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Plasma excitatory amino acids in autism.

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Key words: Infantile autism, amino acids, asparagine, glutamine, glutamic acid, aspartic acid, taurine, calcium, glutamate receptor, vitamin B6, tryptophan, serotonin, inborn errors, nutritional disorders, sodium-chloride-coupled neurotransmitter transporters.

Abstract. Plasma amino acid levels were measured by high pressure liquid chromatography (HPLC) in fourteen autistic children, all below 10 years of age. Mean glutamic and aspartic acid values were elevated (169 ± 142 uM and 22.1 ± 13 uM respectively) together with taurine (90.1 ± 78.7 uM) ($p > 0.1$). All affected children had low levels of glutamine (241 ± 166 uM; $p < 0.01$) and asparagine (22.9 ± 12.9 uM; $p < 0.01$) as compared to normal values (585 ± 25 and 59.2 ± 4.2 uM respectively); eleven children had increased aspartic acid and eight children had high levels of glutamate; seven of these children had a concomitant increment of taurine. The increment of the three above mentioned compounds was observed at the same time only in five children. These findings demonstrate that abnormal plasmatic levels of neurotransmitter amino acids may be found in some autistic children. Increased glutamatemia may be dietary in origen or may arise endogenously for several reasons, among others, metabolic derrangements in glutamate metabolism perhaps involving vitamin B6, defects or blockage of the glutamate receptor at the neuronal compartment, or alterations in the function of the neurotransmitters transporters. Increments of taurine, an inhibitor, is likely compensatory and calcium dependent.

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Palabras claves: Autismo infantil, aminoácidos, asparagina, glutamina, ácido glutámico, ácido aspartico, taurina, calcio, receptor glutamatergico, vitamina B6, triptofano, serotonina, errores innatos, alteraciones nutricionales, transportadores de neurotransmisores acoplados a sodio-cloro.

Resumen: Los aminoácidos plasmáticos se midieron en catorce niños autistas menores de diez años de edad, por cromatografía líquida de alta presión. Los ácidos glutámico (169 ± 142 uM) y aspártico (22,1 ± 13 uM) se encontraron elevados en este grupo así como también si bien en menor grado, la taurina (90,1 ± 78,7). Los valores normales observados en controles apareados por edad y sexo fueron 102 ± 4,3, 8,5 ± 0,5 y 42,3 ± 1,4 respectivamente. Al análisis individual, once niños tenían incremento del acido aspártico, y ocho niños tenían incremento del ácido glutámico, si bien no siempre al mismo tiempo; siete de estos niños tenían aumento concomitante de la taurina. Un aumento correlativo de los tres amino acidos neurotransmisores mencionados se observó solo en 5 de estos niños. La glutamina y la asparagina estaba disminuída en todos. Estos hallazgos demuestran que los niños autistas pueden mostrar aumentos de aminoacidos excitatorios y quizá por compensación de aminoácidos inhibitorios como la taurina. La hiperglutamatemia, podría ser de origen exógeno o podría originarse en forma endógena por variados motivos, tales como un desarreglo metabólico en la utilización del glutamato quizá dependiente de la vitamina B6, un bloqueo del receptor de glutamato en el compartimiento neuronal o alteraciones de los transportadores de neurotranmisores. En todo caso, la medida de aminoácidos neurotransmisores debe realizarse tempranamente en todo niño con sospecha de padecer el sindrome autista.

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INTRODUCTION

Infantile autism in which cognition and/or communication are more or less severely affected (25), and behavior and affectivity are consequently altered (14), is currently recognized as a heterogeneous group of disorders, affecting children with a number of adquired and/or inherited defects (1, 8, 24, 33, 42, 43, 45, 64, 69, 72). The autistic behavior may be considered a clinical sign depending on several etiological factors and the term autistic phenotype is probably more

adequate (48). Familial occurrence had been suggested by twin and familial studies (18, 57) and autosomal recessive inheritance has been indicated in some groups of families (44, 45, 56). Criteria for diagnosis remain on clinical bases (DSM-III-R; Manual of Statistics and Classification of Mental Disorders; American Psychiatric Association). Recently, we have proposed that some autistic children might have mild auriculo facial dysplastic features useful for clinical diagnosis and classification (45).

