m/e 609. The retention time is given in min.

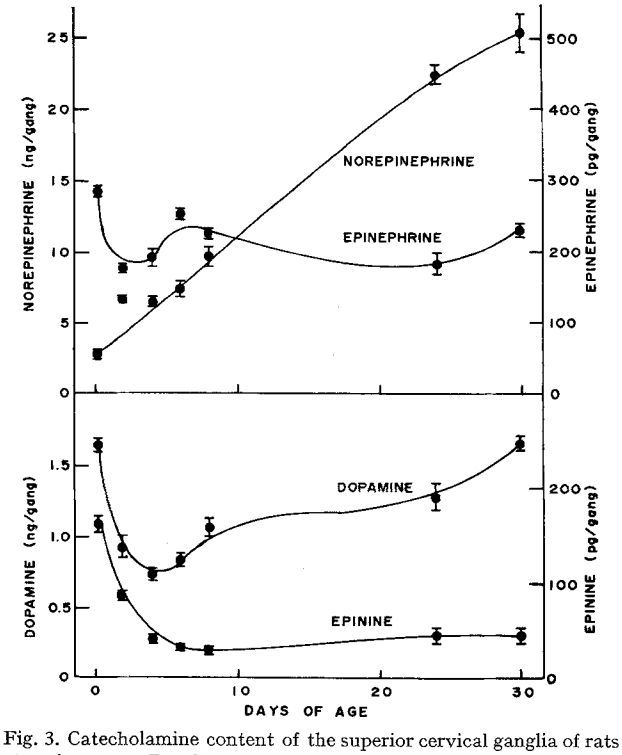

Fig. 3. Catecholamine content of the superior cervical ganglia of rats at various ages. Results are mean values (± SEM) for groups of 6–8 rats. The levels of amines were determined by GC-MS.

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cooled on ice. Immediately after dissection, ganglia from 1 or 2 rats were homogenized in ice cold 100 µl N HCl. To an aliquot of the homogenate was added 100 µl of 0.1 N HCl, containing deuterated epinine (epinine-N-CD_3), epinephrine (epinephrine-α-D_2-β-D_1), nor-epinephrine (nor-epinephrine-α-D_2-β-D_1) and dopamine (dopamine-β-D_2). The amounts of deuterated standards added were about 10% less than the expected amount of the respective endogenous compounds as determined previously without standards. To diminish losses, 50 µl of 0.1 N HCl containing 0.5% ascorbic acid, 0.1% EDTA, and 0.05% α-methyl-dopamine, which did not interfere with the quantitative measurements, was also added. The homogenate was centrifuged at 10.000 × g for 15 min at +4 °C. The supernatant was extracted with 500 µl ethylacetate and the organic layer discarded. Carefully avoiding contamination with material from the acid-ethylacetate interphase, the aqueous fraction was transferred to a 5 ml round-bottom flask and freeze-dried. The dry residue was dissolved in 50 µl acetonitril and then treated at room temperature with 100 µl pentafluoropropionic anhydride (PFPA) for 30 min. After transfer to a 3 ml conical centrifuge tube, the solvent was evaporated in a stream of nitrogen and the dry residue was redissolved in 5–10 µl dry acetonitril. Aliquots (2–3 µl) were injected on a GC-column coupled to a mass spectrometer. The column, 150 cm × 2 mm, was packed with OV-17, 3% on Anakrom Q. The column temperature was 160 °C, and the helium flow was 20 ml/min. The retention times were approximately 1.5 min for epinephrine-PFP, 2 min for norepinephrine-PFP, 3.5 min for dopamine-PFP and 4 min for epinine-PFP. The column effluent was subjected to chemical ionization-mass spectrometry using methane as reagent gas. The mass spectrometer was a Finnigan Model 1015C interfaced with a Programmable Multiple Ion Monitor (PROMIM). The β-hydroxylated catecholamine derivatives were monitored by their most abundant fragments: m/e 604 and 607 for epinephrine-PFP and epinephrine-D_3-PFP and m/e 590 and 593 for norepinephrine-PFP and norepinephrine-D_3-PFP. The other amines were estimated from their MH^+ ions: m/e 606 and 609 for epinine-PFP and epinine-D_3-PFP and m/e 592 and 594 for dopamine-PFP and dopamine-D_2-PFP. A standard curve was prepared using known amounts of epinine, epineprine, norepinephrine, dopamine and their deuterated homologues[15]. When hydrolysed partially, norepinephrine-PFP, can also give rise to a response at m/e 606. Under the conditions of the assay this was not observed. When the disubstituted derivative was made by partial hydrolysis of the fully substituted derivative, its retention time on the gas chromatograph was slightly longer (by 10–15 sec) than that of epinine-PFP so that the 2 compounds could be adequately distinguished. The identification of epinine was confirmed by similar gas chromatographic and mass spectrometric behavior of the trifluoroacetic derivatives of epinine, epinine-N-CD_3 and the endogenous amine.

In another experiment, 24 newborn rats received i.p. 2 ml/kg of a saline solution containing 5 mg/ml methionine-CD_3. The rats were killed 20 min later, the superior cervical ganglia removed, and groups of 16 ganglia from 8 animals pooled, homogenized, and the epinine and epinephrine analyzed by GC-MS as described above, but without addition of the deuterated compounds. The carcasses of the animals were homogenized in 0.1 N HCl, and the relative enrichment of the methionine with CD_3 determined by GC-MS[16].

