Studies on the Role of *N*-Acetylaspartic Acid in Mammalian Brain

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ABSTRACT *N*-Acetylaspartic acid (NAA) occurs at relatively high concentrations exclusively in the mammalian and avian brain and undergoes rapid rise in level soon after birth (Tallan, 1957).

The amount of NAA in brains of mentally abnormal human beings and of young human beings was measured. The route by which NAA is synthesized was shown to involve a direct acetylation of aspartic acid. The degradative activity of the brain toward NAA is slight. Some experiments indicate that NAA in the brain is a physiologically and metabolically active compound.

INTRODUCTION

Recently the occurrence of *N*-acetylaspartic acid (NAA) in the brain of birds and mammals was reported and it was shown that this compound increases in concentration five to six times in the early days of a rat's life (Tallan, 1957). These observations are of interest in the study of the brain since they may give an insight into some aspects of the chemical and physiological development of this organ. Thus, there are instances in which an amino acid is associated with some specific brain function or abnormality. Tryptophan gives rise to serotonin (Udenfriend *et al.*, 1957); the product of dihydroxyphenylalanine decarboxylation gives rise to noradrenaline (Holtz and Westerman, 1956); and phenylalanine itself is involved in phenylketonuria (Wright and Tarjan, 1957).

In an effort to understand the importance of NAA the following questions were posed. (1) Is there a correlation between NAA content of human brain and mental deficiency? (2) Because the content of NAA increases markedly during development, what is the stability of NAA? (3) How is NAA made in the brain?

In order to answer these questions, the NAA content of human brains of

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various ages was measured. Also the stability of NAA was assessed through experiments with rabbit brains which, thus, were found to afford a useful material for studying the synthesis of NAA.

RESULTS AND DISCUSSION

NAA IN HUMAN AND RABBIT BRAIN AT VARIOUS AGES It was shown by Tallan (1957) that a considerable increase in the amount of NAA in the rat brain occurs during the 2nd and 3rd weeks of life. The values obtained in the present work for rabbit brain are reported in Fig. 1. It is seen that there is an increase from about 20 mg. per 100 gm. of tissue to about 95 mg. per 100 gm. of tissue during the first 2.5 weeks of life in the rabbit.

The amounts of NAA in human brain as determined in this investigation are given in Table I. The first twelve values are those for human beings be-

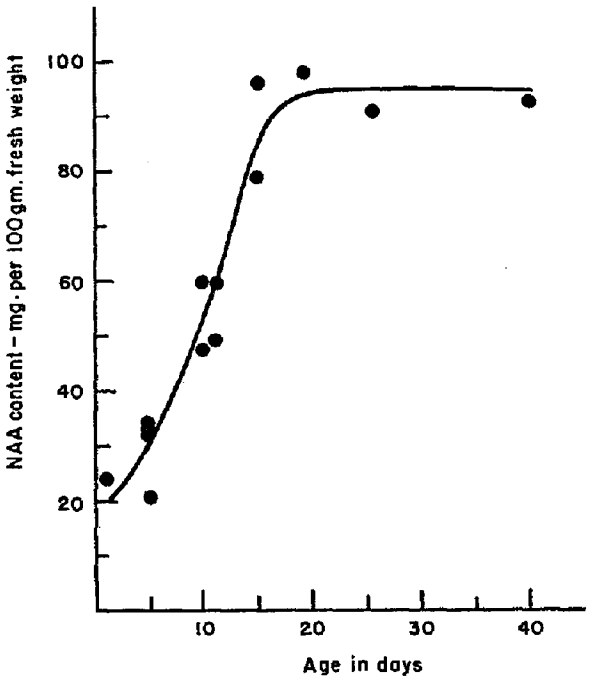

FIGURE 1. The NAA content of rabbit brains as a function of postnatal age. The NAA was determined chromatographically after extraction with 5 per cent TCA.

lieved to be mentally normal. There seems to be some increase with increasing age: the average for the first eight values (subjects not over 15 days old at time of death) is 47 mg. per 100 gm. brain, and that for the other four (between 42 days and 61 years old) is 76 mg. per 100 gm. brain. From these

results it may be concluded that there is probably some increase in the NAA level in human beings during the first few weeks of life, but that it is not as great as for rats and rabbits.

