

Samevattend, kan AMC dus beskou word as 'n uitgebreide kongenitale simmetriese gewrigsaandoening wat die eindresultaat van meer as een patologiese proses verteenwoordig. Baie gevalle wat egter as AMC gerapporteer is, het feitlik net een of twee gewrigte met fleksie-deformiteite gehad, en stem nie ooreen met die kenmerkende kliniese beeld wat oorspronklik deur Stern in 1925 beskrywe is en hierbo genoem is nie.<sup>3</sup> By kenmerkende gevalle, in die afwesigheid van 'n ander bekende spier- of neurale toestand, moet 'n noukeurige stamboom-analise gedoen word. Voorligting vir geval 2 het op outosomale resessiewe basis geskied, maar by geval 1, waar die ouerpaar reeds 5 kinders sonder AMC het, is die oorerwing as dominant beskou. Hierdie geval kon moontlik 'n nuwe mutasie verteenwoordig het. Die moeder is egter gesteriliseer en dus was die informasie dat daar geen herhalingsrisiko was nie, slegs 'n interessante feit.

Ons wil graag professor M. P. Keet bedank vir toestemming en fasiliteite om die pasiënte na te gaan.

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# Neurochemical Aspects of Porphyria

## STUDIES ON THE POSSIBLE NEUROTOXICITY OF DELTA-AMINOLAEVULINIC ACID

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### SUMMARY

It has been proposed that delta-aminolaevulinic acid (ALA), which is overproduced in the inherited hepatic porphyrias, may be responsible for the neurological manifestations of the acute attacks seen in these disorders. Studies were conducted in rats to test the neurotoxicity of ALA. It was found that, after intraperitoneal or subcutaneous injections, ALA is rapidly eliminated via the kidneys. In nephrectomised animals sustained elevation of blood ALA concentration was demonstrated, but despite this, brain uptake was extremely low. Experiments on incorporation of [4-<sup>14</sup>C]-ALA into brain haem yielded similar information. After intraventricular injection of [4-<sup>14</sup>C]-ALA, significant uptake by brain tissue occurred. The subsequent disappearance of ALA was moderately rapid

and was virtually complete within 24 hours. Uptake of [4-<sup>14</sup>C]-ALA was apparently significantly greater in the hypothalamus than in other brain areas. The subcellular distribution of radioactivity did not reveal any preferential uptake by nerve endings. Intraventricular injection of unlabelled ALA revealed definite but transitory neurotoxic effects in doses of 3 micromoles and greater. These include involuntary movements and ataxia. No effect of ALA administration on brain protein synthesis could be demonstrated. It is concluded that ALA does have effects on the nervous system *in vivo*, but the significance of these effects in relation to the pathogenesis of the neurological manifestations of acute porphyria is questionable.

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The clinical manifestations of the acute attack in the inherited hepatic porphyrias are generally recognised as being largely neurological or neuropsychiatric in nature. Virtually nothing is known, however, of the nature of the underlying disturbance.

Various hypotheses have been proposed from time to time. Among these is the idea that porphyrin precursors



which are overproduced in all these disorders may be neurotoxic. Earlier studies by Goldberg and Rimington<sup>1</sup> and Jarrett *et al.*<sup>2</sup> suggested that both delta-aminolaevulinic acid (ALA) and porphobilinogen (PBG) were innocuous substances. More recently, this view has been questioned. Feldman and co-workers<sup>3</sup> have shown that ALA and PBG can cause presynaptic inhibition of neurotransmitter release. Becker *et al.* have found that ALA can inhibit Na<sup>+</sup>K<sup>+</sup>-dependent ATPase in brain<sup>4</sup> and that both ALA and PBG have an inhibitory effect on monosynaptic reflex activity in the isolated frog spinal cord.<sup>5</sup> However, none of this recent evidence can be accepted as conclusive, since it relates only to *in vitro* effects of these porphyrin precursors on neural tissue.

An evaluation of the possible neurotoxicity of ALA and PBG obviously necessitates detailed information on their effects and behaviour *in vivo*. These data are largely unavailable. The present study was undertaken to examine the consequences of administering ALA peripherally, or directly into the nervous system, in experimental animals.

## MATERIAL AND METHODS

The animals employed were young adult female albino rats weighing 150 - 250 g. In each experiment the test and control animals were matched for age and weight. Animals were sacrificed by decapitation at the times indicated in the individual experiments. Tissues were promptly removed, rapidly frozen and kept at -20°C until analysis.

