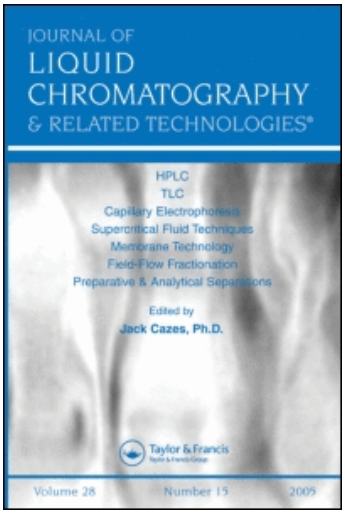

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NON-CODED BIOSYNTHESIS OF N-ACETYLASPARTYL PEPTIDES IN MOUSE BRAIN HOMOGENATES

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ABSTRACT

N-acetylaspartate and labelled glutamate, aspartate, γ-aminobutyrate and taurine were incorporated into a number of low-molecular weight peptides in mouse brain homogenates, as detected by autoradiography on thin-layer chromatography plates. Peptide biosynthesis was dependent on N-acetylaspartate, cAMP, ATP and an ATP regenerating system. The number of peptides varied from 5 to 12 after incubation for 40 min. The original labelled amino acids were determined by re-chromatography of the acid hydrolysates of the peptides, and the probable structures (N-acetylaspartyl-glutamyl-taurine, [N-acetyl]aspartyl-taurine and N-acetylaspartyl[aspartyl,glutamyl]-taurine) were assumed to be present.

INTRODUCTION

At least 30 well-known peptides (excluding hormones) occur in the central nervous system (CNS), and at least as many or slightly more peptides whose structure, function and origin are less clearly understood (1-3). There are two principal biosynthetic groups among the CNS peptides, the first comprising those peptides which are formed through enzymatic degradation (by peptidases)

from larger coded peptides or protein molecules (e.g. endorphins and encephalins), and the second those formed directly from amino acids by the agency of the cytoplasmic peptide synthetases, without any coding by DNA. The biosynthesis of the latter can thus be studied via the incorporation of labelled amino acids, although the amounts involved are generally very small (4-6). γ-Glutamyl-taurine also may be included in this group (7,8). An interesting subgroup of the brain peptides consists of the N-acetylaspartyl peptides (4). We found earlier a couple of acidic, low-molecular weight peptides whose amino acid content and sequence closely resembled those of the N-acetylaspartyl peptides of Reichelt's group, in calf brain nerve ending fractions (9-11), and were able to demonstrate the incorporation of amino acids to a number of N-acetylaspartyl peptides in mouse brain homogenates using labelled amino acids observed to exist in these peptides (12). The present work provides a further characterization of these peptides and this peptide-synthesizing mechanism in mouse brain homogenates.

MATERIAL AND METHODS

The brains of NMRI mice were homogenized immediately after decapitation in 4 volumes of 0.05 mol/l potassium phosphate buffer, pH 7.4, containing 0.1 mol/l KCl (4,13). The incubation medium was essentially that of McIntosh and Cooper (13) and contained (mmol/l) 25 potassium phosphate buffer, pH 7.4, 10 $MgCl_2$, 10 glucose, 5 Na-phosphate buffer, pH 7.4, and 50 KCl, with an addition of 0.5 ATP, 0.1 cAMP, 0.25 creatine phosphate and 20 mg/l creatine kinase. The labelled amino acids used, [^{14}C]aspartic acid (8.4 TBq/mol), [^{14}C]glutamic acid (10.7 TBq/mol), [^{14}C]γ-aminobutyric acid (GABA, 8.6

TBq/mol) and [^{14}C]taurine (4.2 TBq/mol) were products of the Radiochemical Centre, Amersham.

The amino acids N-acetylaspartate (Sigma), glutamate, taurine, aspartate or GABA were each present in a final concentration of 0.25 mmol/l, and the labelled components, except for N-acetylaspartate, were added in concentrations of 9.25 GBq/l. The effects of N-acetylaspartate, cAMP, ATP and the ATP regenerating system on peptide biosynthesis were tested in separate experiments (see Table I). The incubations were performed for 40 min in a 310 K water-bath with shaking. The brains of two mice were used for each incubation. The reaction was

TABLE I

Effect of the Omission of N-acetylaspartic Acid, cAMP, ATP and an ATP Regenerating System on the Biosynthesis of N-acetylaspartyl Peptides from N-acetylaspartate, [^{14}C]glutamic acid, [^{14}C]aspartic acid, [^{14}C]GABA and [^{14}C]taurine.

