Synthetic histidine²-angiotensin II analogues and related intermediate peptide fragments

Compound	Yield (%)	Mp. (°C)	$[\alpha]_D^{25}$ values[a]
I[b]	83	245–247	−14.1° (c 2.0, DMF)
II[c,d]	55	213–214	−67.5° (c 1.0, MeOH)
III[e]	91	208–210	−51.0° (c 1.0, MeOH)
IV[e]	65	168–170	−29.4° (c 1.0, AcOH)
V[f]	85	150–151	−48.5° (c 1.0, MeOH)
VI	90	151–154	−44.1° (c 1.0, MeOH)
VII	85	140–145	−31.7° (c 0.5, MeOH)
VIII	86	135–140	
IX	90	156–160	−48.3° (c 0.5, MeOH); −29.7° (c 0.25, DMF)
X	97	152–157	−47.1° (c 0.5, MeOH); −31.1° (c 0.25, DMF)
XI	87	157–159	−49.1° (c 0.5, MeOH); −33.2° (c 0.25, DMF)
XII	95	159–162	−42.3° (c 0.5, MeOH); −28.8° (c 0.25, DMF)

[a] Unless otherwise stated; [b] $[\alpha]_D^{23}$; [c] $[\alpha]_D^{22}$; [d] reported[7] mp 192–194; $[\alpha]_D^{23}$ −66.0° (c 1.0, MeOH); [e] $[\alpha]_D^{24}$; [f] $[\alpha]_D^{20}$.

ified with benzyl alcohol/*p*-toluenesulfonate/benzene to give H(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OBzl as the tri-*p*-toluenesulfonate (VII). The product VII was desalted on a DOWEX 2-X8 column and the resulting heptapeptide ester, H-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl) His-Pro-Phe-OBzl (VIII) was coupled with Z-(β-benzyl)-L-aspartate[9] via the carbodiimide method in DMF solution. The obtained octapeptide derivative, Z-(β-OBzl)-Asp-(im-Bzl) His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OBzl (IX) afforded, by selective catalytic hydrogenation, the desired product, H-Asp-α-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OH (X), which was isolated in pure form by gel filtration on Sephadex LH-20, using MeOH as the eluent.

Finally, the substituted Asp¹-β-His²-Ile⁵-angiotensin II analogue was synthesized by condensation of the heptapeptide benzyl ester VIII with Z-(α-benzyl)-L-aspartate via the carbodiimide method. The resulting octapeptide derivative, Z-(α-OBzl)-Asp-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OBzl (XI), after removal of the N-carbobenzoxy and *O*-benzyl groups by selective catalytic hydrogenation, gave the analogue H-Asp-β-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OH (XII).

In the course of this work, α-benzyl-L-aspartate was synthesized by a new route, using N-trityl-L-aspartate di-benzyl ester (XIII), [mp 103–104°; $[\alpha]_D^{25}$ + 12.8° (c 2,0, CH_2Cl_2)] as the starting material. Due to the steric hindrance of the trityl group[10], the alkaline hydrolysis of the ester XIII proceeds with removal of the β-benzyl group selectively. Thus, the obtained crude product of N-trityl-α-benzyl-L-aspartate (86% yield) was further detritylated with acetic acid[10] to give α-benzyl-L-aspartate[9] in 85% yield. [mp 173–174°; $[\alpha]_D^{25}$ −15.7° (c 5.0, HCl)]. The latter upon carbobenzoxylation gave Z-α-benzyl-L-aspartate[9] [mp 84–85°; $[\alpha]_D^{25}$ −15.1° (c 5.0, acetone)] in 72% yield.

The biological activities of the new synthetic analogues X and XII will be reported in a forth-coming communication. All new compounds, reported here, gave satisfactory elemental analysis.

Summary. The synthesis of histidine²-angiotensin II analogues, namely H-Asp-α-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OH and H-Asp-β-(im-Bzl)His-Val-Tyr-Ile-(im-Bzl)His-Pro-Phe-OH, are described. Also a new route leading to the synthesis of α-benzyl-L-aspartate, using N-trityl-L-aspartate di-benzyl ester as the starting material, is reported.

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Distribution of Glycerophosphorylcholine Diesterase in Rat Brain

The enzyme glycerophosphorylcholine diesterase (L-3-glycerylphosphorylcholine glycerophosphohydrolase EC 3.1.4.2., GPC diesterase) has been shown to occur in liver[1], kidney[2] and brain[3] of mammals. Its action releases choline from glycerophosphorylcholine, which in turn is released by the breakdown of phosphatidylcholine. Its action in the brain could make a substantial contribution to the pool of free choline which has been thought to be the major source of choline for ACh synthesis. Potentially, therefore, GPC diesterase could influence the synthesis of ACh, but if that were the case it would be expected that its regional distribution in the brain would be closely associated with that of ACh and choline acetyltransferase (EC 2.3.1.6). Both regional and subcellular distributions of the enzyme and their relation to choline acetyltransferase are examined in the present paper.

