Childhood Autism: A Complex Disorder

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Twenty-four–hour urine samples from psychotic and autistic children were precipitated with benzoic acid at pH 4.3. Fractionation of the aromatic complexes thus formed with benzoic acid—protein and peptides and uric acid, proteins and peptides on G-25 columns—yielded filtration patterns that may be of diagnostic value. Peptide material could be extracted from the formed complexes and refractionated on P2 gels. Increased levels of peptide material, especially of N-substituted peptides, could be demonstrated. Several bioactive factors (Reichelt et al. 1981) are under study. Possible etiological factors are discussed, and a working hypothesis is presented.

Introduction

Childhood autism was first described by Kanner (1943) and is, according to DSM-III, an affliction starting before the age of 30 months. Pervasive childhood developmental disorder (DSM-III) has very similar symptoms, but starts after the age of 30 months. There is increasing evidence that autism is a mixed bag of syndromes (Ornitz and Ritvo 1975). Thus, the fragile X syndrome may clinically resemble autism. Likewise, damage to the limbic brain, especially the left temporal lobe, may apparently cause a Klüver-Bucy–like syndrome that is clinically very similar to childhood autism. A genetic disposition is probable (Folstein and Rutter 1977), and a preponderance of boys over girls of 8:1 or 8:2 is usual (Rimland 1964).

We report on the chromatographic patterns obtained with benzoic and uric acid complexes formed with urinary constituents fractionated by G-25 gel filtration columns and as acid-extracted peptides separated on P2 columns. The level of hydrolyzable amino acids, largely peptidic, is found to be considerably increased in the diseased state. A working hypothesis is presented. This work is an extension of earlier reports (Trygstad et al. 1980; Reichelt et al. 1981).

Methods

Table 1 lists the diagnostic symptoms. The diagnosis of definite childhood psychosis with autistic trait demanded one symptom from each category to be present. The children were all referred by psychiatrists with the diagnosis of infantile psychosis with autistic traits. Using the urinary pattern as a guide, we quickly found the three patterns presented. Type

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Table 1. Symptoms of Autistic Syndromes

Emotional	1. Social isolation—marked reduced playing with others
	2. Decreased eye contact: sees through you
	3. Withdrawn and emotionally indifferent to persons
	4. Infants do not conform to body of lifting mothers
	5. Inappropriate emotional discharges (anger, giggling, grief)
	6. Panic attacks caused by trivia
Cognitive	7. Poor discriminatory ability: part–whole confusion
	8. Strong resistance to learning (behavior, tasks)
	9. Poor abstracting ability—concrete thinking
	10. Preservation of sameness (rituals, surroundings)
	11. Lack of fear of real dangers
Attention	12. Appears deaf without being deaf
	13. Extreme focussing on peculiar trivia
	14. Unusual interest in objects that can be put into rotatory movement
	15. Priority of proximal over distal sensors
Language	16. Expresses wishes by gestures
	17. Pronoun reversal
	18. Answering questions with the question
	19. Ecolalia
	20. Mutism
Motor	21. Physically, mostly hyperactive
	22. Peculiar gait, stance (toe walking, striatal toe)
	23. Stereotyped movements and mannerisms
Sensory	24. Decreased sensitivity to pain
	25. Decreased or no postrotatory nystagmus

A: a relatively normal peak from 600 to 900 ml and a stepwise peak increase from 1100 to 1600 ml with a large late peak. Type B_1: large peak from 600 to 900 ml and two small peaks at 1100–1500. Type B_2: only a large 600–900 ml peak (Figure 1); also, the two peaks from 600 to 900 ml are confluent or elute as one peak; no late peak is present. The only consistent difference that could be found was the presence of a normal premorbid period and more aggressive and/or hypermotoric behavior in type A pattern patients. Average age of type A patients on examination was 7.2 ± 3.1 years (n = 79), with a range of 3–18 years. Except for onset before the age 30 months, type A covered all subjects with a diagnosis of childhood-onset pervasive developmental disorder (DSM-III).

