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POTENTIAL BIOCHEMICAL MARKERS FOR INFANTILE AUTISM

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POTENTIAL BIOCHEMICAL MARKERS FOR INFANTILE AUTISM

DISSERTATION

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By

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To My Mother

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PUBLICATIONS AND PRESENTATIONS

Israngkun, P.P., Newman, H.A.I., Patel, S., Duruibe, V., and Abou-Issa, H. (1986) Potential biochemical markers for infantile autism. *Neurochemical Pathology* (in press).

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LIST OF ABBREVIATIONS

ALT ..... Alanine transaminase

AST ..... Aspartate transaminase

BUN ..... Blood urea nitrogen

COMT ..... Catecholamine-O-methyl transferase

CNS ..... Central nervous system

CARS ..... Childhood autism rating scale

DSIP ..... Delta sleep-inducing peptide

DSM-III ..... Diagnostic and statistical manual of mental disorders-III

DHBA ..... Dihydroxybenzylamine

DBH ..... Dopamine-ß-hydroxylase

EEG ..... Electroencephalogram

EDTA ..... Ethylenediaminetetraacetic acid

HPLC ..... High performance liquid chromatography

HVA ..... Homovanillic acid

5-HIAA ..... 5-Hydroxyindoleacetic acid

5-HT 5-Hydroxytryptamine

K_m ..... Michaelis-Menten constant

LHRH ..... Leutenizing hormone releasing hormone

MAO ..... Monoamine oxidase

nm ..... Nanometer

PITC ..... Phenylisothiocyanate

PKU ..... Phenylketonuria

PTC ..... Phenylthiocarbamyl

PTH ..... Phenylthiohydantoin

V_{max} ..... Maximum velocity

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CHAPTER I

INTRODUCTION

Introduction

Autism is a syndrome of childhood characterized by a lack of social relationship, a lack of communication abilities, persistent compulsive rituals, and resistance to change. A child with these characteristics does not relate to surrounding people, preferring instead to play repetitively with an object, a toy, or his or her own body. Language, if present, is severely impaired, but the child is aware of the environment. An emotional upset or temper tantrum may result if the environment is interfered with. The syndrome's onset is usually in infancy, but certainly evident in the first three years of life.

The definition of childhood autism was described by Kanner (1943) after his careful and systematic observations on eleven children with previously unrecognized syndrome. There were a variety of behavioral features which seemed to be characteristic of all children but differ from children with other psychiatric disorders. These features included an inability to develop relationships with people, a delay in speech acquisition, the non-communicative use of speech after it developed, delayed echolalia, pronominal reversal, repetitive and stereotyped play activities, an obsessive

insistence on the maintenance of sameness, a lack of imagination, a good rote memory and a normal physical appearance.

Clinical Diagnosis

The autistic syndromes (Coleman, 1976) are variously referred to as childhood autism (Wing, 1980), infantile autism (Rutter, 1978) and childhood psychosis (Fish and Ritvo, 1979). The criteria for infantile autism currently agreed upon by most authorities are:

1. **Early onset** - Autism is probably present at birth. Very careful history-taking usually elicits the fact that subtle signs did indeed occur during the first year of life. These may be forgotten, overlooked, or denied due to parental anxiety or unfamiliarity with normal development. It is accepted by most clinicians and researchers today that this behavioral disorder began prior to the age of 30 months (American Psychiatric Assoc., 1980; Rutter, 1978).

2. **Severe disturbance of social relatedness** - The central features of this disturbance seem to be a lack of social interaction with other humans and a failure to recognize the uniqueness of other human beings (Wing, 1980; Rutter, 1983). However, the abnormalities of social relatedness become more subtle as the child grows older.

3. **Severe abnormalities of language development** - From a very early age, the autistic child usually shows major problems in the comprehension of human mime, gesture, and speech as well as a deficiency in social imitation. Almost without exception, autistic children are delayed in

their development of spoken language, and approximately one out of every two children fails to develop useful spoken language altogether.

4. **Elaborate repetitive routines** - Autistic children often form bizarre attachments to certain objects, and the majority of the children demand that certain routines be adhered to in a pathologically rigid fashion.