Materials and methods. L-3-glycerophosphorylcholine (1-^{2}^{14}C-choline, GPC), as the cadmium chloride complex was obtained from ICN radiochemicals and was diluted to a suitable specific activity (approx. 400 dpm/nmol)

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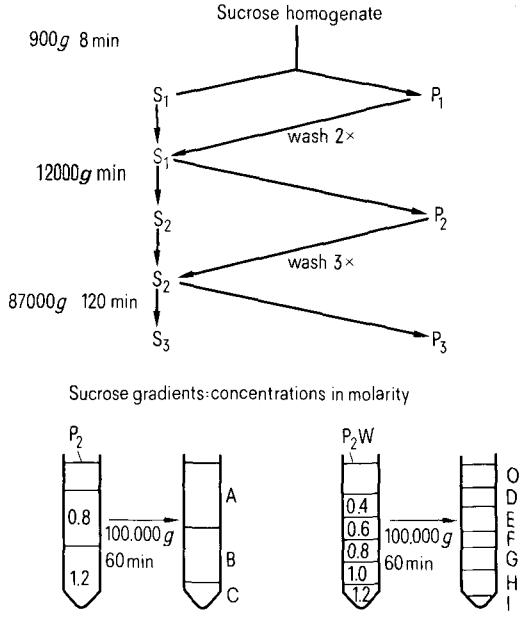
Table I. Distribution of GPC diesterase in regions of the brain

	μmols/g/h	SD (*n*)
Spinal cord	24.3	2.5 (4)
Spinal cord ether treated	31.8	0.43 (3)
Cerebellum	28.6	7.7 (4)
Cerebellum ether treated	25.6	2.0 (3)
Cerebrum	29.9	2.1 (4)
Cerebrum ether treated	35.3	5.8 (4)
Caudate	22.9	5.1 (4)
Caudate ether treated	27.7	7.5 (4)
Cerebral cortex	22.6	7.6 (4)
Human placenta	55.9	24 (4)



Schematic diagram of the subcellular fractionation of sucrose homogenates. The sizes of fractions A to I are to scale.

with unlabelled GPC. The cadmium was removed by passing the GPC through a column of Zerolit 225 resin which retained the cadmium while permitting all the GPC to pass through. The specific activity of this substrate was found by hydrolyzing it and then bioassaying the choline released, using a method modified from BLIGH[4]. Sucrose homogenates (100 mg/ml in 0.32 *M* sucrose) of rat brain were incubated in the presence of 1.0 m*M* $MgCl_2$, 30 m*M* aminomethylpropandiol buffer pH 8.0, and 2.5 m*M* GPC at 39 °C for 10 or 15 min. The reaction was stopped by adding an equal volume of 50 m*M* EDTA. The released choline was then separated from the remaining GPC by passing the incubate (0.1 ml + 0.1 ml EDTA) through a 5 × 0.63 cm column of Zerolit 225 resin in the sodium form. The choline which was bound to the column was eluted with *M* KCl and counted in the premixed scintillator, Unisolve, in a Packard liquid scintillation spectrometer (model No. 3320).

Results. It was found that Mg^{++} ions activated the enzyme but EDTA added directly to the enzyme in a final concentration of 1 m*M* caused an almost complete inhibition. The Michaelis constant (K_m) and optimum pH of this enzyme have been variously reported. Preliminary experiments showed that under the conditions used here the optimum pH was 8.0. A substrate concentration of 2.5 m*M* appeared to be saturating.

The rates of release of choline by homogenates of rat cerebrum, cerebellum, caudate nucleus, cerebral cortex and spinal cord were measured. In addition the samples were incubated after treatment with ether[5] which in most cases gave a small but significant increase in GPC diesterase activity. The values obtained from the different regions ranged with fairly narrow limits from 22 to 35 μmoles/g/h, as shown in Table I.

For comparison, GPC diesterase in homogenates of full term human placentae was also measured. The value obtained (55.9 μmol/g/h) is the highest yet reported for this enzyme, and could account for difficulties encountered by some authors who have used placentae as a source of choline acetyltransferase for assaying choline in tissue extracts[6].

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Table II. Distribution of GPC diesterase in subcellular fractions of the brain

Fraction	GPC diesterase (nmol/g/h)	Recovery (%)		Choline acetyltransferase (nmol/g/h)	Recovery (%)	
P_1	8733	28		811	14	
P_2	12364	38.2		2300	40	
P_3	5676	16.7		684	12	
S_3	5522	16.4		1932	33.9	
A	570	11		375	29.9	
B	2431	62.75		550	43.8	
C	955	26.65		330	26.3	
O	908	25.1		275	19	
D	202	5.5		122	8.5	
E	291	7.65		196	13.6	
F	569	15.9	55.7	231	16	42.8
G	702	19.45		227	15.8	
H	707	20.3[5]		158	11	
I	222	5.65		225	16.0	

Percentage recoveries for each fraction are expressed as a percentage of the amount recovered. Total recoveries were: P_1–S_3, 92.5% for GPC diesterase and 100 for choline acetyltransferase; O–I, 58% for GPC diesterase and 53% for choline acetyltransferase.