ALA, obtained from Sigma Chemical Co., USA, or [4-<sup>14</sup>C]-ALA was administered by the intraperitoneal, subcutaneous or intraventricular routes as indicated below. Intraventricular injections were carried out under light ether anaesthesia according to the method of Noble *et al.*<sup>6</sup> Solutions of unlabelled ALA for intraventricular injection were adjusted as near as possible to pH 7,4 prior to administration.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, UK. The purity of [4-<sup>14</sup>C]-ALA (SA 53 mCi/mmol) was checked chromatographically and found to be greater than 90%. Other reagents employed were all of analytical grade.

Tissues were homogenised in 0,1M trichloroacetic acid (TCA) and ALA and PBG were then determined in the clear supernatant after centrifugation according to the method of Marver *et al.*<sup>7</sup> In the study of [4-<sup>14</sup>C]-ALA uptake by the brain after intraventricular injection, the ventricles were opened and thoroughly washed in physiological saline before homogenisation of the tissue in 0,1M TCA. Total radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer model 3385 on aliquots of the clear supernatant preparation after centrifugation. [4-<sup>14</sup>C]-ALA was determined in the supernatant solutions by column chromatography according to Ebert *et al.*<sup>8</sup> followed by pyrrole formation, extraction of the pyrrole with ethyl acetate<sup>9</sup> and determination of radioactivity in this extract. Haem was isolated with the aid of carrier in the form of washed erythrocytes according to Labbe and Nishida.<sup>10</sup> Samples were prepared for counting by combustion in a Packard sample oxidiser. Incorporation of [<sup>35</sup>S]-methionine and L-[U-<sup>14</sup>C]-lysine into brain

protein was determined by the method of Agrawal *et al.*<sup>11</sup> Regional dissection of the brain was carried out according to Glowinski and Iversen.<sup>12</sup> The technique of subcellular fractionation was essentially as described by Marchbanks and Whittaker.<sup>13</sup>

## RESULTS

### Brain ALA Uptake in Normal Rats

Previous preliminary studies suggested that ALA administered peripherally did not readily enter the nervous system.<sup>14</sup> This was justifiably criticised on the grounds that blood levels were not determined.<sup>15</sup> Accordingly, the first step was to examine concentrations of ALA in blood, brain and liver 4 hours after a single intraperitoneal injection of ALA.

Despite a dose as high as 500 mg/kg, ALA was undetectable in blood, liver and brain in these animals. Unfortunately, the urinary excretion of ALA was not measured in this experiment but there was little doubt that the injected ALA must have been rapidly eliminated by the kidneys. Therefore, it was decided to repeat the experiment in nephrectomised animals.

### Brain ALA Uptake in Nephrectomised Rats

Bilateral nephrectomy was performed under ether anaesthesia on young adult rats. ALA was injected subcutaneously (in order to avoid the intraperitoneal route) on the subsequent day. All the animals were in good condition at the time of the injection. The results are shown in Table I.

Substantial concentrations of ALA were found 4 hours after injection in blood and liver but not in brain in these animals. Initially, as indicated in the table, the ALA concentration in brain was found to be below the limit of detection. When the experiment was repeated, using the whole brain for analysis, the ALA level was found to be 74 nanomoles/g (SD 14) in association with a blood concentration of 0,96 mM (SD 0,28). In the brain, therefore, the concentration of ALA was less than 10% of that found in the liver and the blood. The blood concentration achieved after 500 mg/kg was well in excess of what might be expected in patients with acute porphyria.<sup>15</sup> To assess the effect of barbiturate on the entry of ALA into the brain the experiment was repeated, with the administration of sodium phenobarbitone (60 mg/kg) together with ALA. No significant effect was noted.