NAA IN MENTALLY DEFICIENT HUMAN BRAIN The mentally deficient human beings whose brains were studied were between 4 and 26 years of age at the time of death.

The amounts of NAA found in the brains of the mentally deficient human beings varied widely, from 33.7 to 128.1 mg./100 gm. brain. The average, 83,

TABLE I

NAA IN HUMAN BRAIN AT VARIOUS AGES

Subject	Age	NAA in parietal lobe mg./100 gm. brain	NAA in parietal lobe μg./mg. protein
Normal	−11 hrs.*	38.9	5.8
	¼ hr.	62.5	11.9
	4 hrs.	57.2	9.1
	18 "	36.6	7.6
	24 "	41.4	7.5
	6 days	40.8	7.0
	14 "	54.0	8.1
	15 "	42.9	10.1
	42 "	57.8	8.4
	90 "	69.5	10.0
	3 yrs.	100	9.2
	61 "	75.7	—
Mentally deficient	4 yrs.	105	9.4
	7 "	75	10.7
	9 "	67.6	10.8
	9 "	128.1	15.0
	11 "	69.4	9.3
	16 "	99.0	12.6
	26 "	33.7	4.8

* This subject was removed from the uterus approximately 11 hours prior to the time when normal delivery was expected.

is probably not significantly different from the average found for normal human beings. It seems not unlikely that the great difference found for the mentally deficient individuals is significant, but the significance is not evident from the information available at present.

The members of the mentally deficient group were all diagnosed as having an undifferentiated mental defect.

The variation noted in the brains in the group of mentally deficient could have resulted from an unusual amount of degradation of NAA in some instances. The stability of NAA was evaluated in the brains showing extremely high and low NAA levels (the 9 and the 26 year old). Duplicate values showed an average of 134 and 44 mg. per 100 gm. after 3 months storage at −20° as

compared to 128 and 34 mg. per 100 gm. immediately after the brain had been sampled. Homogenization of the 26 year old's brain sample and incubation for 15 minutes at 37° (60 μg. NAA, 1 gm. gray matter of frontal lobe in total volume of 5 ml.) resulted in the loss of 11 per cent of the NAA. Similar treatment of another brain showed 6 per cent loss of NAA. These results indicate that no appreciable errors were introduced by the sampling procedure, but they do not explain the low level in the 26 year old's brain.

STABILITY OF NAA IN RABBIT BRAIN In examining rabbit brain for the stability of NAA the organ was divided into sagittal halves, and one half was used as the control for individual experiments. These experiments showed that the NAA content of the brain did not change more than 5 to 10 per cent while stored at −10° for several days or while a minced half-brain was kept a 25° for 1 hour. Normal levels of NAA (90 to 105 mg. per 100 gm. brain) were also found in the brain whether the entire rabbit was stored at 4° for 24 hours after death, then sampled and analyzed or whether the brain was removed immediately and stored about 2 weeks at −20° before analysis. These data were obtained prior to the analyses of human brain; they extend the observation on the stability of NAA in brain to another species.

STABILITY OF NAA DURING HYPOGLYCEMIA Under the extreme stress of convulsions induced by insulin, the content of NAA is unaltered. Thus, Cravioto *et al.* (1951) reported that the aspartic acid content of the rat brain rises several times over the normal level in a hypoglycemic animal (insulin-induced). This was confirmed when adult Sprague-Dawley rats that had died in convulsions 3 hours after receiving 300 units of insulin per kilo of body weight were shown to contain three times the normal amount of aspartic acid in their brains (Table II). However, essentially no change had occurred in the NAA level. Thus it seems that glutamic acid is a preferred energy source in the hypoglycemic brain and that NAA, which occurs at about three-fourths of the concentration of glutamic acid plus glutamine, is not utilized. On the other hand, the shortage of acetyl groups during hypoglycemia may account for the lack of an increase in NAA which would be expected if NAA results from the acetylation of aspartic acid. Since the NAA content of the brain did not change, either NAA may play a vital role, carefully protected metabolically, or it is so far off the main pathways that it is not metabolically functional. Some indication that the latter is not the case will be presented. It may be added that hypoglycemic mice showed a 20 per cent rise in aspartic acid, as compared to 320 per cent in rats.