B_1 and B_2 fitted infantile autism criteria completely (DSM-III). At the time of examination, type B_1 was 6.4 ± 4.5 years old (n = 16), and type B_2 was 7.9 ± 5 years old (n = 50). The difference between groups is further discussed in the Results section.

As is quite clear, the diagnostic criteria are very close to DSM-III, but contain a few added items. A 24-hr urine sample was collected after voiding the morning urine of the first day. The urine was kept frozen if possible; if not, 0.5 g thymol was added and the urine was kept at 4°C. No difference was observed between the frozen and the cooled urine samples if the latter were delivered for processing within 48 hr. We chose a 24-hr urine, as considerable variation in hormone excretion over 24 hr is known to be usual. In disease, changes in circadian rhythm may be expected. The children were without

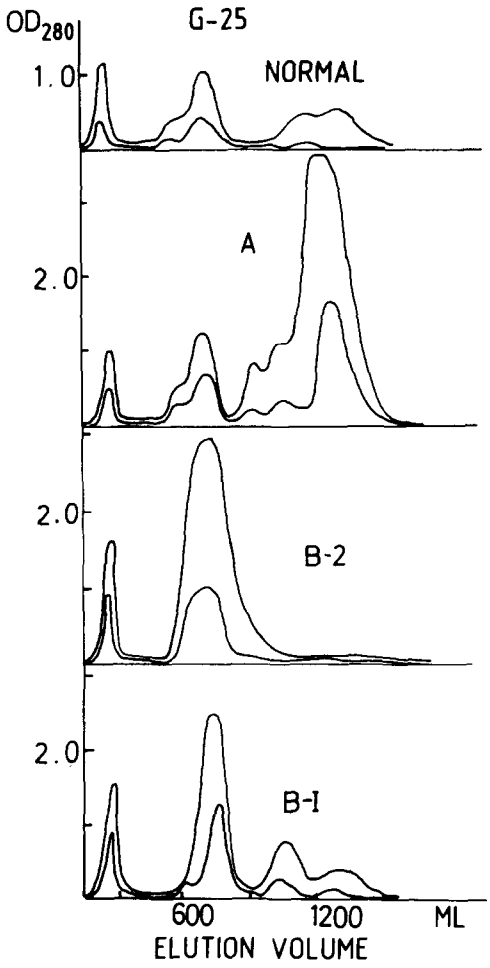


Figure 1. The Sephadex G-25 gel filtration patterns obtained running the benzoic acid-precipitated glycoprotein–peptide–benzoic acid or uric acid complexes as described. The range of the normal group and typically high and low total compound containing samples are demonstrated for the patient groups. For statistical data, see Table 2. Twenty-four–hour complete urine samples are the starting material. Of type A, 59 fitted the diagnosis of delayed-onset infantile autism and 20 that of childhood-onset pervasive developmental disorder. Types B_1 and B_2 are both infantile autists by the DSM-III criteria and are apparently present from the neonatal period onward. The peak from 600 to 900 ml is made up of glycoprotein–peptide–benzoic acid complexes; the later peak of similar complexes with purines is mainly uric acid. The $UV_{280\ nm}$ trace therefore indicates the level of aromatic complexes formed. The aromatic interaction with the gel is thought to cause retention even of macromolecules on the G-25 columns.

drugs at the time of urine collection and had been so for a minimum of 14 days. No dietary restrictions were observed. To avoid institutional bias, urines were collected from all three Scandinavian countries and also from autistic patients at home with their families.

Normal controls were children in the hospital for minor anomalies (hypospadia) and school children and members of an athletic club all within the same age range (details on the latter groups before and after exercise, etc., will be published separately).

