A relationship of n-acetylaspartate biosynthesis to neuronal protein synthesis / D. D. Clarke, S. Greenfield, E. Dicker, L. J. Tirri, and E. J. Ronan Chemistry Department, Fordham University, Bronx, NY 10458, U.S.A.

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A preliminary report was presented at the 1972 Meeting of The American Society for Neurochemistry, Seattle, Washington.

This investigation was supported by Research Grant NS-07890, National Institutes of Health, Public Health Service. Dr. Ronan is recipient of NASA Traineeship NS-07890.

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Abbreviations used: NAA, N-acetylaspartic acid; TCA, trichloroacetic acid; HOAc, acetic acid.

ALTHOUGH N-acetylaspartate (NAA) has been found to occur in large quantities in brain, 5-7 μmol/g (TALLAN et al., 1956; TALLAN, 1957; GEBHARD & VELDSTRA, 1964), its functional and metabolic importance is as yet unclarified. Many investigators reported that within short periods of time after the administration of labelled precursors, very little radioactivity is incorporated into the aspartyl moiety of NAA, as compared to that in other amino acids, suggesting that endogenous NAA might be metabolically inert (JACOBSON, 1958; MARGOLIS et al., 1960; BERL et al., 1961; O’NEAL & KOEPPE, 1966; REICHELT & KVAMME, 1967). However, the slow labelling of NAA indicated to us that (1) NAA is not metabolically inert and (2) its metabolism is not directly related to that of free aspartic acid, but rather to that of protein bound aspartate.

In accordance with this hypothesis, we investigated the incorporation of label from sodium-[1-14C]acetate, [1-14C]ethanol, and [2-14C]glucose into the aspartyl moiety of NAA for time periods up to 2 h. We also sought evidence for the existence of an enzyme system capable of synthesizing NAA-t-RNA which would relate the biosynthesis of NAA to that of protein bound aspartate.

MATERIALS AND METHODS

In vivo experiments

Female albino mice (25-35 g, CF-1 strain, Carworth Laboratories; Becton & Dickinson & Co., New City, NY) were fasted for 18 h, given water ad libitum, and then injected with 15 μCi of a labelled precursor. At predetermined times after the administration of either sodium-[1-14C]acetate, [1-14C]ethanol, or [2-14C]glucose, (S.A. 5-6 mCi/mmol), obtained from New England Nuclear, Boston, MA, the animals were rapidly frozen by being dropped head first into isopentane, cooled at −150°C by liquid nitrogen. Each brain was removed, weighed, and homogenized with three aliquots of 5% trichloroacetic acid (TCA), 3, 1, and 1 ml respectively. The homogenates were centrifuged for 15 min at 50,000 g. The supernatants containing the free amino acids and small peptides were neutralized to pH 7.0 ± 0.2, and brought to a volume of 7-5 ml with deionized water. During the whole procedure the samples were kept between 0-4°C.

Aliquots of these neutralized samples were chromatographed on AG 1 × 4 columns (BioRad Laboratories,
Richmond, CA), acetate form, by a modified procedure of Berl et al. (1968) (Table 1). NAA was eluted with 0.1 N HCl, and then hydrolysed in 2 N-HCl at 100°C. Each sample was dried, neutralized to pH 7.0 ± 0.2, brought to a volume of 2.5 ml, and 2.0 ml was chromatographed on another AG 1 × 4 column. The aspartate moiety of NAA was eluted with 0.3 N-acetic acid. Concentrations of the amino acid were determined by the ninhydrin method of Moore & Stein (1954), on a Technicon Autoanalyzer, using the sample plate technique. In each experiment a mixture of standard amino acids and peptides was chromatographed in parallel, thus resolution and recoveries checked. The radioactivity in a separately measured aliquot was determined by liquid scintillation spectrometry. After exhaustive dialysis for 72 h, the dialysis residue containing t-RNA's was chromatographed on a column of Bio-Gel P-10 (20 cm), eluted with homogenizing solution, and the u.v. spectra at 260 nm was taken for each fraction. The radioactivity of each fraction was determined on a separate aliquot by liquid scintillation spectrometry.