A distinctive metabolic marker is lacking in autism, perhaps an expression of its clinical heterogeneity and/or variability of findings over time, but several authors have demonstrated metabolic disturbances in autistic children especially related to levels of serotonin, chronic metabolic acidosis and lactic acidosis (9, 12, 43, 45, 52, 55) The serotonin and amino acid content in platelets of autistic children have been described (58).

Some children appear to benefit from treatment with vitamin B6, the apoenzyme moiety for decarboxylase enzyme activity (32, 40, 41, 45, 54). No formal explanation is available for those piridoxine responsive autistic individuals. Co-factor responsive inborn errors of metabolism have been known for years and certain defects of amino acid metabolism respond to vitamin B6 therapy (2, 36, 39, 59, 63).

In view of this relationship, we decided to explore the plasma amino acid levels in autistic children.

PATIENTS AND METHODS

Fourteen children, all below 10 years of age, fulfilling psychiatric criteria for infantil autism (DSM-III-R), were selected on the basis of order of arrival to our clinic. Agreement to participate in this study was obtained after informed consent, indicating that the results might not have immediate application. Rating of the autistic phenotype was done following Freeman (20); most children were within the moderate to severe autistic behavior. Physical findings, other psychiatric observations, rutinary laboratory evaluation, blood chemistry, genealogic data and segregation analysis on these patients have been previously published (45). A heparinized blood sample was obtained and plasma was inmediately separated by centrifugation, and kept frozen for variable periods of time before amino acid determinations within a month by HPLC (6). Briefly, after 5-sulfosalicylic acid deproteinization of plasma samples, free amino acids were first derivatized with orthopthaldehyde and then chromatographed on C-18 reversed-phase columns. Sixteen amino acids were readily separated and quantified, with a run time of about 60 minutes, and a sensitivity of at least 10 pmoles. Parents were asked to bring to the clinic a child matched by age and sex with the patient, to be used as control; this child was usually non-related, although a proband's cousin was at times used as control. All children were otherwise healthy,

had no intercurrent illnesses and were not taking any medication. Taurine was not measured in four cases for technical and logistic reasons, since measuring taurine requiered a modification of the method and further blood sampling. Lactic and pyruvic acids were measured by the NAD/NADH comsumption test (Sigma Chemical Co.). Consanguinity, isonimia, common ancestry, common geographical origen or ability to respond to vitamin B6 therapy, were not criteria for the selection. Diet was ad-libitum for both patients and controls.

RESULTS

Amino acid levels in controls were similar to those previously reported by others using HPLC (16).

Aspartic acid was elevated as compared to control values ($p<0.05$). Glutamic acid and taurine, were elevated however differences with control values were not significant due to a wide variance. Glutamine and asparagine were below normal limits in all patients ($p<0.01$) (Table I).

Individual analysis demonstrated elevation of aspartic acid in eleven children. Eight children had increased glutamic acid of which seven had also above normal taurinemia. Only five children had concomitant increment of the three above mentioned neurotransmitter amino acids.

Other findings were a mild lactic acidemia; lactic and pyruvic acids values were 1.92 ± 0.77 (s.e.m.) mM and 0.125 ± 0.045 mM respectively in autistic children and 0.83 ± 0.17 and 0.06 ± 0.01 respectively in controls ($p<0.01$; Student t-test). Gamma aminobytiric acid (GABA), was also measured in our patients and was only mildly decreased (patients: 18.3 ± 3.4 uM; controls 20.7 ± 1.7 uM; $p>0.10$).