5. **Abnormal perceptual responses to sensory stimuli** - Disturbances of perception are most likely due to an underlying neuropathophysiological process that is common to all autistic patients (Tanguay, 1978). It results in faulty modulation of external sensory input (Ornitz and Ritvo, 1968; Ornitz et al., 1970), distortion of the normal hierarchy of receptor preferences (Goldfarb, 1956), and an impaired ability to use internal sensory input to make discriminations in the absence of feedback from motor responses (Hermelin and O'Connor, 1970).

The failure of adequate modulation of sensory input constitutes a striking and unique aspect of autism (Ornitz and Ritvo, 1968; Goldfarb, 1956). Behaviorally, there are hyporesponsive or hyperresponsive states which alternate in the same child (Goldfarb, 1961; 1963). Sound stimuli elicit the most characteristic abnormal perceptual response encountered in autistic children. Many children cover their ears to shut out even ordinary noise levels. Abnormal sensitivity to pain and abnormal responses to visual stimuli are also often encountered in autism. Autistic children frequently want to smell people and objects, a characteristic that is not commonly displayed in either normal developing or in mentally retarded persons. Clinical experience suggests that perceptions relating to auditory and tactile stimuli are more impaired in autistic children than are perceptions of visual and especially olfactory stimuli.

Autistic children may also demonstrate the following complicating handicaps: A majority (67 to 81 percent) of all autistic children are operationally mentally retarded (Lotter, 1966; Rutter, 1978; Wing, 1980; Gillberg, 1984). This retardation is not caused by motivational factors in the child and remains relatively stable over the years with or without improvement in the autistic behavior problems. Speech-language competence on the whole is closely correlated with the IQ level. Severely retarded children are less likely to have any speech, whereas more operationally intelligent autistic children are the ones most likely to demonstrate a wide variety of elaborate repetitive routines and to exhibit savant capabilities in circumscribed areas. Some autistic children have an early onset of seizures. Indeed, infantile spasms are often followed by the development of autistic syndrome even in infancy (Taft and Cohen, 1971; Riikonen and Amnell, 1981). Rather more common, however, is the development of any type of epilepsy at or near the time of puberty. One third of all autistic children (Rutter, 1970; Gillberg, 1984) develop seizures. It is a more common phenomenon among the mentally retarded autistic children, but it can occur at all levels of intelligence (Corbett, 1983). Blindness and deafness are also encountered among autistic children (Rapin, 1979). Based on clinical experience, it would seem that deafness is more likely to show a primary connection with autism than blindness is. Throughout childhood, most autistic children show deviance reactions to sound, whereas deviance to light appear to be less conspicuous, at least in later childhood. Furthermore, among autistic individuals there are more clinically proven cases of deaf rather than blind people (Coleman and Gillberg, 1985).

Although autism resembles schizophrenia in emotional withdrawal, endogenous initiation of activity independent of external inputs, and tendency to repeat the same behavior and mannerism (Trygstad et al., 1980), the syndrome is clearly distinct from other mental disorders (Rutter, 1978). Autism is estimated to be present at 12 in 10,000 live births and in select populations can be as much as 16 in 10,000 live births (Wing, 1966; 1976). Lotter (1966) found the number to be 4 in 10,000 live births and the male to female frequency was 4 to 1.

The classification and diagnosis of infantile autism depend on the individual investigator's ideas concerning etiology and the underlying meaning of various symptoms. Since Kanner (1943) first classified autistic persons as being different from those with other neurological disorders but having basically normal intelligence, any child who met Kanner's criteria but exhibited neurologic signs and mental retardation was ruled out as being autistic. As more knowledge was gained about the syndrome, it became well accepted that autism could be complicated by neurological dysfunction or mental retardation or both (DeMyer et al., 1981). Diagnostically, the important development of the 1970's for the classification and diagnosis was the new Diagnostic and Statistical Manual of Mental Disorders-III (DSM-III, American Psychiatric Assoc., 1980) which was multi-axial and listed infantile autism as a subclass under pervasive developmental disorders. Under this method, a child is considered autistic if he/she meets all of the following criteria: onset before age 30 months, serious lack of social response, language deficit (gross), speech peculiarity, and no delusions/hallucinations. The other method that has received considerable research attention is the Rimland's E-2 checklist (Rimland, 1971). Only those with a score of +20 and