### Incorporation of Radioactivity from [4-<sup>14</sup>C]-ALA into Liver and Brain Haem in Normal Rats

Incorporation of [4-<sup>14</sup>C]-ALA into liver and brain haem *in vivo* was compared with results obtained by incubating these tissues *in vitro* with [4-<sup>14</sup>C]-ALA. The dose of [4-<sup>14</sup>C]-ALA was 0,1  $\mu$ Ci/g body weight. Tissues were homogenised in 9 volumes of saline with washed erythrocytes (*in vivo* experiment) or 10 mM tris-saline buffer, pH 7,4 (*in vitro* experiment). Incubation



TABLE I. MEAN TISSUE CONCENTRATIONS OF ALA AND PBG IN NEPHRECTOMISED RATS 4 HOURS AFTER SUBCUTANEOUS INJECTION OF ALA

Dose of ALA (mg/kg)	ALA			PBG		
	Blood (mmol/l)	Brain (nmoles/g)	Liver (nmoles/g)	Blood (mmol/l)	Brain (nmoles/g)	Liver (nmoles/g)
0	ND	ND	ND	ND	ND	ND
250	0,25	ND	908	ND	ND	138
500	0,54	ND	1 164	ND	ND	159

ND = not detectable.

Each result represents the mean of 5 observations.

mixtures comprised homogenate with added [4-<sup>14</sup>C]-ALA (0,25  $\mu$ Ci/ml) and ferric chloride (0,6 mM). Incubations were carried out aerobically with shaking at 37°C for 1 hour. The results are shown in Table II.

TABLE II. COMPARISON OF THE INCORPORATION OF [4-<sup>14</sup>C]-ALA INTO LIVER AND BRAIN HAEM *IN VIVO* AND *IN VITRO*

Experiment	Radioactivity of haem		
	Liver (dpm/4 mg)	Brain (dpm/4 mg)	Liver : brain ratio
<i>In vivo</i> *	178 $\times$ 10 <sup>3</sup>	271	657
<i>In vitro</i> *	26 $\times$ 10 <sup>3</sup>	721	36

\* See text for details.

The liver : brain ratio in the *in vitro* experiment was very much lower than that obtained *in vivo*. It must be pointed out that the conditions of incubation which were used in the *in vitro* experiment may not have been ideal for maximal incorporation of labelled ALA into haem, either in liver or in brain. Nevertheless, the values obtained would appear to confirm the finding in the preceding experiment that the 'blood-brain barrier' in the normal rat is relatively impermeable to ALA.

### Distribution and Metabolism of [4-<sup>14</sup>C]-ALA Following Intraventricular Injection

The fact that significant amounts of ALA cannot be detected in brain following acute elevation of the blood concentration of ALA, does not rule out the possibility that this substance might gradually accumulate in the nervous system in patients with acute porphyria, until a toxic level is reached. Accordingly, it was decided to experiment with direct introduction of ALA into the nervous system.

Table III shows the uptake and disappearance of [4-<sup>14</sup>C]-ALA from rat brain at intervals after intraventricular injection of 5  $\mu$ Ci. Two hours after injection approximately 23% of the administered radioactivity had been taken up by the brain, but less than half of this was recoverable as ALA. After 4 hours the total radioactivity present had halved and ALA accounted for less than one-third. Twenty-four hours after injection only 0,7% of the administered dose was still present and ALA constituted less than 10%.

TABLE III. UPTAKE BY BRAIN AND DISAPPEARANCE OF [4-<sup>14</sup>C]-ALA AFTER INTRAVENTRICULAR INJECTION OF 5  $\mu$ Ci

Time after injection (hours)	Percentage of original dose in brain			
	Total radioactivity		Radioactivity extractable as ALA	
	Mean*	SD	Mean*	SD
2	23,2	6,6	9,1	1,8
4	11,6	5,8	3,5	0,6
24	0,7	0,2	0,05	0,01

\* Mean of 5 observations.

The regional distribution of radioactivity 2 hours after intraventricular injection of [4-<sup>14</sup>C]-ALA is shown in Table IV. A significantly higher concentration was found in the hypothalamus compared with other brain regions.

TABLE IV. REGIONAL DISTRIBUTION OF RADIOACTIVITY IN BRAIN 2 HOURS AFTER INTRAVENTRICULAR INJECTION OF [4-<sup>14</sup>C]-ALA (5  $\mu$ Ci)

Brain region	Radioactivity (dpm/mg tissue)	
	Mean*	SD
Cerebellum ... ..	1 236	184
Pons ... ..	994	306
Hypothalamus ... ..	3 750	963
Midbrain ... ..	1 277	380
Striatum ... ..	819	104
Cortex ... ..	1 190	282

\* Mean of 5 observations.

It was also of interest to know whether ALA is concentrated in any particular subcellular fraction following uptake into the brain. Table V shows the subcellular distribution of radioactivity in rat brain 2 hours after intraventricular injection of [4-<sup>14</sup>C]-ALA. Most of the radioactivity was found in the soluble fraction and there was no evidence of a peak in the synaptosomal or 'nerve ending' fraction.