Urine Precipitation

The benzoic acid precipitation described by Chalmers et al. (1960) was used as modified by us (Trygstad et al. 1980). A 24-hr urine sample was adjusted to pH 4.3 with HCl and precipitated with 10% by volume of ethanol saturated with benzoic acid. The pH was then again adjusted to 4.3 and the sample was left overnight in the cold room (4°C) with one stirring. The supernatant was carefully decanted, and the rest centrifuged at 3000 × *g* for 20 min at 4°C. The precipitate was washed with ethanol, containing 2% methyl isobutyl ketone by volume, by stirring and recentrifugation until the OD_{280} of the supernatant was between 0.3 and 0.4 (with a 1-cm lightpath). Further washing apparently

decreases recovery of benzoic acid–glycoprotein–peptide complexes (Reichelt et al. 1981). Insufficient washing causes benzoic acid to dominate the middle peak in the gel filtration chromatogram (Figure 1).

Gel Filtration

First, the precipitate from a 24-hr urine sample was extracted with 40 ml of NH_4HCO_3 buffer, 0.1 M, pH 8.5, and centrifuged at 3000 × *g* for 20 min. The supernatant was then applied on to a Sephadex G-25 fine-gel column of 2.6 × 160 cm. The gel filtration was carried out in the cold room with the same bicarbonate buffer at a rate of 30 ml/hr. Fractions of 15 ml were collected, and continuous $UV_{280\ nm}$ recording was provided by an Isco UA-5 monitor. The supernatant must be applied to the column immediately after centrifugation, or in some cases, uric acid may slowly dissociate from complexes formed and precipitate out. Without complex formation, very little benzoic acid or uric acid is soluble in the NH_4HCO_3 buffer.

Second, the fractions containing material identified by the $UV_{280\ nm}$ trace were lyophilized and reextracted with 0.5 M acetic acid in a volume not exceeding 10 ml. The supernatant was then applied to a Biorad P2 gel (100–200 mesh) column of 1.6 × 90 cm and developed in 0.5 M acetic acid at a rate of 18 ml/hr. Fractions of 3 ml were collected and the UV absorbtion of the eluate measured at 280 and 254 nmol on an Isco UA-5 monitor. To detect peptidic material, 10% aliquots of each fraction were dried overnight, hydrolyzed in 0.2 ml 2-*M* KOH for 2 hr in a boiling water bath, neutralized with HCl, and the ninhydrin color developed in an acetate/cyanide buffer (Reichelt and Kvamme 1973). The sensitivity was 0.060 $OD_{570\ nm}$ units, which is equivalent to 10 nmol amino acid. With a molecular absorption coefficient at 570 nmol close to unity for most amino acids, a reasonably accurate estimate of the amount of amino acid liberated by hydrolysis of peptide bonds and/or *N*-substituted peptides can be obtained. Cyclic peptides and pyroglutamyl peptides can also be detected by this method. That the released amino groups are amino acids has also been checked by amino acid analysis on the Chromaspeck amino acid analyzer. Further purification of the bioactive peptide fractions has been carried out as described (Reichelt et al. 1984). It should be noted that high-pressure liquid chromatography (HPLC) of peptides at this early stage is impossible due to the enormous complexity of the urinary material. The bioactivity assays and their purification will be described in a separate paper.

Material corresponding to the void volume of the P2 gels (not shown) was run by crossed-immunoelectrophoresis against antibodies to whole serum, and the number of precipitation lines counted (see Table 3) (Weeke 1975b). Further tests of the protein nature were carried out by rocket technique on agarose plates against commercially available antibodies (see Table 3) (Weeke 1975a). The sample was applied in 10 μl buffer to each well. Acrylamide gel electrophoresis also demonstrated the glycoprotein-like nature of the proteins (unpublished data). The lowest protein standard used 10 μl of a 0.003 mg/ml albumin. Coloring was carried out using Coomassie brilliant blue B (Weeke 1975).