Omission of	Incorporation into N-acetylaspartyl peptides of [^{14}C]glutamate, [^{14}C]taurine %	Incorporation into N-acetylaspartyl peptides of [^{14}C]aspartate, [^{14}C]GABA, [^{14}C]taurine %
N-acetylaspartate	16	10
ATP, creatine phosphate, creatine kinase	24	32
cAMP	73	66
cAMP, ATP, creatine phosphate, creatine kinase	59	48

Values given as percentages of the corresponding control values, obtained in the presence of all the cofactors indicated. Incorporation was checked by determining the radioactive products on the TLC plates using a Berthold gas counter (LB 1240).

stopped by adding ice-cold perchloric acid to a final concentration of 0.1 mol/l and centrifuging the precipitable proteins. The precipitates were washed three times, and the combined supernatants then neutralized with KOH and the potassium perchlorate centrifuged. The neutral, protein-free extracts were lyophilized and the residue taken up in acidified water and used immediately for thin-layer chromatography (TLC). One fraction was hydrolysed in 6 mol/l HCl at 373 K for 20 h, the HCl then evaporated and the residue taken up again in water.

The two-dimensional thin-layer chromatograms were developed on 0.25 mm-thick silica gel plates (Kieselgel G) with 70 % ethanol and 75 % phenol in water, dried carefully and then submitted to autoradiographic analysis. Some of the dried plates were sprayed with ninhydrin (see ref. 9) and the spots scanned semiquantitatively with a densitometer.

Autoradiography was performed according to Randerath (14). The TLC plates, containing about 740 Bq of $[^{14}C]$, were treated with 7 % 2,5-diphenyloxazol in ether and covered with Kodak X-Omat films, enveloped tightly in black paper and stored at -203 K for one week. The films were then developed with Agfa Rodinal and fixed with Agfa Agefix. The radioactive spots on the TLC plates were also scanned with a Berthold gas counter (LB 1240) using standard samples for the quantitative determination of the label in the spots.

RESULTS

The mouse brain homogenates incorporated labelled aspartic and glutamic acids, GABA and taurine to form a number of hydrolysable compounds. Autoradiographic detection of these compounds was highly sensitive, since even

femto molar amounts of the labelled substances could be localized on the X-films. Peptide biosynthesis was dependent on an energy source (ATP) and an ATP regenerating system and cAMP, and almost entirely dependent on the presence of N-acetylaspartate (Table 1). The residual activity in the absence of exogenous N-acetylaspartate could be explained by its relatively high endogenous concentration (5-6 mmol/kg brain tissue, 15). There is thus good reason to assume that the compounds formed may include several N-acetylaspartyl peptides, although the label could also have been incorporated into other types of peptides.

Visual comparison of the X-films from the hydrolysed and non-hydrolysed samples after incubation revealed 5-12 hydrolysable labelled products and a number of non-hydrolysable products (Table 2), but the concentrations of new radiopositive compounds were in general very low.

TABLE II

Examples of the Numbers of Labelled Products Formed from Different Combinations of the Four Labelled Amino Acids.

Labelled amino acids added	Number of labelled products Hydrolysable	Non-hydrolysable	Formed in hydrolysis
[^{14}C] GABA	12	6	3
[^{14}C] aspartate [^{14}C] taurine	5	2	2
[^{14}C] glutamate [^{14}C] aspartate [^{14}C] taurine	8	4	2
[^{14}C] glutamate [^{14}C] aspartate [^{14}C] GABA [^{14}C] taurine	10	5	3