DISCUSSION

Mean values of glutamate and taurine proved not to be significantly increased, due to the wide variance, but as a whole there was a 166 % increment above the mean normal values; on individual basis eight children demonstrated significative ($p<0.01$; Student t-test) increments of glutamate (Table I). Regression analysis for glutamate as the independent variable and the other compounds studied here, shows increment of taurine with the highest glutamate values (Fig 1a); taurine had a 46.9 % increment in 70 % of the patients examined. A better fit for this correlation is a sector of a hyperbole, suggesting that the increments of taurine is dependant upon the highest glutamate values. Glutamine and asparagine, were below normal values in 100 % of these patients ($p<0.01$; Student t-test) (Table I) and the lowering correlates with the highest glutamate levels (Fig 2a-d), indicating perhaps consumption of glutamine and asparagine in favor of glutamate and shifting of the equilibrium towards aspartate, a less

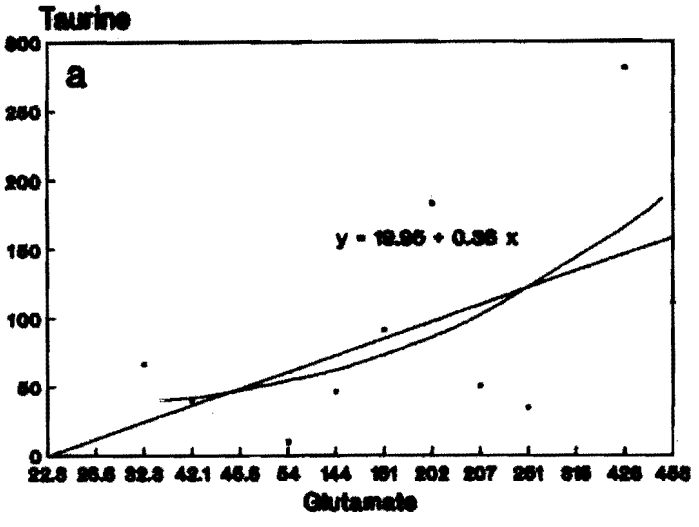
TABLE I

EXCITATORY AMINO ACIDS IN AUTISTIC CHILDREN

Patient	Glu	Asp	Gln	Asn	Tau
VES	22.3	14.0	14.0	19.4	?
CS	26.6	7.4	73.9	19.4	?
ZNN	32.3	26.2	507.0	45.0	66.1
OSB	42.1	28.2	397.0	37.9	39.7
MH	45.6	9.4	501.0	34.1	?
DM	54.0	22.8	484.0	47.9	9.5
APP	144.0	19.8	287.0	19.4	45.5
OM	151.0	27.9	218.0	19.8	91.1
MIY	202.0	8.7	139.0	9.0	183.5
JGU	207.0	9.5	140.0	15.6	50.1
EPD	251.0	8.4	42.8	12.0	34.1
JLC	316.0	52.7	108.0	12.1	?
WPS	426.0	45.6	189.0	19.5	280.8
CM	453.0	19.0	51.0	9.7	110.8
X	169.5	22.1	241	22.9	90.1
s.e.	142.3	13.0	166	12.5	78.7
Normal	102.0	8.5	585	59.2	42.3
s.e.	4.3	0.5	25	4.2	1.4
p	n.s.	<0.01	<0.0001	<0.0001	n.s.
diference	+166%	+260%	-41.2%	-38.7%	+46.9%

toxic compound. Aspartic acid was elevated 260 % above the mean normal values in most (eleven = 71 %) of the children examined and its increment also correlates with glutamate (Fig 1b).