HYDROLYSIS OF NAA Examination of the hydrolytic activity toward NAA was made by incubating 75 mg. of an acetone powder of adult rabbit brain with 1 mg. of NAA in 2 ml. at 37° (pH 8.2). The release of aspartic acid as measured by the enzymatic procedure was linear over a period of 4 hours, at which time 54 per cent had hydrolyzed. This amount of brain is large in

comparison to that usually employed and indicates that the hydrolysis of NAA should not interfere seriously with the study of NAA formation.

INCORPORATION OF C^{14} PRECURSORS INTO NAA IN VIVO To demonstrate that the mouse maintains the cerebral NAA in a metabolically active state, uniformly labelled C^{14}-glucose was administered by intracerebral injection (Haley and McCormick, 1957). In one mouse, three hourly injections were

TABLE II
RAT BRAIN IN HYPOGLYCEMIA

	Aspartic	NAA	Glutamic + aspartic
Control	2.44	7.27	11.75
	2.72	6.52	9.49
	—	—	11.32
Insulin-treated	8.10	6.87	12.31
	8.50	7.03	12.75
	—	—	10.42

Values given as micromoles/gram fresh weight of brain. The aspartic acid was measured enzymatically and the NAA chromatographically. The value for micromoles of glutamic + aspartic was obtained by using a "molecular weight" of 143 for the controls and 137 for the insulin-treated; these amino acids appear in a common chromatographic fraction.

given and, in another mouse, four; the brains were extracted with 5 per cent trichloroacetic acid 1 and 5 hours, respectively, after the last injection. The specific activity of the NAA fraction obtained from chromatography is shown in Table III. Also shown are the results from the intracerebral administration of one dose of 1-C^{14}-sodium acetate given 1 hour before death. These data

TABLE III
INCORPORATION OF C^{14} INTO NAA BY MOUSE BRAIN IN VIVO

Source of C^{14}	NAA				Glutamic-aspartic Total c.p.m.
	Total μmol	Total c.p.m.	$\frac{\text{c.p.m.}}{\mu\text{mol}}$	$\frac{\mu\text{mol}}{\text{gm. brain}}$	
1. UL-C^{14}-glucose 3 injections	2.33	1490	640	5.30	1734
2. UL-C^{14}-glucose 4 injections	1.91	1355	710	4.57	732
3. 1-C^{14}-acetate	2.32	1025	442	6.11	—
4. 1-C^{14}-acetate	1.94	1123	578	5.10	4110

Inject the following intracerebrally:
1 and 2. Uniformly labelled glucose in isotonic saline at 1 hour intervals, each portion being 0.05 ml. containing 3.13×10^6 c.p.m. Specific activity = 25.7 mc./mmole. See text for further details.
3 and 4. 0.05 ml. of 1-C^{14}-acetate in isotonic saline, thereby administering 3×10^8 c.p.m. Specific activity 1 mc./mmole. A second injection of 0.05 ml. was made and each mouse went into convulsions and died immediately.

show that glucose and acetate act as precursors for NAA *in vivo*. The chromatogram also gives a fraction containing glutamic plus aspartic acids—the radioactivity of this fraction is reported since some reference to the relatively constant total radioactivity of NAA may be of interest.

The rather uniform distribution of radioactivity in the NAA when UL-C^{14}-glucose was given (Table IV) indicates that glycolysis and the tricarboxylic acid cycle are involved. When 1-C^{14}-acetate entered the NAA molecule it did so predominantly in the acetyl portion, where 80 per cent of the radio-

TABLE IV

DISTRIBUTION OF C^{14} IN NAA FOLLOWING INTRACEREBRAL ADMINISTRATION OF UL-C^{14}-GLUCOSE AND 1-C^{14}-ACETATE

Source of C^{14}	Total c.p.m. in		Ratio: $\frac{\text{Aspartic moiety}}{\text{NAA}}$
	NAA	Aspartic acid portion of NAA	
1. UL-C^{14}-glucose	511	420	0.82
2. UL-C^{14}-glucose	835	538	0.64
3. 1-C^{14}-sodium acetate	883	218	0.25
4. 1-C^{14}-sodium acetate	786	145	0.18

The labelled NAA is a portion of that reported in Table I.

activity occurred. This is a strong indication that a direct acetylation of aspartic acid occurs in the process of NAA formation.