Results

Figure 1 illustrates the range of the normal patterns of aromatic glycoprotein–peptide complexes found in urine using the method described. In autistic children, we find three patterns, arbitrarily named A, B_1, and B_2. The peaks at 600–900 ml contain glycopro-

tein–peptides and benzoic acid (Reichelt et al. 1985; Sælid et al. 1985). The late peaks contain various purine bases, mainly uric acid in complexes with glycoproteins and peptides (Reichelt et al. 1981; Sælid et al. 1985). The complex formation with aromatic compounds probably explains the retention of the glycoproteins on the G-25 gel filtration.

Of the children who satisfied the DSM-III criteria of childhood infantile autism with onset before the age of 30 months, we found that 59 had pattern type A, 16 type B_1, and 50 type B_2. However, anamnestically, it became quite clear that in those with type A pattern, all but one had taken ill after a lucid period or a period of normal development. The rest of our type A children (20 children) fitted the diagnosis of childhood-onset pervasive developmental disorder (DSM-III). In all type A children except one, the parents reported stomach upsets and persistent crying. Anamnestic data of retrospective and low reliability inidicated a relationship to increased intake of grain, especially bread/porridges. Average age of onset of type A patients was 1.8 years, with a range from 5 months to 8 years of age. Childhood schizophrenics could not be separated chemically from the type A patterns (unpublished data). We also have problems in clinically differentiating this disorder from childhood-onset pervasive developmental disorder.

All of the type B_2 patients were clearly identical to infantile autism as described by DSM-III and Rimland (Rimland 1964), and all were reported ill from the early neonatal period onward. All of our highly functioning autists (with language and attending ordinary school) were in this group. Again, anamnestically, reports on the use of cow's milk in various mixes were apparently related to the aggravation of the disorder (36 of 49). We lack information on one patient. However, these data were collected after the event and may be less than reliable. Other researchers have also noticed intestinal upsets and frequent crying and unrest in the infants during the first years of life (Rimland 1964).

Type B_1 was also closely allied to type B_2 in that the disorder was present from the early neonatal period (no lucid interval). However, although the urinary pattern was different (having late peaks), we could not find any significant difference in the anamnestic data or symptoms. Two children with left temporal lobe cysts on computer tomography and with clinically autistic-like symptoms had a pseudonormal chromatogram. Another with a normal pattern had atrophy of the left temporal lobe.

The quantitative data for the different groups can be seen in Table 2. The area (in square centimeters) enclosed by the $UV_{280\ nm}$ trace and the abscissa is taken as a measure of the amount of complexes found. The peaks from 600 to 900 ml are different from the normal, with $p < 0.01$.

The lower half of Table 2 gives the measure of nanomoles of amino acid groups found after alkaline hydrolysis in the normal and disordered children (see also Figure 3). Again, the difference from the normal is significant at $p < 0.01$, using the two-tailed Wilcoxon ranking test. The typical P2 gel filtration curves on which Table 2 is based can be seen in Figures 2 and 4. The ninhydrin color obtained by hydrolysis of 10% aliquots of each fraction of 3 ml obtained and read at 570 nm is shown. Again both the pattern and the level of compounds found is clearly different from normal. Also, the variability from one patient to the next is more apparent on this further fractionation and is more in accord with the variability found clinically in the patients. The typical K_{av} values for the peak 3 peptides on P2 gels were: type B_2—0.81, 0.67, 0.89, 1.18, 0.47, 0.56; type B_1—0.80, 0.67, 0.56; and type A—0.76, 0.63, 0.89, 1.15, 0.47, 0.56, where K_{av} = (elution volume − void volume)/(total volume − void volume).

As an example of the variation, the peak maximum at 153 ml for type A (K_{av} = 0.76) varied for 79 patients, 153 ± 4 ml (± SD).