The wide variance observed in our patients regarding glutamatemia might be dietary in origen and clinical findings, as pointed out by some researchers, could also be related or worsened by the dietary ingestion of neurotoxic amino acids (35). In our patients, extreme values of glutamate and aspartate correlate with the lowest values of asparagine and glutamine (Fig 2b and 2d), suggesting perhaps abnormal glutamate homeostasis in autism. Since there are doubts on the efficiency of the blood-brain barrier against neurotoxic amino acids (7,19) the



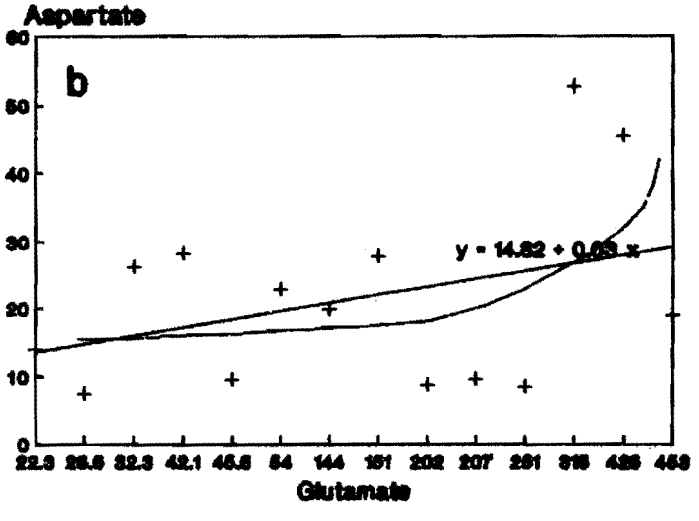


Fig 1. 1a: Tendency to a positive correlation between plasma glutamate levels and taurine in fourteen autistic children. Arbitrary smoothing of the line suggest exponencial increment above the mean glutamate values for autistic individuals.
1b: Tendency to a positive correlation bewtween plasma glutamate levels and aspartate in fourteen autistic children. Exponential increment of plasma aspartate levels seems to occur one s.e. above the mean for autistic individuals, suggesting shifting of the equilibrium towards aspartate, a less toxic compound.

dietary ingestion of glutamic acid would have to be monitored in further studies of this type and the clinical consequences of the dietary restriction or loading of excitatory amino acids should be evaluated.

Glutamate plays a central role in many biochemical pathways both peripherically and centrally, and hyperglutamatemia, although not seen in every one of our patients, perhaps due to heterogeneity, timing of sampling or unforseen dietary variables, seems to be a central finding here, and most likely related to the autistic phenotype. Glutamate may be derived from excesive utilization of glutamine via glutamina amidotransferase, glutamine synthetase and glutamate synthase or from asparagine, via aspartic acid and alfa-ketoglutarate. Allosteric inhibition of asparagine and glutamine synthetases, indirectly related to hyperglutamatemia, might also explain low levels of these compounds in our patients. We however, did not measure the activity of these enzymes. Glutamine is requiered for the synthesis of nucleotides and for water efflux into the neuronal gap (68), aspects of brain metabolism which to our knowledge, have not been studied in autistic individuals.

Increments in plasmatic levels of glutamate might also be related to a blockage in glutamate utilization for which vitamin B6 is requiered. Vitamin B6, reportedly efficient in the treatment of some autistic children (40, 41), is an important coenzyme. Its active forms are piridoxal phoshate and piridoxamine phosphate.

The piridoxine coenzymes are extremely versatile, functioning in a large number of different enzymatic reactions in which amino acids or amino groups are transformed or transferred. The most common type of enzymatic reaction requiering piridoxal phosphate is transamination: the transfer of the alfa-amino group of the amino acid to the alfa-carbon of its corresponding alfa-keto acid, in most cases, alfa-ketoglutarate, leaving behind the corresponding alfa-ketoacid analog of the amino acid and causing the amination of the alfa-ketoglutarate to L-glutamic acid. Such reactions, in normal conditions freely reversible, are catalyzed by enzymes known generically as aminotransferases or transaminases, a large number of which are known. Most require alfa-ketoglutarate as one amino group acceptor. There are therefore specificity for the substrate couple alfa-ketoglutarate-L-glutamate. The specificity for the other substrate couple is less rigid, although usually there is one showing gratest activity for which the enzyme is named. As an example, a prominent transaminase in animal tissues is aspartate transaminase. All the transaminases appear to have the same prosthetic group, piridoxal phosphate and share a common reaction mechanism. Altered reversibility of the transamination step, would explain the lowering of certain amino acids as we have previously reported (45), but this should correlate with