Epinephrine-bitartrate, norepinephrine-tartrate-HCl and dopamine-HCl were purchased from Calbiochem, Los Angeles, Cal., USA; epinine-HCl, from Aldrich Chemical

Co., Inc., Milwaukee, Wisc., USA; methionine-CD_3, epinephrine-α-D_2-β-D_1, norepinephrine-α-D_2-β-D_1 and dopamine-β-D_2 were obtained from Merck, Sharp and Dohme, Kirkland, Quebec, Canada. OV-17, 3% on Anakrom Q, 90–100 mesh, from Analabs, North Haven, Conn., USA; trifluoracetic anhydride and pentafluoropropionic anhydride from Pierce Chemicals, Rockford, Ill., USA. Epinine-N-CD_3 was generously donated by Jansen Farmaceutica (Beerse, Belgium) and α-methyl dopamine was the gift of Dr J. Daly, NIAMD, NIH.

Results and discussion. Epinine in the superior cervical ganglia of rats was detected and identified by GC-MS. The mass spectra of the PFP derivatives of epinine and epinine-CD_3 are shown in figure 1. The amounts of epinine and other amines present in the homogenates of superior cervical ganglia were determined from the relative peak heights of the unlabelled compounds and the added deuterated standards at appropriate retention times for the gas chromatographic columns and m/e in the mass spectra. A typical tracing from the GC-MS recording, after injection of an aliquot of the derivatives derived from the homogenate of sympathetic ganglia to which deuterated standards had been added, is shown in figure 2.

Norepinephrine is the major catecholamine present in sympathetic ganglia (figure 3). The amount of norepinephrine present progressively increases with age as the ganglia and the animal grow in size. Dopamine levels are highest at birth, decline during the next few days, and then increase slightly as the ganglia become larger. The amounts of the N-methylated derivatives of these catecholamines are considerably lower. Epinephrine levels are highest at birth but its levels are maintained only slightly below those found at birth for at least 30 days. Thus the relative amount of epinephrine compared to norepinephrine and ganglia size decreases with age, from 10% of norepinephrine at birth to less than 1% at 30 days of age.

Epinine, the N-methylated derivative of dopamine, is present in very small amounts. Its level is highest at birth, at which time its levels are about half those of epinephrine, and rapidly declines during the first 4 days of life. The amount of epinephrine present is usually less than one-tenth those of dopamine (figure 3). 20 min after i.p. injection of methionine labelled with deuterium on the methyl group (CD_3), labelled epinephrine and epinine are found in the superior cervical ganglia. The relative enrichment of epinephrine with CD_3 approaches that of methionine found in the carcass, but that of epinine is only 4% of epinephrine (table).

The results of these experiments establish that epinine is present in the superior cervical ganglia and can be formed in vivo. The amounts of epinine present, however, are very small, and even at their highest are less than half those of epinephrine. The relatively low enrichment with CD_3 after injection of methionine-CD_3 indicates that the rate of epinine formation in the ganglia is very slow compared to that of epinephrine and that formation of epinephrine from epinine is at most a minor pathway.

Relative enrichment with CD_3 of epinine and epinephrine after administration of methionine-CD_3

	Relative enrichment
Methionine (carcass)	26 ± 3%
Epinephrine (SCG)	19 ± 4.8
Epinine (SCG)	0.74 ± 0.12

Newborn rats received 10 μg/g methionine-CD_3 i.p. The rats were killed after 20 min. The relative enrichments with CD_3 of the indicated compounds were determined by GC-MS.

In vitro binding of citrinin to serum protein[1]

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Summary. In vitro study shows that the mycotoxin citrinin binds to human serum albumin.

The binding of toxins to plasma proteins and subsequent biological activity has been observed earlier[3]. Such observations have been made with the food-borne mycotoxins, aflatoxin and ochratoxin[4–6]. The present communication is a report on the binding of the mycotoxin citrinin to human serum protein.

Material and method. Pure crystalline citrinin (isolated from cultures of Aspergillus candidus or Penicillium citrinum) was used in this study. Human serum was centrifuged out from pooled blood samples of healthy adult volunteers and used fresh. 3 different preparations were then obtained: a) 1 ml of serum was dialysed at 4–7 °C overnight against 1 l of barbitone-sodium barbitone buffer (ionic strength –0.05; pH 8.6) and the dialysed serum was ready for electrophoresis; b) 0.2 ml of chloroform solution of citrinin (200 μg) was taken in a small beaker and dried. To the dry toxin, 1 ml of serum was added and incubated at 37 °C for 2 h with intermittent gentle agitation. This was then dialysed and kept ready for electrophoresis; c) a solution of citrinin was prepared in barbitone buffer (200 μg/ml) just before electrophoretic run. Paper electrophoresis was carried out on a strip (10 × 30 cm) of Whatman No. 3 paper. 10 μl each of a), b) and c) was spotted separately but alongside and placed for electrophoretic run in barbitone buffer at 180 V and 5 mA for $3^1/_2$ h at room temperature (29–31 °C).

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