**Isolation of N-[1-14C] acetyl aspartic acid**

The dialysis residues suspected to contain NAA-t-RNA, were hydrolysed with ammonium hydroxide, pH 10.5 (Press et al., 1959), for 20 min at 37°C, lyophilized, and made up to a volume of 30 ml with deionized water. These solutions were then chromatographed on AG 1 × 4 columns, acetate form. NAA was eluted with 0.1 N-HCl (Table 1) and the radioactivity determined by liquid scintillation spectrometry.

**Distillation of volatile radioactivity**

The fraction eluted, from the above column, which was known to contain NAA from calibration studies with authentic NAA, was hydrolysed with 2 N NaOH for 2 h at 95°C. To this hydrolysate was added 1 ml of concentrated sulphuric acid and 1 ml of glacial acetic acid to act as carrier, prior to distillation. The resulting acetic acid-H₂O distillate, was counted by liquid scintillation spectrometry.

**Electrophoretic procedure**

Dialysis residues containing t-RNA's were hydrolysed with ammonium hydroxide as previously described, lyophilized and reconstituted with deionized water. Aliquots were then chromatographed electrophoretically on parallel Whatman No. 3 paper strips at 2°C for 1.5 h at 1000 V in 0.01 M-formate buffer pH 2.85. NAA was located with bromcresol green (Smith, 1960) and the parallel strip was covered with Ilford Industrial X-Ray film. After 30 days, the film was developed.

**Protein and nucleic acid estimations**

Protein concentrations were estimated by the method of Warburg & Christian (1941), by measuring extinctions at 260 nm and 280 nm.

<table>
<thead>
<tr>
<th>Neutrals 0.04 N-HOAc</th>
<th>Glutamate 0.1 N-HOAc</th>
<th>Aspartate 0.3 N-HOAc</th>
<th>NAA 0.1 N-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 2.0 ml</td>
<td>A 4.0 ml</td>
<td>A 3.0 ml</td>
<td>A 5.0 ml</td>
</tr>
<tr>
<td>B* 5.0 ml</td>
<td>B 1.0 ml</td>
<td>B* 6.0 ml</td>
<td>B 3.0 ml</td>
</tr>
<tr>
<td>C 1.0 ml</td>
<td>C* 5.0 ml</td>
<td>C 2.0 ml</td>
<td>C* 6.0 ml</td>
</tr>
<tr>
<td>D 2.0 ml</td>
<td>D 2.0 ml</td>
<td>D 3.0 ml</td>
<td>D 2.0 ml</td>
</tr>
</tbody>
</table>

This elution scheme for AG 1 × 4; acetate form, is a modification of that reported by Berl et al. (1968). In those experiments where the quantification of the aspartyl moiety of NAA is desired, the major fraction*, C, of 0.1 N-HCl is hydrolysied in 2 N-HCl at 100°C (see Methods) and the neutralized hydrolysate is chromatographed on another AG 1 × 4 column.
The biosynthesis of N-acetylaspartate

Nucleic acid concentrations were estimated by using the extinction coefficient of \( E_{260}^{\text{nm}} = 0.023 \) at 260 nm.

RESULTS

After the administration of each labelled precursor, the specific activity of aspartate increased rapidly with time reaching a peak within 15-20 min and then fell sharply. On the other hand, the specific activity of the aspartyl moiety of NAA rose very slowly and continued to rise even after the specific activity of free aspartate had declined to less than 1 per cent of the peak values (Figs. 1a–c).

Incubation of a rat brain microsomal supernatant fraction with sodium-[1-\( ^{14} \)C]acetate resulted in the incorporation of radioactivity into a non-dialysable fraction, which cochromatographed with the soluble 260 nm absorbing material (Fig. 2). The coincidence of the 2 curves indicated that the radioactivity is probably associated with the high molecular weight 260 nm absorbing material, t-RNA.