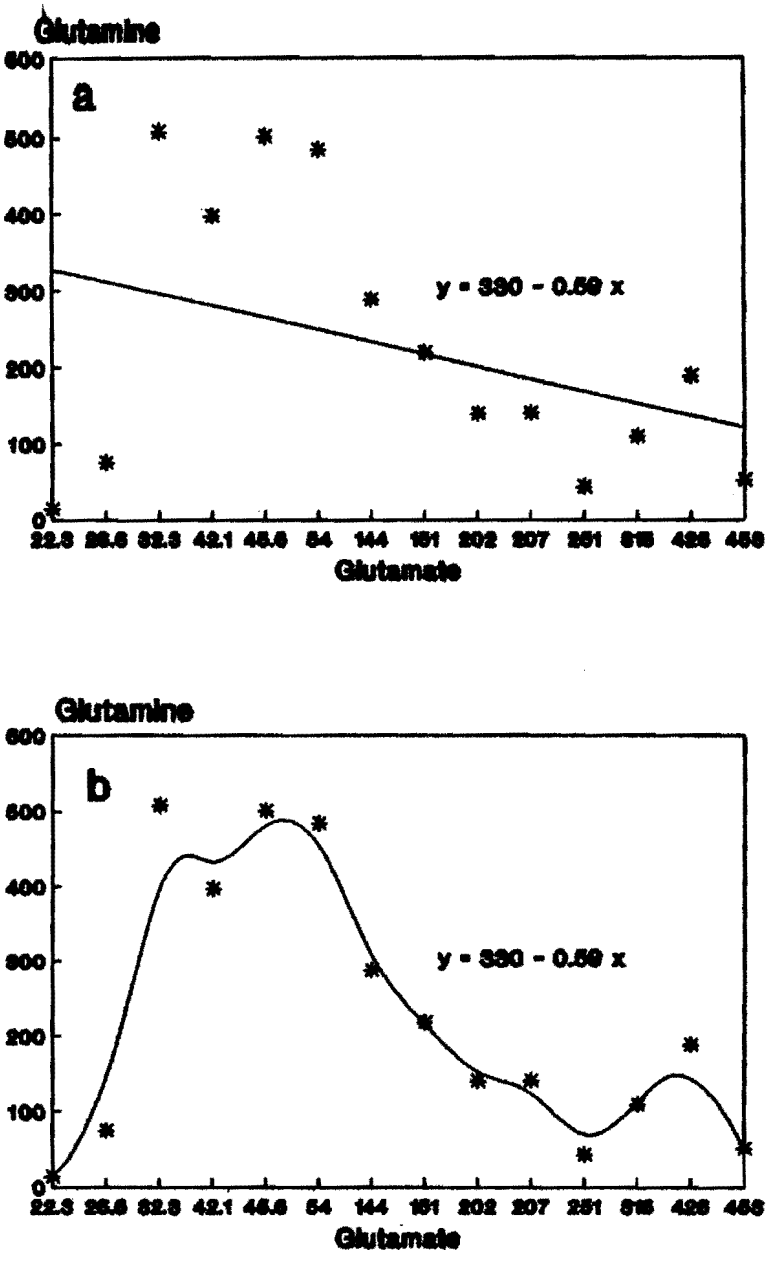
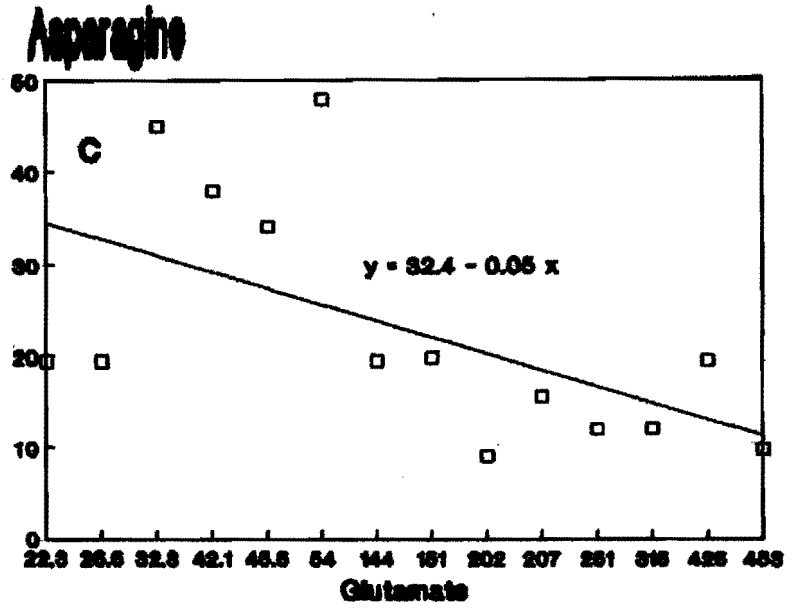