INCORPORATION OF C^{14}-ACETATE AND C^{14}-ASPARTATE INTO NAA IN VITRO

Confirmation of these incorporation experiments was sought under *in vitro* conditions to ensure that the reactions are catalyzed by brain constituents themselves. The incorporation of 4-C^{14}-aspartic acid as well as 1-C^{14}-sodium acetate was shown by incubating each precursor with a portion of the minced sagittal half of an adult mouse brain. Of each half brain, 110 to 120 mg. (about 60 per cent of the total) was incubated in a reaction mixture similar to that

TABLE V

COMPARISONS OF INCORPORATION OF 1-C^{14}-ACETATE INTO NAA BY RABBIT BRAIN AT VARIOUS AGES

Age of rabbit	Total c.p.m. in NAA
5 days	1025, 1400
10 "	3930, 3680
20 "	1665, 2470

Brains were cut into sagittal halves, one half minced and a portion weighed into a tube containing: NAA, 65 μg.; glucose, 40 μmol; NaCl, 18 mg.; 0.1 M phosphate buffer, pH 7.3; ATP, 5 mg.; DL-aspartic acid, 0.36 mg.; 1-C^{14}-sodium acetate, 100 μg., 4.1×10^5 c.p.m. Total volume was 2 ml. Incubation with occasional shaking was for 3 hours at 39°. Each tube contained 111 to 120 mg. of brain. Three rabbits were used making duplicate determinations on each brain.

in Table V. In the presence of 5.4×10^6 C.P.M. of 1-C^{14}-aspartic acid the NAA obtained gave 994 C.P.M. In other experiments, the presence of 0.5 mg./ml. of CoA resulted in a 20 per cent increase in labelling of NAA in the presence of 4-C^{14}-aspartic acid. The main conclusion is that brain tissue is able to perform the incorporation of a specific precursor into NAA.

When the brain was further disrupted by homogenization to an essentially cell-free state, neither the incorporation of acetate nor aspartate could be observed. Similarly, no net synthesis occurred. Further attempts will be made to elucidate the enzyme systems involved in the acetylation of aspartic acid. It would appear that an enzyme system in a soluble form has been achieved recently by Goldstein (1959).

The rate at which a 10 day old rabbit brain incorporates acetate into NAA may be compared with the rate in 5 day and 20 day old brains as representa-

TABLE VI

RATE OF INCORPORATION OF ACETATE-1-C^{14} INTO NAA IN MOUSE BRAIN

Time	Specific Activity C.P.M./μmol NAA	NAA		Per cent C^{14} in aspartic moiety
		Total (μmol)	μmol/gm. fresh weight	
30 min.	428	3.58	8.09	16
60 "	675	2.51	5.71	—
60 "	404	2.39	5.70	—
120 "	806	1.71	4.39	18
60 min. + malonate	254	2.98	8.06	21
0 (2 animals, averaged)	—	—	7.20	—

An intracerebral injection of 1-C^{14}-sodium acetate (2×10^7 C.P.M., 1.52 mc./mmole) dissolved in 0.9 per cent NaCl was given, after adjusting solution to pH 7.41. In the last case, malonic acid was included at 1.335 M and pH adjusted to 7.38. In all cases 0.05 ml. (0.5 mg. NaOAc) was administered; of eleven mice injected, six died immediately. The animals were without food for about 10 hours prior to injection.

tive of the times before and after the period of the naturally occurring rapid rise of NAA level in the brain. The results in Table V show a marked correlation with the period of NAA increase in the brain. The youngest was least active, the oldest more active than the youngest, but the 10 day old rabbit brain, representing a brain in the midst of the period of rapid production of NAA, was most active of all.

It is of some interest to estimate the time it would take for the brain to increase the NAA to the adult level. Assume that the young mouse makes the NAA three times faster than the adult (Table V), and that the rate of NAA synthesis is approximated by the rate of acetate incorporation shown in Table III. Then the amount of NAA formed from the 5th to the 20th day is calculated to appear in about 40 to 50 days, a time of the same order as the 15 day period in which the increase actually occurs.