The nature of the protein component found in the void volume and partly precipitated

Table 2. Quantitative Data

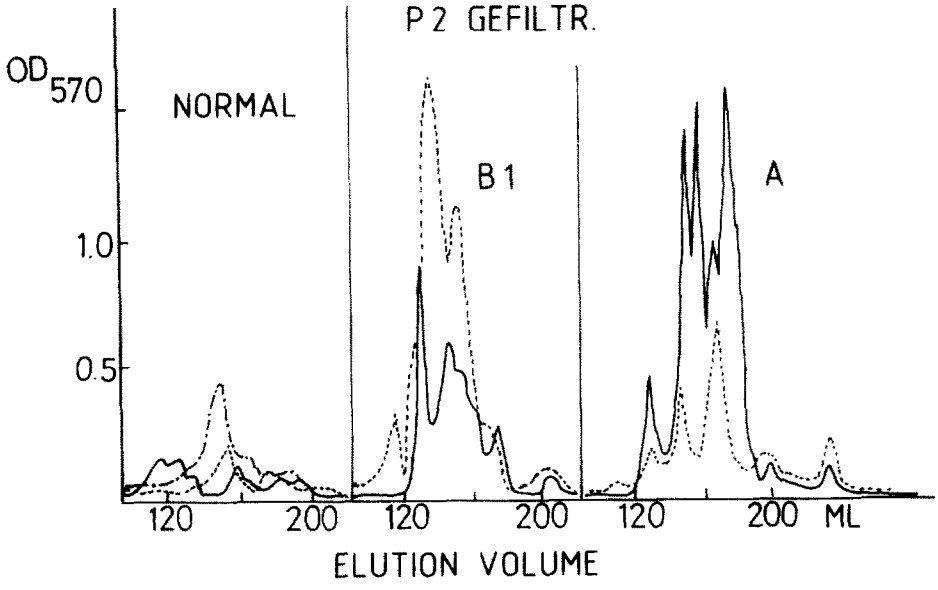
Diagnosis	n	Peak 600–900 ml	Range	1100–1500 ml	Range
UV_{280} Trace-enclosed peak area (cm^2)					
Normal					
age 8–18	27	7.9 ± 3.7	2.8–12.6	4.5 ± 2.8	0.0–11
Type A	79	14.3 ± 8.4[a]	6.0–23.0	64.1 ± 27[b]	38.0–147
Type B_1	16	23.2 ± 6.7[b]	18.0–48.0	8.8 ± 4.0	4.4–23
Type B_2	50	28.4 ± 7.5[b]	12.0–54.0	—	—
Micromoles amino acid following hydrolysis					
Normal					
age 8–18	27	6.9 ± 3.6	2.5–10.5	7.4 ± 2.1	0.0–12
Type A	79	39.3 ± 21[b]	11.0–94.0	39.3 ± 21[b]	28.0–149
Type B_1	16	35.2 ± 12.2[b]	9.2–67.0	5.6 ± 4.2	1.0–27
Type B_2	48	26.9 ± 15.2[b]	8.0–81.0	—	—

The area in cm^2 enclosed by the $UV_{280\ nm}$ trace is shown in the first half of the table (± SD). The 600–900-ml peak contains glycoprotein–peptide–benzoic acid complexes. The 1100–1500 peak has similar complexes, but with various purines, mainly uric acid. The micromoles of amino acid are all calculated from the P2 gel filtrations for material smaller than 2000 daltons in mol wt separated in 0.5 M acetic acid. Age range of patients: type A, 3–18 years; types B_1 and B_2, 3–24 years.

[a]Significant at the $p < 0.025$ levels.

[b]Significant at the $p < 0.01$ level.

Figure 2. The P2 gel filtration pattern of the middle peak (600–900 ml) material from G-25 runs. The columns were run in 0.5 M acetic acid, which dissociates the complexes retarded on the G-25 columns. This step separates out proteins (not shown) and peptides and amino acids, whereas the aromatic compounds, benzoic acid, and purines are retained. The level of ninhydrin material released after hydrolysis of 10% aliquots of each fraction measured at 570 nm as ninhydrin-colored material is shown (10 nmol = 0.06 OD_{570} units in sensitivity). Typical high and low values for the patients and three randomly picked normals are shown. Note considerable microheterogeneity.