### Effect of Intraventricular Injection of ALA on Behaviour in Rats

In the preceding experiments tracer amounts of [4-<sup>14</sup>C]-ALA were administered intraventricularly. The next



TABLE V. SUBCELLULAR DISTRIBUTION OF RADIOACTIVITY IN BRAIN 2 HOURS AFTER INTRAVENTRICULAR INJECTION OF [4-<sup>14</sup>C]-ALA (5  $\mu$ Ci)

Subcellular fraction	Percentage of total radioactivity	
	Mean*	SD
Whole homogenate	100,0	
Nuclear pellet	4,8	1,9
Supernatant	61,7	7,3
Crude mitochondrial fraction	25,4	4,0
Myelin	6,0	1,3
Synaptosomes	5,2	1,7
Mitochondria	4,1	2,4

\* Mean of 5 observations.

step was to examine the effect of intraventricular injection of larger amounts of unlabelled ALA. With a dose of up to 2 micromoles delivered in 30 microlitres of buffered solution (pH 7.4) no discernible gross behavioural effects were noted. The animals recovered quite normally from the anaesthetic and no untoward effects could be observed.

At higher dosages, however, definite effects were noted. The animals took longer to recover from anaesthesia. With doses of 3 and 5  $\mu$ moles 'jumping seizures' were prominent during the recovery phase, i.e. the animals made involuntary forward jumps during this time but later (after approximately 1 hour) they appeared quite normal. A few of the control animals also showed this phenomenon, but to a much lesser extent. A dose of 10  $\mu$ moles did not produce these 'jumping seizures' but ataxia was marked for several hours after recovery from anaesthesia. In a group of smaller animals weighing about 130 g this dose proved uniformly fatal within minutes after injection.

The solutions containing 5 or 10  $\mu$ moles/30  $\mu$ l were undoubtedly hypertonic, which raised the question as to whether any of the observed effects might be attributable to this reason. Hypertonic saline solutions (1,8% and 2,7% NaCl) were, however, found to have no untoward effects when injected intraventricularly.

### Effect of ALA Administration on Brain Protein Synthesis

Apart from possible direct effects of ALA on neural tissue, the possibility was also considered that this amino acid might be indirectly responsible for pathological changes in the nervous system, e.g. through interference with protein synthesis, as has been shown in experimental hyperphenylalaninaemia.<sup>11</sup>

Tables VI and VII show the incorporation of radioactivity from [<sup>35</sup>S]-methionine and L-[U-<sup>14</sup>C]-lysine respectively into the acid-soluble fraction and into protein in rat brain after administration of ALA. In the case of [<sup>35</sup>S]-methionine ALA was administered subcutaneously and the animals were nephrectomised on the preceding day. In the case of L-[U-<sup>14</sup>C]-lysine, ALA was administered intraventricularly. No significant diffe-

rence was found between normal animals and those treated with ALA with regard to the incorporation of radioactivity either into the free amino acid pool or into brain protein.

TABLE VI. INCORPORATION OF [<sup>35</sup>S]-METHIONINE INTO BRAIN PROTEIN IN NEPHRECTOMISED RATS AFTER SUBCUTANEOUS ADMINISTRATION OF ALA

Dose of ALA (mg/kg)	Radioactivity			
	Acid-soluble fraction (dpm/mg brain)		Protein (dpm/mg)	
	Mean*	SD	Mean*	SD
0	8 762	1 952	378	59
250	8 838	1 9 0	290	13
500	8 850	1 477	340	36

\* Mean of 5 observations.

TABLE VII. INCORPORATION OF L-[U-<sup>14</sup>C]-LYSINE INTO BRAIN PROTEIN IN RATS AFTER INTRAVENTRICULAR ADMINISTRATION OF ALA

Brain region	Radioactivity of brain protein (dpm/mg)*		
	No ALA	ALA 1 $\mu$ mole	ALA 3 $\mu$ mole
Cerebellum	467	551	452
Pons	546	513	547
Hypothalamus	464	521	496
Midbrain	455	450	356
Striatum	366	364	397
Cortex	523	442	545

\* Tissues of 5 animals pooled.