above are labeled autistic and those who scored -42 to +20 are called autistic type children. The reliability of this checklist is questioned because it relies solely on the parents scoring the items based on the child's behavior in the first 5 years of life. Since the parents' response to the questionnaire may take place at a much later time, there is a possible attendant loss of recall of their child's behavior patterns. The most recent classification system is Childhood Autism Rating Scale (CARS) introduced by Schopler et al. (1980). They use the broadest concept of autism encompassing the features found in other methods but insist in the inclusion of sensory peculiarities as a primary diagnostic feature of autism. It is clear then that a crucial question still remains about which behavioral functions critically define the differences between infantile autism and related conditions. It is now strongly suggested that degree of social distance and degree of communication of speech are the most consistent differentiating rating items (DeMyer et al., 1981). Since none of the rating scales currently used can differentiate normal from autistic children 100 percent of the time, it is important then that these instruments would be more effectively used in conjunction with other methods.

Current research has demonstrated that the etiology of autism may be an organic impairment. The cause of this impairment is unknown, but several different causes may produce the fundamental central nervous system (CNS) defect which gives rise to the symptoms of autism. The fact that autism is present so early in life is in itself suggestive of a biological problem. Although perinatal complications have not been clearly linked to autism, rubella during pregnancy does cause a higher incidence of autism in the offspring (Chess et al., 1971). Other conditions in children which affect the

CNS such as meningitis (Knobloch and Pasamanick, 1975), encephalitis (DeLong et al., 1981), tuberous sclerosis (Valente, 1971), and phenylketonuria (Friedman, 1969) have been reported to be associated with autistic patterns of behavior. When autistic children are evaluated and followed, they frequently show signs of neurological dysfunction. In terms of intelligence, most, though not all, autistic children show IQs below the average range (below 80). As already mentioned, epilepsy is more common in autistic than in normal children, becoming frequently evident clinically in adolescence or young adulthood. Electroencephalogram (EEG) studies done on autistic children have shown abnormalities even in those children who do not demonstrate a seizure disorder clinically (Tanguay, 1976).

Various soft neurological signs have been described at times with autistic children. Soft signs refer to neurological abnormalities which do not pinpoint a specific area of neurological damage. Such signs are often associated with CNS damage or dysfunction. Mobility problems such as toe-walking or hand flapping may be viewed in this category (Vilensky et al., 1981).

With regard to etiological and therapeutic considerations, none of the checklists or categorical systems which have been proposed in the last decade seem to be of ultimate usefulness. Thus, continued work toward devising a more useful diagnostic system is required. Since, as previously indicated, autism is associated with biological dysfunction, a search for the biochemical markers is crucial.

Biochemical Studies

Most of the biochemical investigations in autism have been concerned with neurotransmitters, though a few other types of biochemical studies have also been done. The neurotransmitters can be viewed as chemical mediators which affect muscular contraction and nerve activity. These mediators have been investigated in a number of psychiatric conditions such as depression and schizophrenia since it is known they affect moods, emotions, and thought processes. A mediator's excess or deficiency or even an imbalance between two different mediators may cause disturbed behavior.

One of the first biochemical studies done on autistic patients was the examination of the levels of serotonin (5-hydroxytryptamine, 5-HT) in the blood. 5-HT is present in several different body tissues: the entero-chromaffin system of the gastro-intestinal tract, the spleen, the platelets in blood, and the central and peripheral nervous systems.

When 5-HT is measured in the blood, it is not a direct measure of the CNS serotonin. Serotonin synthesized outside the CNS does not cross into the brain because of selective active transport (blood brain) barriers. CNS 5-HT is made de novo from tryptophan. Bertaccini (1960) has shown that a total removal of the gastrointestinal tract in the rat has very little effect on brain 5-HT, despite decreasing serum, spleen and lung 5-HT levels. Thus, serotonin in the brain is not directly measurable. In the blood, almost all the serotonin is in platelets. However, the platelet in the blood may be used as a partial model for a neuronelevatedrst study of serotonin in autistic patients was done by Schain and Freedman (1961), who reported elevated levels of endogenous serotonin in whole blood. Other studies confirmed that serotonin in whole blood tends to be eleveted in cases "variously diagnosed