RATE OF NAA FORMATION IN VIVO An investigation into the rate of acetate-NAA exchange, *in vivo*, was made by injecting C^{14}-acetate into the cerebrums of several mice and measuring the NAA activity at various times thereafter. In Table VI, the specific activity of the NAA is seen to reach half of the 120 minute value within 30 minutes and then to rise more slowly. When malonate was included with the C^{14}-acetate the incorporation was inhibited by 50 per cent. Another effect of acetate injection was to depress the concentration of NAA in the brain. This effect is not the result of injury alone, since injection of 0.05 ml. of 0.9 per cent NaCl showed a 10 per cent depression after an hour as compared to 40 per cent when acetate was also injected. Since malonate inhibits this depression of NAA the brain must react in a complex way to acetate injection. The inhibition of the tricarboxylic acid cycle by malonate is a well known fact but cannot account simultaneously for both the decreased acetate incorporation into NAA and the depression of the NAA level of the brain.

Intracerebral injection of acetate also had a pronounced effect on the mortality rate of the animals. Injection procedure as such normally does not result in death of the mouse. For example, 0.9 per cent NaCl solutions containing India ink or glucose (Table III) were injected into the same mice repeatedly without causing death, but when sodium acetate (10 mg./ml.) was injected, a 50 per cent mortality resulted from the first injection; following a second injection all mice died immediately. An adverse effect of acetate on nervous tissue was reported by Lorente de Nó (1947) and may be taken with the above evidence to mean that acetate seriously interferes with the metabolic function of the brain. This change in the total amount of NAA in the brain is an indication that metabolic activity of NAA can be induced and studied apart from the incorporation of radioactive compounds.

CONCLUSIONS

N-Acetylaspartic acid has been studied in relation to its level in the brain of human beings and other animals. The amount in the brains of mentally deficient human beings was not correlated to a specific abnormality but does seem to be both unusually high and low in this group. When the amount of NAA was measured in relation to the age of young human beings the correlation with age was not as high as it was in the case of the rat (Tallan, 1957) and rabbit.

The stability of NAA was noticed in various experiments involving storage of brains and the incubation of various brain preparations.

The biosynthesis of NAA occurs in the mouse brain by direct acetylation of aspartic acid. This route is indicated by the high level of radioactivity in the acetyl portion of NAA when C^{14}-sodium acetate was administered intracerebrally.

The concentration of NAA in the brain of young rabbits undergoes a four-fold increase between the 5th and 15th day *postpartum*. The rate at which 1-C^{14}-acetate was incorporated into NAA by brain slices of the 10 day old rabbit was two to three times higher than the rate for younger or older animals, and thus was correlated with the change in concentration.

The intracerebral administration of acetate results in a 40 per cent depression in the NAA concentration of the mouse brain. Since the NAA level remained normal in hypoglycemic rats and in a mouse that died of suffocation (animal enclosed so that death occurred in 30 to 40 minutes) this reaction to acetate is the first indication that NAA is related to metabolic processes in the brain.

It may be added that NAA was tested on the crayfish stretch receptor nerve, as described by Wiersma *et al.* (1953). No stimulation or inhibition of discharge of the nerve could be noticed at 10^{-3} to 10^{-5} M NAA. Mr. John Wolf kindly performed this test.

Materials

N-Acetylaspartic anhydride was synthesized according to Barker (1953) and used as the standard source of *N*-acetylaspartic acid.

Uniformly labelled glucose (25 mc./mmole) and 1-C^{14}-sodium acetate (1 mc./mmole) were obtained from California Foundation for Biochemical Research. The 4-C^{14}-DL-aspartic acid (1.2 mc./mmole) was purchased from Tracerlab. Uniformly labelled L-aspartic acid (2 mc./mmole) was received from Nucleonic Co. The CoA and ATP were purchased from Pabst Laboratories.

Insulin used was Eli Lilly brand iletin, a zinc form. Swiss mice and New Zealand rabbits were obtained from Mission Animal Supply, San Gabriel, California.