Fig 2. 2a: Negative correlation between plasma glutamate and glutamine levels.
2b: Curve plotting indicates that glutamine falls with extreme values below and above the normal mean for glutamate.



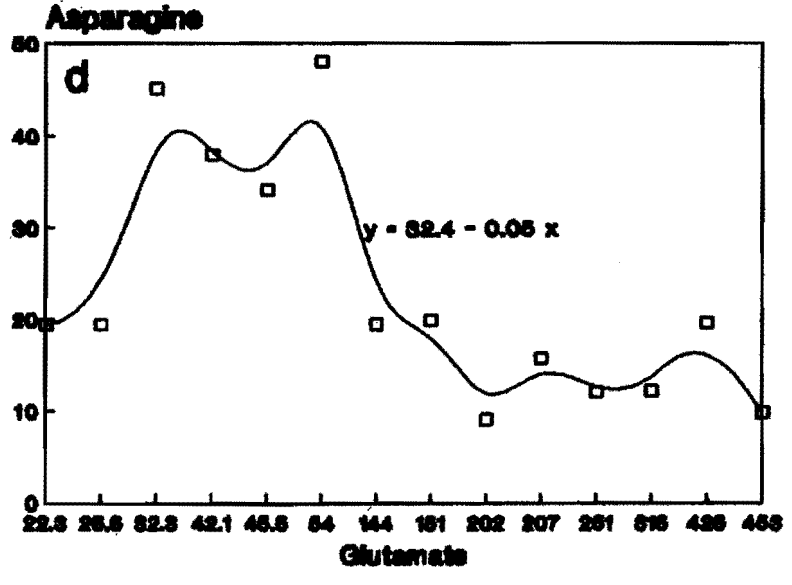


Fig 2. 2c and 2d: Similar regresion line and curve are observed between glutamate and asparagine (see text).

the increment of its corresponding alfa-ketoacid analogs, which has not been measured in autistic individuals, and perhaps contributes with the presence of metabolic acidosis. In our patients, plasma bicarbonate was below 18 mEq/lt and the mean value for the anion gap was above this figure, revealing in this group of children the presence of a mild metabolic acidosis.