The radioactivity was released from the 260 nm absorbing material by mild alkaline hydrolysis with \( \text{NH}_4\text{OH} \) (Preiss et al., 1959) and was shown to migrate electrophoretically with authentic NAA (Fig. 3). A darkened band appeared on the developed film at 3.35 cm toward the cathode which coincided with the known location of NAA.

In other experiments the fractions containing the released radioactivity were lyophilized and then chromatographed on AG 1 \( \times \) 4 columns (Fig. 4). The fraction which eluted with 0.1 N-HCl, which is known to contain NAA was hydrolysed with concentrated NaOH. After acidification and steam distillation, the radioactivity was shown to be volatile, i.e. it was found in the acetic acid–\( \text{H}_2\text{O} \) distillate. Of the 1550 c.p.m.
Table 2. The effect of heat-treatment on the incorporation of sodium-[1-14C]acetate into NAA by rat brain supernatant

<table>
<thead>
<tr>
<th>Sample</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>'100,000 g' extract, heated</td>
<td>29</td>
</tr>
<tr>
<td>'100,000 g' extract, unheated</td>
<td>758</td>
</tr>
</tbody>
</table>

Prior to incubation with sodium-[1-14C]acetate, the unheated sample was maintained between 0 and 4°C while the experimental sample was heated for 10 min at 95°C. Then 3.75 µCi of sodium-[1-14C]acetate was added to each sample and they were incubated at 37°C for 20 min. The reactions were stopped by the addition of 2 ml of H2O-saturated phenol. After dialysis and mild alkaline hydrolysis (see Methods) the fraction containing NAA was separated on AG 1 × 4, acetate form (Table 1) and the radioactivity was determined by liquid scintillation spectroscopy, and expressed as c.p.m. above background.

Table 3. The effect of the addition of heated extract on the incorporation of sodium-[1-14C]acetate into NAA by rat brain supernatant

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg Nucleic acid added</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.68</td>
<td>261</td>
</tr>
<tr>
<td>2</td>
<td>1.36</td>
<td>393</td>
</tr>
<tr>
<td>3</td>
<td>2.04</td>
<td>441</td>
</tr>
<tr>
<td>4</td>
<td>2.73</td>
<td>561</td>
</tr>
<tr>
<td>5</td>
<td>4.77</td>
<td>648</td>
</tr>
</tbody>
</table>

The initial protein concentration of each sample was 3-40 mg. The samples were maintained between 0 and 4°C prior to incubation. Increasing amounts of heat treated extract were then added to samples 2-5, and incubations were performed with sodium-[1-14C]acetate as described in the legend of Table 2.

Table 4. The effect of the addition of coenzyme-A on the incorporation of sodium-[1-14C]acetate into NAA by rat brain supernatant

<table>
<thead>
<tr>
<th>Sample</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3256</td>
</tr>
<tr>
<td>2</td>
<td>2036</td>
</tr>
</tbody>
</table>

Each sample contained 10.4 mg protein, and 1.5 mg nucleic acid. Prior to the incubation with sodium-[1-14C]acetate, 12.5 µg Co-A was added, and incubated as described in the legend of Table 2.

Table 5. The incorporation of [U-14C]aspartate and sodium-[3H]acetate into NAA by rat brain supernatant

<table>
<thead>
<tr>
<th>Moiety</th>
<th>d.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-14C]Aspartate</td>
<td>335</td>
</tr>
<tr>
<td>[3H]Acetate</td>
<td>57,135</td>
</tr>
</tbody>
</table>

The incubation and work-up procedure is as previously described (Table 2) except, 4.5 µCi [3H]acetate and 0.6 µCi [U-14C]aspartate as used.
ment was also performed, using tritiated acetate and [U-14C]aspartic acid. Both of these labelled compounds were incorporated and found in the NAA fraction (Table 5).