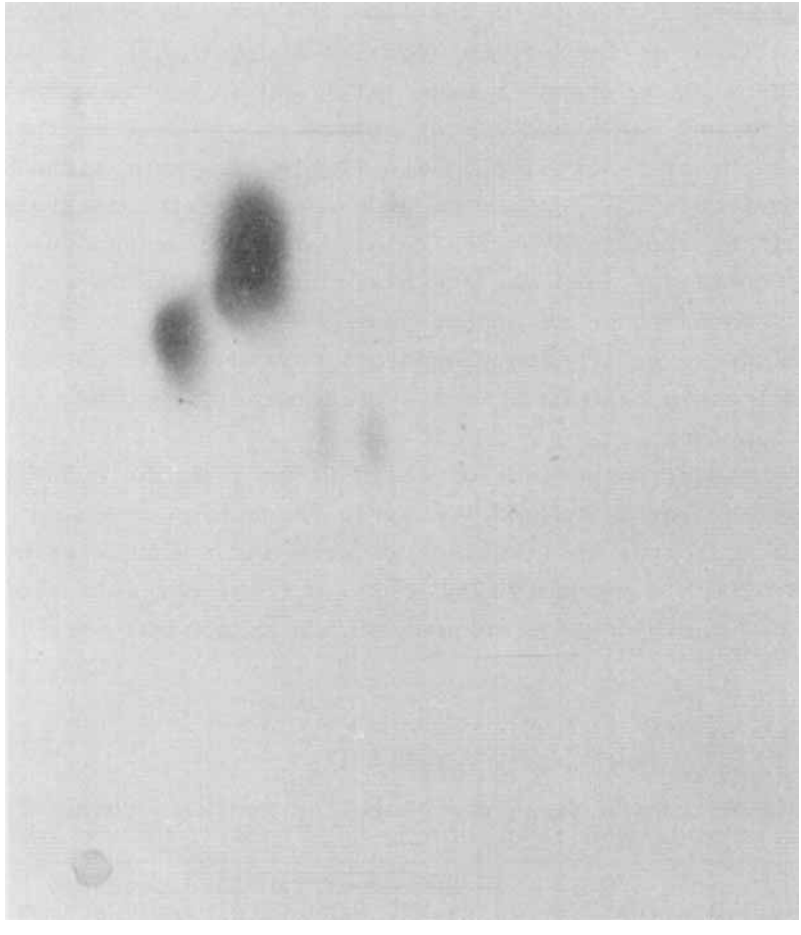


Figure 1. Autoradiographic X-film of the TLC chromatogram from a brain extract after incubation for 40 min at 310 K in the presence of N-acetylaspartate, [^{14}C]taurine, [^{14}C]-glutamate and [^{14}C]aspartate. The TLC plate was covered with an X-film for one week at -203 K in the dark and then developed.

Some labelled products also were formed during acid hydrolysis. Typical results from the X-films are given in Figures 1 and 2. The best results (illustrated in Figure 2 A) were obtained when all four labelled amino

TABLE III

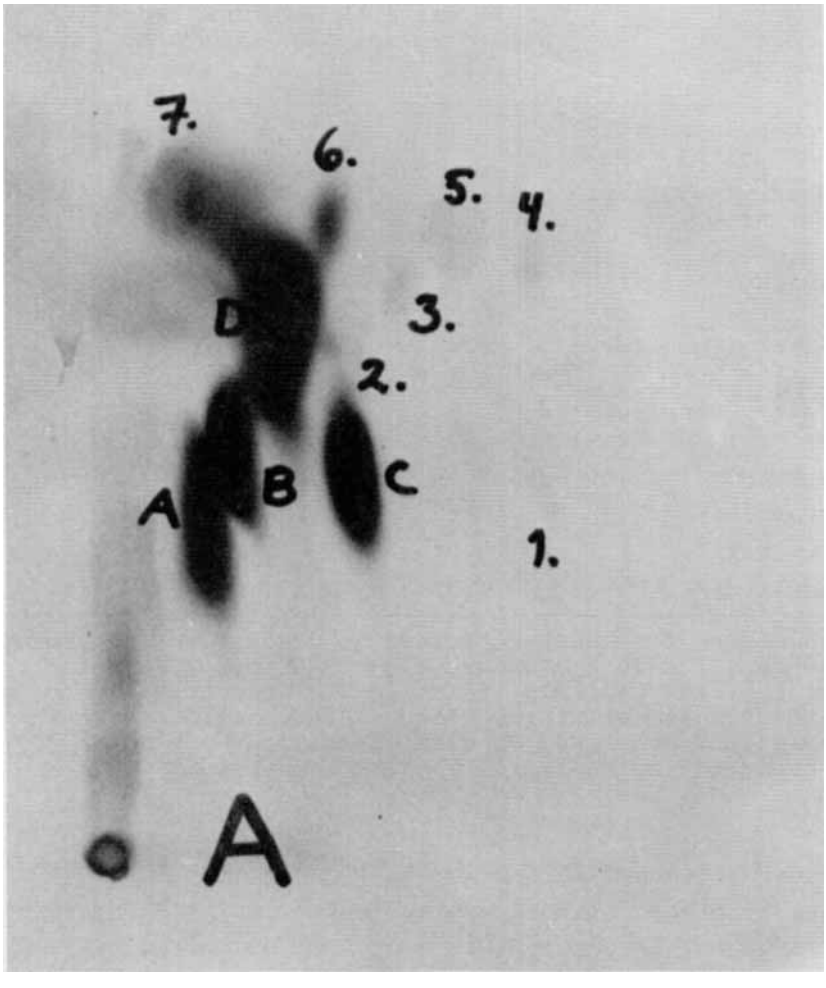
R_f Values for Certain Brain Peptides and Standard Amino Acids Obtained in Two-dimensional TLC.