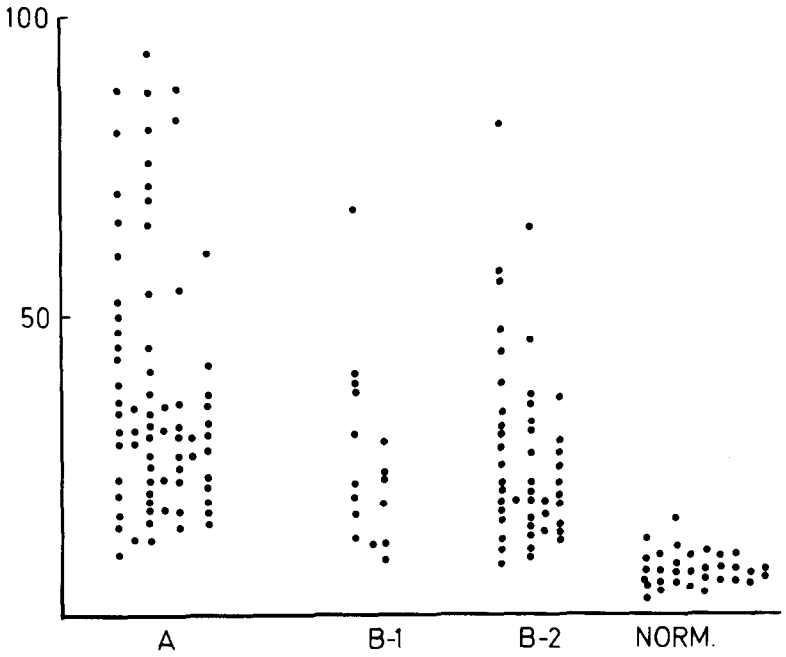


Figure 3. The scatter diagram of the micromoles of hydrolysis-released amino acids found in the 600–900 peak of material from the G-25 runs. For statistics see Table 2.

Figure 4. To the left is the P2 gel filtration pattern obtained by running the material from the 600–900-ml peak on G-25 of two infantile autists, type B_2. To the right is the pattern obtained for a patient with attention disorder without hyperactivity. B_2 infantile autists and attention disorder without hyperactivity cannot be distinguished on G-25 columns alone. Clinical and especially skin conductance data are very different, however (Mørkrid et al. 1986).

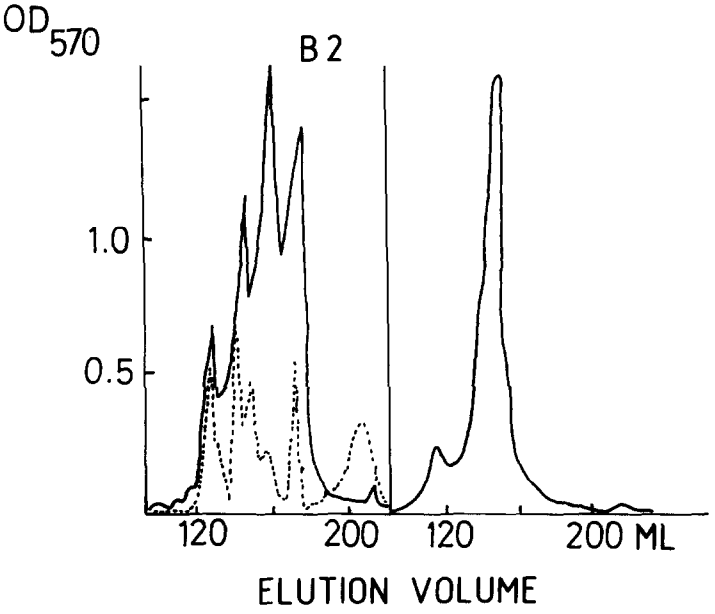


Table 3. Immunologically Determined Proteins from the 600–900-ml and 1100–1500-ml G-25 Fractions after P2 Gel Filtration for Type A Patients (n = 11)