Subcellular fractions were prepared from sucrose homogenates of rat cerebrum, according to the procedure summarized in the Figure. P_2, the crude mitochondrial and synaptosome (pinched-off nerve ending) pellet, was resuspended in either 0.32 *M* sucrose or H_2O; in the latter case it was designated P_2W. The fractions obtained by this procedure have been characterized by TUČEK[7]. Subfraction B (obtained from P_2) contains synaptosomes, while O (obtained from P_2W) is soluble material; D contains synaptic vesicles while F, G and H are nerve ending membranes and I, mitochondria.

Each fraction and subfraction was examined for GPC diesterase and choline acetyltransferase activity. The choline acetyltransferase was assayed using the method described by HEBB et al.[8]. The results obtained were expressed as nmols/g/h and are given in Table II. A considerable proportion of GPC diesterase activity was bound to membranes and in particular to nerve-ending membranes; 38% of the total was present in the P_2 fraction. Subfractionation of this showed that 24% (i.e. 63% of P_2) was in the synaptosomes. Fractionation of P_2W showed that much of the activity in the synaptosome was in, or associated with, the nerve-ending membrane. It has already been shown that GPC diesterase in the liver is associated with plasma membranes[9].

GPC diesterase was also incubated in the presence of 10 m*M* hemicholinium-3, an inhibitor of ACh synthesis in vivo, but no inhibition of GPC diesterase was observed even when the substrate concentration was lowered.

Discussion. The assay of GPC diesterase described here is simple and reliable and gives values for brain close to those previously reported. There is, however, a significant difference between the optimum pH and substrate concentration used here and those reported by other authors[1-3]. It has not been possible to establish the reason for this but it may be associated with the Mg^{++} concentration of the original tissue, a question that can only be resolved by using purified enzyme preparations in place of the crude homogenates used in the present work.

The distribution of GPC diesterase in the rat brain is very uniform and is not specifically localized in any area, thus differing from many of the enzymes which are associated with neurotransmitter synthesis. It is, therefore, somewhat surprizing to find that its subcellular distribution is associated with nerve-ending particles, and in this respect its distribution closely resembles that of choline acetyltransferase. This would tend to suggest that the enzyme is associated with the transmitter function of the nerve ending and possibly the nerve-ending membrane. The amount of the enzyme in the brain leaves no doubt that it could be a major contributor to the pool of free choline in the brain. But it could be argued that this pool would then be evenly distributed through the brain like the GPC diesterase. Where this enzyme occurs in cholinergic neurons the choline released could well be utilized for the synthesis of ACh, although some, at least, is likely to be used to form choline phosphate[10], which is a precursor of phosphatidylcholine. The uniform distribution in the brain would tend to indicate that it is present in most if not all nerve endings. In addition to releasing choline this enzyme also releases energy in the form of glycerol-3-phosphate, and this could be of importance in membrane function. The work reported here does not indicate which of these possibilities is the most likely and it may be that the release of choline and a source of energy have an equal importance in the transmitter function of the nerve ending.

Summary. The distribution of glycerylphosphorylcholine diesterase in the rat brain has been examined. The enzyme was evenly distributed throughout the brain but was localized in the synaptosome (nerve ending) fraction which was prepared by ultracentrifugation.

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Regional Distribution of Lactate Dehydrogenase Isoenzymes in Adult Rat Brain

The special functions of different brain parts must be based on specific biochemical differences. Recently[1] we were able to show that there are significant regional differences in levels of substrates and adenine nucleotides in adult rat brain. These differences may be related to different enzymatic patterns of the glycolytic sequence. We have now determined the activity of lactate dehydrogenase (EC 1.1.1.27; LDH) and its isoenzymes in cerebral cortex, thalamus, cerebellar cortex and pons, because the total activity, and especially isoenzymatic pattern of this enzyme, can give useful information about major pathways of glucose utilization[2-4].

Experimental. Pyruvate-Na, α-oxobutyrate and NADH were obtained from Boehringer Mannheim GmbH. DEAE Sephadex A-50 (3.5 mEq/g) was purchased from Pharmacia (Uppsala, Sweden).

The investigations were carried out on adult male Louis rats. Non-anesthetized animals were decapitated, their heads were opened and the brains removed. After weighing, tissue samples were homogenized in ice-cold hypotonic (10 m*M*) potassium phosphate buffer (pH 7.0); the tissue/buffer ratio was 1:50 (w/v). Homogenates were centrifuged at 25,000 × *g* at 0°–4 °C for 1 h. Supernatant solutions were divided into 2 parts: one part was used for the measurement of the total and LDH_1 activity and the other part (0.5 ml) was mixed with 1 ml DEAE Sephadex A-50 (20 mg/ml phosphate buffer, pH 6.0)[5] and allowed to stand for 10 min; resin was removed by centrifugation (4,000 × *g*, 10 min). DEAE Sephadex adsorbs anodic LDH isoenzymes[4,5] i.e. LDH_1 and LDH_2.

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