## DISCUSSION

These studies clearly show that after intraperitoneal or subcutaneous injection of ALA in the experimental animal, this amino acid is rapidly eliminated via the kidneys. In an attempt to simulate the situation in acute porphyria, we administered ALA to nephrectomised animals and were able to demonstrate that 4 hours after injection of 500 mg/kg the mean blood level ranged from 0,54 to 0,96 mM in different experiments. This is considerably higher than the blood concentration expected in porphyric patients during acute attacks.<sup>15</sup>

Despite this sustained elevation of ALA concentration in the blood, uptake into the brain in the present study was negligible in comparison with liver uptake of ALA. This suggests that the blood-brain barrier, at least in the normal animal, is relatively impermeable to ALA. Consequently, if elevated blood levels of ALA in patients with acute porphyria do lead to accumulation of this amino-ketone in the nervous system, then either the blood-brain barrier must be much more permeable to ALA under these circumstances or, alternatively, ALA must slowly accumulate in the nervous system when circulating blood levels are chronically elevated.



There is, of course, no reliable information available on the concentration of ALA in the nervous system in acute porphyria. What evidence is available suggests that plasma concentrations may be ten times those found in the cerebrospinal fluid.<sup>16</sup> The possibility that barbiturates, which are well known to be precipitating agents of acute attacks, might enhance uptake of ALA into the nervous system, was also examined in the present study. No significant effect was found.

Kramer *et al.*<sup>15</sup> have suggested that ALA may accumulate in the nervous system in patients with acute porphyria and subsequently interfere with nerve conduction. Our experiments using intraventricular injection of [4-<sup>14</sup>C]-ALA would seem to indicate that while ALA is readily taken up from the cerebrospinal fluid into the brain, it is fairly rapidly metabolised and removed. This process is virtually complete in 24 hours. Therefore it is unlikely that ALA slowly accumulates in neural tissue, either as a result of uptake from the blood or as a result of endogenous production in the nervous system.

The significance of the preferential uptake of radioactivity by the hypothalamus following intraventricular administration of [4-<sup>14</sup>C]-ALA is not at all clear. Unfortunately the percentage of this radioactivity present in the hypothalamus as ALA was not determined. Conceivably, if ALA does gain access to the brain in significant amounts in acute porphyria, the highest concentrations could be expected in the hypothalamus. This in turn could possibly underlie the presumed inappropriate secretion of anti-diuretic hormone seen in acute porphyric patients with hyponatraemia. However, this is highly speculative and the central problem of whether or not ALA does reach toxic levels in the nervous system in acute porphyria remains.

Intraventricular administration of relatively large amounts of ALA produced definite neurotoxic effects of an immediate but transitory nature. These include involuntary movements, mainly of the hind limbs, and prolonged ataxia. In small animals weighing about 130 g, 10  $\mu$ moles of ALA was uniformly fatal within minutes, apparently owing to respiratory failure. Animals which survived, on the other hand, seemed to suffer no residual ill-effects after 24 hours. There is no doubt, therefore, that ALA is potentially neurotoxic if introduced into the nervous system in sufficient concentration. Whether or not this could occur in patients is, as indicated previously, highly debatable. A further consideration is whether or not the types of neurological effects noted in our experiments have any relevance to the neurological lesions of acute porphyria.

Alterations in the free amino acid pool in the brain can interfere with brain protein synthesis, as has been shown

in experimental hyperphenylalaninaemia.<sup>11</sup> Studies were done to test the possibility that ALA might conceivably induce neurological damage via such a mechanism. However, no significant effect of elevated blood ALA concentration or of intraventricular administration of ALA was demonstrable.

In conclusion, the present study has shown that ALA when introduced directly into the nervous system by intraventricular injection can exert transitory neurotoxic effects. Nevertheless, there are major objections to the hypothesis that ALA is the neurotoxic factor in acute porphyria. Chief among these is the finding that in experimental animals there is a substantial blood-brain barrier to ALA and the demonstration that ALA introduced by intraventricular injection is relatively rapidly removed. It may be that it is not ALA but rather some pyrrolic compound which is the elusive factor. There are a number of possibilities which require examination. On the other hand, the solution to the mystery may lie in a study of haem biosynthesis in neural tissue. It is entirely plausible that the metabolic lesion which is present in the liver in the inherited hepatic porphyrias is also present in the neurons. If this is so, it could be that acute attacks represent crises of 'haem starvation' occasioned by various stresses on the nerve cell, including drugs such as the barbiturates. Studies on these possibilities are in progress.

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