Compound	R_f value in TLC obtained after ninhydrin reaction		autoradiography	
	R_{f_I}	$R_{f_{II}}$	R_{f_I}	$R_{f_{II}}$
N-acetylaspartyl-glutamyl-taurine	0.75	0.31	0.77	0.32
Aspartyl-glutamyl-taurine	0.80	0.36	-	-
N-acetylaspartyl-taurine	0.84	0.32	0.83	0.19
N-acetylaspartyl-aspartyl-glutamyl-taurine	0.80	0.21	-	-
N-acetylaspartyl-glutaminyl-taurine	0.61	0.33	0.80	0.28
Aspartyl-glutamate	0.66	0.28	0.70	0.40
Aspartate	0.60	0.11	0.54	0.12
Glutamate	0.64	0.20	0.62	0.18
Taurine	0.71	0.23	0.74	0.21

The plates were heat-activated at 378 K for 1 h before the run. I = 70 % ethanol, II = 75 % phenol in water. The peptides and amino acids in TLC were detected by the ninhydrin reaction, the N-acetylated peptides after hydrolysis in 2 mol/l HCl, and in autoradiography after development of the X-films.

acids were added to the incubation mixture, and therefore this special case was subjected to further analysis.

When the hydrolysable compounds on the silica gel plates under the spots on the X-films (Figure 2 A) were scraped down, extracted in water, hydrolysed and rechromatographed, the labelled amino acids formed in acid hydrolysis could be identified using R_f values from standard samples (Table 3). The results from the 7 main spots in Figure 2 A, where all the four amino acids