Vitamin B6 is also requiered by glutamate decarboxylase, which is needed for the synthesis of gamma-aminobutyric acid (GABA) an important neuronal inhibitor (29, 30). Increments of glutamate via a blockage of this reaction would lower levels of GABA centrally (plasmatic levels of GABA were only mildly decreased in our patients (v. supra) and a neurochemical situation for a hyperexcited neuronal state would arise. The sodium-chloride-coupled-GABA-transporter has been found to be more sensitive to proteolisis in rat brain when availability of GABA diminished (38). If this situation occurs in autism the inhibitory effect of GABA would not be obtained. Due to the hyperexcited state, glucose consumption would be present, leading to by-products such as lactic acid, perhaps contributing with the mild acidemia observed in our patients.

Both glutamic acid and taurine are released by hyperexcited (15, 23, 53, 62) and ischemic neurons (22, 28). This release is calcium dependent and has been more clearly demostrated for glutamic acid (15, 47,60) than for taurine (13, 31, 34, 37, 49, 50, 51, 66, 67, 68, 70). The therapeutic benefitial effects of B6 and magnesium in some autistic individuals (27) could be related to favoring glutamate utilization and GABA anabolism whereas magnesium competes with calcium, helping to sequestrate it inside the mitochondria, favoring taurine excretion and hence helping to diminish neuronal excitability.

Glutamate and tryptophan, (the latter was significatively decreased in our patients (47.6 ± 5.5 uM) as compared to controls (59.3 ± 3.2 uM; ($p < 0.01$) (45)), could give rise to serotonin, thus explaining reported increments of the same in autism (11, 12, 52, 55, 58), and correlation among these compounds should be established in future studies. Serotonin could also increase by other mechanisms: i) via altered phosphorylation of sinapsine I which intervenes in the presynaptic secretion of neurotransmitters (5, 17, 46); ii) via altered relation to its receptor (65).

Altered glutamate receptor or altered sodium-chloride-coupled glutamate trasporter may explain altered brain function in autism and increased levels of the excitatory amino acids; three different glutamate transporters have recently been cloned, which have not significant homology to the members of the neurotransmitter transporter superfamily (26). Our findings perhaps justify studies of glutamate receptors and transporters in autism, especially because the idea of altered neuronal

receptors is compatible with the possibility of inmunological related autism. As occurs in diabetes (71), correlation of autism with infectious disorders has been reported several times (10, 24, 61).

Reported diminished cerebellar vermis size in autism (3), observed in at least one of our patientes, might well be related to glutamate toxicity since glutamate, if not efficiently removed, causes death of neuronal cells; neuronal loss due to glutamate toxicity, perhaps sectorial (4), could be an on going situation in autism.

Others (21, 73) have failed to confirm altered levels of glutamic acid in similar groups of autistic children. However, Zavala et al., (personal communication, 1995) using ionic exchange column chromatography, have recently confirmed our findings in a population with the same ethnic background as that studied by us.

The neurochemical picture in autism, has been difficult to establish in view of the fact, that functional brain studies requiring invassive sampling are seldom possible to be performed in vivo, and confront heavy ethical and practical difficulties. Positron emission tomography scanning (PET scanning) is an exception, not available in many institutions. Even post-morten studies in autistic individuals, usually with a normal lifespan, are very difficult to obtain. In addition, consent for clinical studies such as the one here presented, involving only blood sampling, are frequently difficult to obtain from children whose parents view them only as experimental procedures.

Therefore, peripheral biochemical findings, however scarce and difficult to repeat with the same subjects, have to be interpreted under the light of present knowledge almost generally derived from experimental data in lower animals, from which extrapolation to humans may not be exact. Moreover, statistic prove of the tendencies observed might be difficult to obtain with the small amount of patients studied. Uniformity of methodologies may lead to the possibility of pooling data from different investigators.

Fibroblasts and/or lymphoblasts cell lines developed from children with autism, selected on the bases of genealogical (common ancestry, isonimic ancestors, affected sibs or twins) or biochemical findings (40, 41, 45), may help in exploring the biochemical alterations suspected and suggested here. Molecular biology studies should be highly contributory in this type of families.

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