DISCUSSION

It has been reported previously by us as well as by other investigators, that there is low incorporation of radioactive activity from a variety of labelled precursors into the aspartyl moiety of NAA (JACOBSON, 1958; MAR-GOLIS et al., 1960; BERL et al., 1961; O'NEAL & KOEPPE, 1966; REICHELT & KVAMME, 1967; CLARKE et al., 1972). In addition we found that this incorporation increases slowly with time and continues to rise, up to 2 h. In sharp contrast to this, the specific activity of free aspartate peaks rapidly and then falls to very low values within the first 30 min of the experiment (Figs. 1a-c).

Considering the precursor-product relationships of ZIELVERSMDT et al. (1943), and GOODMAN & NOBLE (1968), our data suggest that the free pool or pools of aspartate could not be the direct precursor of the aspartyl moiety of NAA. Rather, by relating NAA to the large pool of protein bound aspartate, the observed labelling pattern could be explained.

The time required for the synthesis of polypeptides and their degradation would be consistent with the appearance of significant label in the aspartyl moiety of NAA only after 30 min to 1 h following the administration of the labelled precursor. To relate the biosynthesis of NAA directly to the protein bound aspartate, two possibilities can be conceived. First, that the activated aspartyl-t-RNA is N-acylated to yield NAA-t-RNA prior to its insertion into the polypeptide, and alternatively, that aspartate is N-acylated after the completion of the polypeptide or during its degradation. We have presented some direct evidence for the existence of NAA-t-RNA. A rat brain microsomal-free supernatant preparation, containing mixed t-RNA's, incorporated label from sodium-[1-14C]acetate into a form that was not washed out by prolonged dialysis. The incorporated radioactive co-chromatographed with the 260 nm absorbing material which is consistent with NAA-t-RNA formation (Fig. 2).

After mild hydrolysis with NH4OH (PRESS et al., 1959), the liberated radioactive was found in the acidic fraction which eluted with NAA on an AG 1 × 4 acetate column (Fig. 4). Also, on paper electrophoresis, it migrated to the same distance as authentic NAA (Fig. 3).

The volatile nature of the radioactivity after hydrolysis with NaOH, suggested that the label was located in the acetyl moiety of NAA (see Results). An experiment with tritiated acetate and [U-14C]aspartate, gave evidence that NAA was indeed synthesized (Table 5). Heating the supernatant prior to incubation with labelled acetate, resulting in virtually complete loss of incorporating activity (Table 2). The addition of heated extract to unheated material stimulated the incorporation of labelled acetate into non-dialysable form, which, after mild hydrolysis with NH4OH, was eluted with NAA on AG 1 × 4 columns (Table 3). The stimulatory effect of co-enzyme A (Table 4) along with the heating experiments gives strong support to the enzymatic formation of NAA-t-RNA.

Thus our data present a strong argument for the postulate that the N-acylation of aspartate occurs on aspartyl-t-RNA. However we cannot completely rule out the possibility, that to some extent, aspartate is N-acylated after the completion of the polypeptide chain. Therefore the in vivo degradation of NAA-t-RNA as well as proteinaceous material would liberate labelled NAA from the TCA insoluble fraction into the soluble amino acid pool, where it can be detected by our experimental methods.

Other brain preparations from cat (KNIZLEY, 1967) and rat (GOLDSTEIN, 1969) demonstrated the enzymatic synthesis of NAA. In these procedures the reactions were stopped by the addition of dilute perchloric acid and the acid was then removed by the addition of potassium hydroxide. This treatment with base, could have liberated newly synthesized NAA from NAA-t-RNA (PRESS et al., 1959), and not give any indication that NAA-t-RNA was in fact, first synthesized. In view of this base treatment our results are consistent with what has been previously reported in the literature.

A SPECULATIVE FUNCTION FOR NAA-t-RNA

Our data demonstrate that NAA-t-RNA formation is truly enzymatic and by relating the biosynthesis of NAA to that of aspartate, bound in polypeptides or aspartyl-t-RNA the results of the in vivo labelling experiments can be rationalized.