Methods

DETERMINATION OF NAA For measuring the NAA content of brain the chromatographic method of Tallan, Moore, and Stein (1956) was used in which the NAA was eluted in a sodium acetate gradient. It was determined, by comparison, that the picric acid could be replaced in the extraction of brain with 5 per cent trichloracetic acid (TCA) or perchloric acid. This change made it possible to add the extract (adjusted to pH 5.5–6.5) directly to the column of Dowex 1-X8. The chromatographic fractions were examined for NAA by a ninhydrin procedure (Moore and Stein, 1954) after hydrolysis in 2 N HCl for 60 minutes in a boiling water bath. This procedure resulted in 90 per cent recovery whereas Tallan reported that 90 per cent hydrolysis occurred in 30 minutes under these conditions. The necessity for 60 minute hydrolysis in our laboratory was established on different preparations of NAA as well as on one kindly supplied by Dr. Tallan.

The mice and rabbits were killed by breaking the neck and bleeding, respectively. The entire brain was homogenized in 5 per cent TCA unless otherwise noted. The

routine procedure for human brain was to remove a specific portion of the parietal lobe within 25 hours after death, freeze the sample, shave off portions of the gray matter (excluding the white matter) while still frozen, weigh, and homogenize in ice cold distilled water using a TenBroeck homogenizer. Samples of the homogenate were taken immediately for extraction with 5 per cent TCA and for protein determination. The NAA in the acid extract was determined by chromatography. Protein was measured by the Folin method (Lowry *et al.*, 1951) using crystalline bovine serum albumin as a standard. It was estimated that a 5 to 8 per cent accuracy in NAA determination was maintained by the chromatographic procedure.

DETERMINATION OF LABELLED NAA The acid extract was chromatographed as described. The NAA zone was measured chemically and by radioactivity using aliquots of each fraction. Radioactivity determinations were made at infinite thickness because of the sodium acetate. To determine the distribution of radioactive carbon in the NAA the remainders of the fractions were pooled and desalted by passing the solution through a Dowex-50 column (resin recharged with HCl, sample added, and washed through with H_2O). The NAA was not retarded and came through with the acetic acid which was removed by lyophilization. The residue was dissolved in water, hydrolyzed in boiling 2 N HCl for 1¼ hours, and evaporated to dryness several times to drive off the acetic acid liberated from the NAA. The radioactivity of this residue (aspartic acid moiety of NAA) was compared to that of the unhydrolyzed NAA. Attempts to recover the labelled acetic acid by ether extraction proved unsatisfactory.

ENZYMATIC DETERMINATION OF ASPARTIC ACID The enzymatic determination of aspartic acid is based on the sequence of two reactions (1) aspartic α-ketoglutaric transaminase, producing oxaloacetic acid from aspartic acid and (2) malic dehydrogenase catalysis of reduction of oxaloacetic acid by DPNH. Aspartic acid concentration is calculated from the decrease in optical density at 340 mμ which accompanies oxidation of DPNH using 6.22 as a millimolar extinction coefficient. This method was developed prior to the knowledge that it had been described previously (Pfleiderer *et al.*, 1955); therefore, the following modifications of the published method exist. The transaminase was prepared according to Green *et al.* (1945) and carried through the heating step that follows the first ammonium sulfate fractionation. This preparation was the source of both enzymes since it contained a very active malic dehydrogenase activity in addition to the transaminase.

With this transaminase preparation the recovery of known amounts of aspartic acid averaged 90 per cent. With this correction factor, duplicate determinations of the aspartic acid in a TCA extract of rabbit brain were made and compared to the amount determined by ion exchange chromatography (100 cm. column of Dowex-50) (Moore and Stein, 1951). The chromatographic method showed 2.31 μmoles aspartic acid and the enzymatic method 2.37 μmoles. Furthermore, when the acid-hydrolyzed extract was determined by the two methods the chromatographic procedure gave 6.09 μmoles and the enzymatic procedure 5.98 μmoles aspartic acid; all values are per milliliter of extract. Thus the enzyme method is entirely satisfactory and in addition is more rapid than chromatographic procedures. One precaution was necessary, namely, the TCA or perchloric acid had to be removed (ether extraction or potassium

precipitation, respectively) to achieve reproducible recovery of aspartic acid by the enzyme method.

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