as early infantile autism, atypical ego development, symbiotic psychosis and certain cases of childhood schizophrenia." (Yuwiler et al., 1970; Ritvo et al., 1970). Although these studies observed an increase number of platelets in autistic patients, they did not account for all of the increase in serotonin. Further, circadian rhythm of serotonin levels in autistic patients compared to controls did not account for the high level of serotonin either (Yuwiler et al., 1971). Studies of serotonin in other bodyfluids, platelets and sera, also confirmed the high levels of serotonin in autistic patients (Campbell, 1975; Hoshino et al., 1979). Although some autistic patients had high levels of serotonin, only 25% of the autistic population has values exceeding those of the control groups (Goldstein et al., 1976).

In an attempt to understand the basis for elevated levels of serotonin in autistic patients, the mechanism of uptake and binding of serotonin in platelets were undertaken. Siva Sankar et al. (1963) found the uptake of serotonin to be reduced, whereas Yuwiler et al. (1975) found it to be normal, and Rotman et al. (1980) found the uptake to be elevated. The disagreements in the uptake of serotonin among these studies could be due to the different criteria for autism employed as well as the incubation periods. Incubation times, 30-90 minutes, utilized by these investigators encompassed both the active and passive transports of serotonin into the platelets. A recent study on the kinetic of platelet serotonin uptake in autistic and developmental language disorder subjects found V_{max} values to be significantly higher than those of controls, but there were no differences in the K_m values among the three groups (Katsui et al., 1986). Efflux of serotonin from platelets was determined by Boullin et al. (1970; 1971), who employed the Rimland E-2 checklist (Rimland, 1971) for clinical diagnosis.

They found the efflux of serotonin out of the platelets to be increased. Yuwiler et al. (1975), using other clinical criteria for diagnosis, found the efflux to be within the normal range. Since there are such a conflict in the level of serotonin in autistic patients, studies of serotonin metabolites had been done. 5-HIAA in cerebrospinal fluid (CSF) was also found to be either increased (Cohen et al., 1974), decreased (Cohen and Young, 1977; Winsberg et al., 1980), or normal (Gillberg et al., 1983). The differences in the results could be due to the problem with the probenecid methodology. It was pointed out by Cowdry et al. (1983) that the administrative doses of probenecid influenced the levels of 5-HIAA in the CSF. The complete blockage of 5-HIAA from the CSF was probenecid dose dependent.

At the present time, the mechanism that causes high serotonin levels in many autistic children and relatively low levels in another small subgroup of autistic children is unknown. It is likely that it will be best understood when the underlying biochemical or structural mechanism causing the disorder in the particular autistic child is worked out. For example, high serotonin levels in the blood do not necessarily mean high serotonin levels in the brain. One child with leukodystrophy had a number of well documented high blood levels all through his life, but was found to have a low level of 5-HT in the CNS on autopsy (Coleman, 1977). In the case of patients with low serotonin levels, it is now understood that one factor in the low blood serotonin level in phenylketonuric children is due to interference with the metabolic pathway by the presence of an abnormally large number of other aromatic amines of the phenylalanine pathway (McKean et al., 1962).

Another subject of interest in the biochemistry of autistic children has been the catecholamines. Marked reduction of dopamine metabolites has

been found in the CSF of autistic children (Cohen et al., 1974; Shaywitz et al., 1975). A dysfunction in noradrenergic transmission may also be associated with adult or childhood schizophrenia (Wise and Stein, 1973). Cohen et al. (1974) reported that autistic children exhibit pronounced disturbances in those functions associated with noradrenergic pathways such as abnormal patterns of arousal, heightened or diminished sensitivity to noise, certain odors, tastes and changes in temperature or posture, disorganized behavior and unstable perception of both pleasure and pain. Intact sympathetic function has also been questioned because of the reported increase fluctuation in pulse rate in autistic patients (MacCulloch and Williams, 1971). The catecholamine pathway is a metabolic pathway where enzyme errors are known to occur that adversely affect the brain. An error in the first enzyme in the pathway, phenylalanine hydroxylase, causes the disease phenylketonuria (PKU). A significant number of these patients have autistic symptoms. An immaturity in the second step of the pathway can cause a neonatal tyrosinemia, which includes, among its symptoms, later learning disabilities and other CNS problems (Menkes, 1972). Following these two amino acids in the pathway are the three active amines - dopamine, norepinephrine and epinephrine. The first two are vital for good brain function.