Antibody tested	Frequency	600–900-ml Peak	1100–1500-ml Peak
Lysozyme	10/11	+	+
Alpha-1-acid glycoprotein	11/11	+	+
Albumin	6/11	Traces	Traces
IgG-γ chain	11/11	+	+
IgG-Fc fragment	9/11	+	+
Prealbumin, alpha-1-aminotrypsin, kappa-free light chains, lamda-free light chain, transferrin, ceruloplasmin, fibrogen, plasminogen, Bence-Jones protein, haptoglobin, B_1Ap_1-C-C_3 complement, IgE, neurophysin		All negative	All negative

In crossed-immunoelectrophoresis against antibodies raised to whole serum, we found one precipitation line for the 600–900-ml peak material and four for the late peak (Weeke 1975). In normal urines, alpha-1-acid glycoprotein and lysozyme are the dominant protein components (n = 7). Probably several mucopolysaccharides are present, too.

after acetic acid extraction of the peaks of material found on G-25 filtration is listed in Table 3. The rocket technique against commercially available antibodies was used to identify the proteins. It is interesting that only in autism have we found antibody chains in our peaks. Usually, alpha-1 acidic glycoprotein is the dominant protein found in normal individuals and those suffering from depression and schizophrenia (Reichelt et al. 1981; Sælid et al. 1985).

The P2 fractionated compounds are peptides because they yield an increase in ninhydrin colorable material on hydrolysis. This is confirmed by amino acid analysis. When purifying the factors present (Reichelt et al. 1981), we end up with active fractions containing only one *N*-terminal, usually *N*-substituted, amino acid with a clear-cut 205 nmol absorbtion on HPLC runs. Two such isolated peptides have been published (Reichelt et al. 1978, 1984). The bioactive compounds and their structures will be published separately.

Discussion

The aromatic complexes found clearly indicate three subtypes of the autistic syndrome. The pattern found with infantile autism type A and also in pervasive childhood development disorder is very similar to slow-onset schizophrenia both on G-25 and P2 runs (Reichelt et al. 1981, 1985). The type B_2 autism G-25 chromatographic pattern is difficult to differentiate from schizophrenia type 2 (acute onset) and from several other psychiatric disorders (anorexia type 1, temporal lobe epilepsy, attention disorder without hyperactivity). However, P2 gel filtration provides the additional information necessary. Attention disorder without hyperactivity has the most similar pattern on P2 gel filtration. In Figure 3, the P2 pattern of autism type B_2 can be compared with that of attention disorder without hyperactivity (Hole et al. 1986). Fortunately, spontaneous changes in skin conductance and basal skin conductance and habituation are at the opposite functional ends in the two groups, thus facilitating diagnosis (Mørkrid et al. 1986).

It is not very surprising that the urine reflects metabolic and/or possibly central nervous system (CNS) states. In normal mammals, the urinary peptide levels were found to reflect the endogenous metabolism more than the dietary input (Ansorge and Hansson 1967; Noguchi et al. 1982), and the excreted peptides were resistant to enzymatic breakdown

(Hansson and Ansorge 1967; Noguchi et al. 1982). ICV injection of homocarnosine results in extensive recovery of this dipeptide in the urine (Ziesler et al. 1984). Increased urinary levels of releasing factors in hyperfunctional or active states are known. Thus, leutinizing hormone–releasing hormone (LHRH) surges have been found in prepubescent children (Root et al. 1977) and also during the menstrual cycle (Bourguignon and Franchimont 1977). In diseased states, very large increases in urinary peptide secretion have been noted. Thus, hydroxyproline peptides are found in states of increased collagen breakdown (Weiss and Klein 1969), and large increases in peptide levels have been found in Fanconi's syndrome (Asatoor et al. 1977). In dermatomyositis, increased peptide secretion has been reported (Gross and Maskaleris 1973). An insulin-releasing peptide that is active only at high glucose levels has been found in congenital generalized lipodystrophy (Reichelt et al. 1984), and a tripeptide that blocks the cephalic phase of digestion (Coy et al. 1981; Schally et al. 1982) has been described (Reichelt et al. 1978).