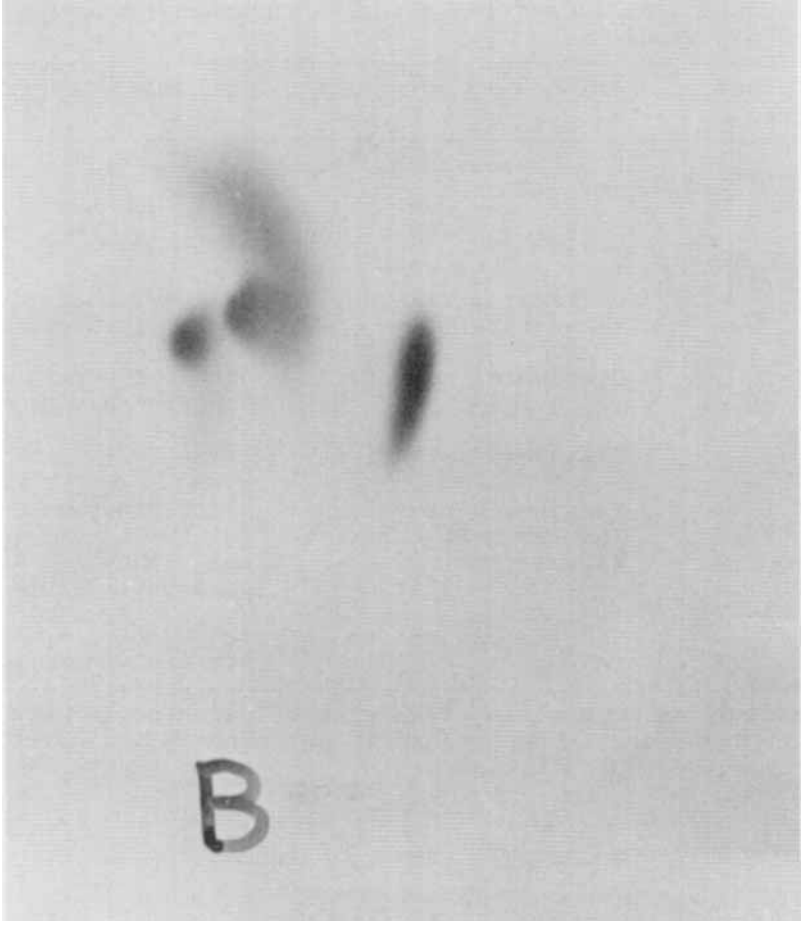


Figure 2. Autoradiographic X-films of an unhydrolysed (A) and a hydrolysed sample (B, opp. page), similar to that in Figure 1, but with four labelled amino acids, [^{14}C] aspartate (=A), [^{14}C] glutamate (=B), [^{14}C] GABA (=C) and [^{14}C] taurine (=D). Figures 1-7 denote hydrolysable compounds. Hydrolysis was performed in 6 mol/l HCl at 373 K for 20 h.

TABLE IV

Amino Acid Composition of the Hydrolysable Products 1-7 of Figure 2 A, Identified after Re-chromatography with Standard Samples.

Compound of Figure 2	Amino acid composition	Probable structure
1	GABA, aspartate, (glutamate)	?
2	Glutamate	Glutamine
3	Glutamate, taurine, (aspartate)	N-acetylaspartyl-glutamyl-taurine
4	Glutamate, aspartate	Glutamyl-aspartate, Aspartyl-glutamate
5	Glutamate, (taurine)	?
6	Taurine, glutamate (glutamine), aspartate (asparagine)	N-acetylaspartyl-(glutamyl [glutaminyl], aspartyl [asparaginyl])-taurine
7	Taurine, aspartate (GABA)	(? N-acetylaspartyl)-aspartyl-taurine

Compounds or structures given without parenthesis were easily reproducible and those in parenthesis indicate alternative possibilities.

(glutamate, aspartate, GABA and taurine) were labelled, are given in Table 4. In some cases a probable structure could be given, based on structural analysis of the corresponding ninhydrin-positive compounds after hydrolysis of the N-acetyl group of the N-terminal aspartic acid with 2 mol/l HCl (16, see also ref. 10), and also using R_f values for the above peptides and some synthetic peptides in some cases (Table 3).

DISCUSSION

Reichelt and Kvamme (4), Reichelt and Edminson (5) and Reichelt et al. (6) reported 30 or more peptide compounds synthesized in the mammalian brain. A large number of these were N-acetylaspartyl peptides, but their exact sequence remained largely unresolved. The peptides studied in our previous work (9-12), whose biosynthesis is reported in more definite terms here, belong to this same group, and the possibility of the presence of γ-glutamyl-taurine (7,8) is not excluded.

The peptides in this group have some properties in common: 1) they are relatively small, having in general 2-6 amino acid residues, but only a few amino acids (glutamate, aspartate, glycine, serine) occur, appearing in various sequences, 2) they are in general acidic compounds (cf. ref. 9) and 3) they include the only known peptide structures in which GABA and taurine occur. The exact structure of these compounds is still largely unknown, because of their very low concentrations and the large number of different molecules in the brain or in brain subfractions.

The biosynthesis of these peptides required the addition of extra N-acetylaspartate, a feature which can also be seen indirectly in the paper of Reichelt and Kvamme (4). Omission of the N-acetylaspartate from the incubation mixture effectively prevented peptide biosynthesis, and there is thus good reason to assume that the peptides formed may be largely of the N-acetylaspartyl type. ATP and an ATP regenerating system also were essential for synthesis, but the omission of cAMP caused only a slight reduction in biosynthetic ability.

The number of radiopositive hydrolysable compounds formed during incubation for 40 min varied from 5 to 12

when different combinations of labelled amino acids were used, but the results were adequately reproducible with a constant amino acid pool. The non-hydrolysable products were apparently metabolic derivatives of the labelled amino acids, e.g. succinic acid, which was identified among the products formed from [^{14}C]GABA. The acid hydrolysis also produced some unidentified labelled products, which may be amines, carboxylic acids or other degradation products.

Determination of the structure of the peptides was hampered by the very low concentration of the radiopositive compounds (cf. ref. 5). Pico or femto molar amounts lie far below the sensitivity limit of the ninhydrin reaction or dansylation procedure. The estimated structures presented here were arrived at on the basis of the very large amount of work done at the nanomolar level upon structurally analogous compounds after the ninhydrin reaction (10,12), and are therefore very tentative, and offer only some indication of the actual situation.

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