The study of plasma norepinephrine levels in autistic patients in both the reclining and standing position was found to be elevated (Lake et al., 1977). These levels are of course outside the CNS and their relevance to brain function is unknown. Urinary epinephrine in contrast compared to controls was found to be lowered (Young et al., 1978; 1979). Studies of both uptake and efflux of dopamine from platelets did not yield a specific finding (Boullin

and O'Brien, 1972). Cerebrospinal fluid homovanillic acid (HVA), the end product of dopamine, was significantly lowered from that of controls (Gillberg et al., 1983). Enzymes of the catecholamine pathways have also been studied in autistic patients. The three enzymes studied, dopamine-ß-hydroxylase (DBH), catecholamine-O-methyl transferase (COMT) and monoamine oxidase (MAO) are all part of the catecholamine pathway. DBH is a possible index of the activity of the sympathetic nervous system in an individual. DBH is stored with norepinephrine in the granular vesicles of nerve endings of the sympathetic nervous system, as well as in the chromaffin granules of the adrenal medulla. At the time that norepinephrine is released into the blood, DBH is released with it, and that is why it is possible to measure it in the plasma. DBH levels in autistic patients were found to be lower than normal (Lake et al., 1977; Young et al., 1980). DBH levels are very complicated to interpret because there is a familial factor. Members of the same family tend to have similar levels, and occasionally there are some families where the levels of DBH cannot be determined at all. Catecholamines are inactivated by O-methylation via COMT which is available in red cells. Studies of this enzyme level was found to be in a normal range (O'Brien et al., 1976; Belmaker et al., 1978; Giller et al., 1980). An important degradation enzyme in the biogenic amine pathway is MAO, which is involved in both the catecholamine and serotonin pathways. A number of studies have been done on this enzyme in platelets, plasma, and fibroblasts showing the results to be within the normal range (Boullin et al., 1976; Roth et al., 1976; Cohen and Young, 1977).

It is clear from the foregoing discussion that biogenic amines are not suitable as specific markers for infantile autism. The difficulty in inter-

preting the results is probably due to the lack of diagnostic specificity and by numerous other factors such as age, sex, intellectual status, and activity level that also affect the various body biochemicals. So it is essential to continue searching for the markers that is more useful in detecting the syndrome as early as possible.

Neuropeptide Studies

In recent years, both the mechanism of action of endorphins and the neurophysiology of underlying mental diseases point to the importance of small peptides in studies of abnormal behavior disorders. Hyperfunction of any pathway or part of the CNS could result in an overflow of peptides to the body fluids and urine. Peptides which may act as neurotransmitters (Emson, 1979) are involved in sleep (Urban and De Wied, 1978), sexual behavior (Moss and Foreman, 1976), learning and recall processes (Bohus et al., 1978) and others. It is also well established that peptides, when exogenously administered, can effect neurotransmitter release, uptake, and metabolism, and also have behavioral consequences in animals (Reichelt et al., 1981).

At lower phylogenetic levels peptides are involved in bacterial chemotaxis (Aswanikumar et al., 1978), initiation of phagocytosis (Stabinsky et al., 1978) and macrophage activation (Tanaka et al., 1977). The nervous system of mollusks has been found to contain a cardio-excitatory peptide (Price and Greenberg, 1977). More complex and sequential events also seem to be controlled by peptides. This includes the inhibition of ovulation in rodents (Kent, 1978).