Where do the peptides come from? We do not know. However, increased peptide levels indicate that there may possibly be an insufficiency of breakdown capacity or peptidase levels. The well known releasing factors seem to be regulated by peptidase levels governed by peripheral feedback (Griffiths et al. 1975; Griffiths 1975; Bauer 1976; Kuhl et al. 1978). Feedback control is usually the critical step in any chain of processes. Peptidase deficiency is also known to cause glutathionuria when glutamyl transferase of the gamma-glutamyl cycle is inhibited (Griffiths and Meister 1979).

However, peptides could derive from external sources. Peptidase deficiencies of the gut mucosa may cause insufficient breakdown of protein fragments, as is found in celiac disease (Bronstein et al. 1966). Peptides are taken up across the mucosa (Gardner 1983; Matthews and Burston 1984). In a case of cystic fibrosis, very large increases in the urinary levels of pyroglu peptides have been found (Wauters and Van de Kamer 1978; Knudtzon J, personal communication). Furthermore, gluten (Zioudrou et al. 1979), alpha- and beta-casein in milk (Loukas et al. 1983; Brantl and Teschemacher 1979) at duodenal pH and by enzymatic digestion with pepsin, chymotrypsin, and trypsin form large numbers of peptides of the pyroglu type and also exorphins. Phosphorylated peptides from milk casein are very resistant to enzymatic breakdown (Mellander and Følsch 1972) and are taken up across the mucosa. It is therefore possible that combined effects of exogenous peptides and endogenous peptides are seen, which are both due to peptidase deficiencies. Peptides generally are good inhibitors of enzymatic peptide degradation, and the exogenous peptides may thus further inhibit peptide breakdown endogenously. Beta-endorphin inhibits enkephalinase (Hui et al. 1982), and substance P inhibits angiotensin-converting enzyme (McGeer and Singh 1979). Peptides injected outside the CNS may also reach the brain (Kastin et al. 1978; Ehrensing and Kastin 1980). Actual finding of gluten fragments in brain has also been reported (Hemmings 1978). The possible relationship of gluten to schizophrenia has been forcefully argued by Dohan (1983). A clinically verified case is also known (Jansson et al. 1984). Gluten related to autism has been reported (Marchi et al. 1974) and in an NIMH sponsored investigation (Coleman 1976).

It should be noted that using independent techniques, increased urinary peptide secretion in childhood autism has been found (Israngkun et al. 1983, 1984). They also found bioactivities similar to those reported by our group (Reichelt et al. 1981; Reichelt 1982). A preliminary report on the possible specificity of the urinary patterns to disease was written by K. L. Reichelt (Trygstad et al. 1980).

Gilberg et al. (1982) reported on the urinary patterns found in mainly institutionalized (83%) children with autism. Pattern A was found in 54%. However, it is difficult to compare the data, as our procedures are different. If we wash the precipitate until no

$UV_{280\ nm}$ is left in the supernatant, no 600–900-ml peak is found. We have therefore standardized the procedure. As described, we also have found that the late peak is made up mainly of glycoproteins, peptides, and uric acid, and it is indeed the aromatic coating that causes retention on gel filtration. It is also vital that the ethanol should contain 2% by volume of methyl isobutyl ketone. Finally, 24-hr urine samples for 145 patients of 1.3 ± 0.7 liters are rather different from 0.7–1 liter collected over 48 hr. Due to very high variation, we think that schizophrenia type 1 may be part of the type A family. We did not find any B_2 patients before also including autists living at home (Reichelt et al. 1984). Some B_2 patients can only be differentiated from normals by the P2 peptide chromatography.

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