From our data the postulate that N-acylation of aspartate occurs on aspartyl-t-RNA suggests that NAA-t-RNA might be an initiator of protein synthesis, since an N-blocked amino acid could only be incorporated into a protein or a polypeptide chain at the N-terminus. The fact that N-blocked amino acids may serve as chain initiators has been shown in bacteria by MARKER & SANGER (1964). They showed that methionine, after binding with its specific t-RNA is formulated and used as a chain initiator. In mammalian systems LIEUW (1970) demonstrated that N-acetyl-seryl-t-RNA participated in initiation of histones. More recently review articles by LUCAS-LENARD & LIPMANN (1971) and HASSELKORN & ROTHMAN-DENES (1973) discussed the role of methionyl-t-RNA as an initiator of protein synthesis in isolated reticulocytes.
Once NAA has initiated protein synthesis and the polypeptide chain is completed, NAA may be cleaved from the polypeptide chain in a similar way as the N-formyl-methionine initiator in E. coli (Wallner, 1963). Proteins which lose their N-acylated terminal groups would therefore be the source of endogenous NAA. The finding of acetylated peptides in brain of various species such as N-acetyl-t-aspartyl-glutamate (Reichelt & Kvamme, 1967; Curatolo et al., 1965; Auditoire et al., 1966; Miyamoto et al., 1966) suggest that this deacylase activity is localized in glia (1972), as well as van den Berg (1972) presented evidence that the small compartment is probably associated with NAA-histamine complex intermediate. However the mechanism for the formation of this complex is unknown. This NAA-histamine complex would not be consistent with our in vivo isotopic labelling data if it were in equilibrium with the free pool of NAA. If NAA-t-RNA is involved with the histamine dependent synthesis of N-acetyl-aspartyl-glutamate then the inconsistencies would be reconciled. The pH dependent recovery of NAA observed and reported by Reichelt et al. (1971) would suggest that NAA-t-RNA is indeed involved. They observed that the levels of NAA were lowered by the presence of histamine and that they would return to near normal if accidental overtitration with potassium hydroxide had occurred.

It is well known that NAA is present in high concentrations in brain of most vertebrates, while only trace amounts can be detected in other tissues. Since metabolism of NAA is very fast in non-nervous tissue (Benuck & D'Adamo, 1968), this would explain the failure to find NAA in those tissues without ruling out the possibility that it was in fact synthesized.

Nadler & Cooper (1972) have suggested that NAA is largely localized in neurons. This has been based on its distribution in grey vs white matter (Tallan, 1957), as well as in different types of tumors. Nadler & Cooper (1971), also observed that N-acetyl-[U-14C]aspartate is rapidly metabolized in brain tissue. Furthermore this labelled aspartyl moiety resulted in glutamate having a higher specific activity than glutamate. In other words it is metabolized via the 'small compartment' of the citric acid cycle. Balazs et al. (1972), as well as van den Berg (1972) presented evidence that the small compartment is probably associated with glia.

Evidently there must be deacylase activity in brain to explain the observations of Nadler and Cooper. In addition, D'Adamo's laboratory has reported direct measurements of this activity in brain (1973). If we assume that this deacylase activity is localized in glia and is absent from neurons, the high levels of NAA in brain as well as the rapid metabolism of exogenous NAA can be explained.

NAA has been found to be the terminal amino acid in certain proteins which are not found in neuronal tissue, for example, G-Actin (Gaetjens & Barany, 1966). This may be an indication that NAA-t-RNA functions as a chain initiator not only in neurons but also in glia and other non-neuronal cells; however, due to the high deacylase activity, free NAA would not be detected in these tissues.

Acknowledgement—The authors wish to express their gratitude to Dr. W. T. Norton for his constructive criticisms, and comments in preparing this manuscript, and to Ms. Renee Sasso for typing this manuscript.

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