Many of the peptides isolated from the CNS on the basis of specific biological activity have been shown to have multiple physiological effects. Substance-P, originally purified by following sialogogic activity (Leeman et al., 1977), is probably a transmitter in dorsal root ganglia (Otsuka et al., 1975) and stimulates the release of 5-HT from the substantia nigra (Reubi et al., 1978). Neurotensin has both vasodilatory activity and affects temperature regulation (Bissette et al., 1978). The hypophysiotropic hormone somatostatin also amplifies pentobarbital-induced sedation, reduce neuronal activity (Kastin et al., 1978) and stimulates monoamine turnover (Garcia-Sevilla et al., 1978). Enkephalins and endorphins modulate neurotransmitter metabolism (Biggio et al., 1978; Arbilla and Langer, 1978; Moroni et al., 1978) and the release of other peptides - substance-P in the spinal cord (Jessell and Iversen, 1977) and vasopressin from the pituitary (Bissette et al., 1978). The opiate agonists may also be involved in the development of obesity (Margules et al., 1978) and are powerful antidipsogens (De Caro et al., 1979).

Enkephalins and endorphins, in addition to their analgesic effects (Terenius, 1978, Guillemin, 1978), induce limbic seizures (Henriksen et al., 1978), and reduce activity levels in goldfish while increase activity in rats (Segal et al., 1977). Consolidation and retrieval processes are modified by oxytocin and vasopressin (Bohus et al., 1978), while angiotensin II disrupts retention performance following neostriatal injections (Morgan and Routtenberg, 1977). These examples indicate that peptides are capable of initiating complex physiological and behavioral processes, and may function endogenously as mediators coordinating and integrating information.

Peptides in the CNS are characterized by their low overall concentrations, high potency, and marked specificity. They probably possess both transmitter and hormonal-like properties and are potential transmitter candidates. Peptides in the CNS also have apparent low rate of de novo synthesis in vitro. These characteristics seem to be true for all peptides presently known, although the concentrations of certain peptides may be fairly high when related to specific isolated structures, or when sequestered in certain synaptosomal populations (Fink et al., 1972; Barnea et al., 1975).

The hypothalamus may be considered to be a mixing box in which multisignal integration results in the formation and release of peptides. The hypothalamic peptidases that use oxytocin or leutenizing hormone releasing hormone (LHRH) as substrate are found in soluble and particulate forms, the activities and degree of solubilization varying with sex, castration, and treatment with gonadal steroids, indicating a feedback mechanism (Griffiths and Hooper, 1974; Griffiths et al., 1975). This type of feedback regulation and intracellular distribution may play an important role in the regulation of levels of peptides, both in the hypothalamus and in other parts of the brain.

From the above discussion, it can be concluded that peptides are present in the CNS, and act as possible neurotransmitters, hormones, and modulators of electrophysiological and secretory processes in neurons. Many CNS oligopeptides contain neurotransmitter candidates and have behavioral, extensive metabolic effects, and considerable informational capacity (Reichelt and Edminson, 1976). Peptides in the CNS are synthesized either by a ribosome-independent synthesis or by ribosomal synthesis followed by peptidase-dependent splitting off of active components. There are a

numerous numbers of peptidases in the CNS that are partially under specific and extensive feedback control.

In view of these observations, it is possible to propose, as a working hypothesis, that peptides: (1) may be the integrated response to multisignal inputs to key cells, such as ganglion cells and neurosecretory cells in the CNS; and (2) possibly reflect the temporal and geometrical sequence of events in the neuron by their composition. The model would have two different forms. One in which the release of peptide is the result of converging pathways, and another in which peptide synthesis is caused by the confluence of inputs impinging on the cell.

Master neurones which determine the firing rate of sets or patterns of neurones are known (Gillette et al., 1978). These could be peptide regulated as has been found for the spontaneous firing rate of ganglion cells in Aplysia and snails (Ifshin et al., 1975). Other examples of centrally commanded behavior are respiratory pumping in Aplysia and the frequency shifts in the output of the electric organ of electric fish following the approach of another member of the species. In the former case, the activity is triggered by a single, spontaneously active interneurones (Byrne and Koester, 1978). The pacemaker command nucleus in the medulla of the electric fish varies its postsynaptic potentials in order to avoid jamming of its own orientation system (Feng and Bullock, 1978).

From the above discussion, it could be proposed that any specific or general overloading of the input systems to a peptidergic master neurone will lead to an increased formation or release of a peptide(s), representing the hyperactivated systems. In normal circumstances, the peptide inactivation capacity of the system will be sufficient to avoid release from the

CNS. However, in the case of genetically determined low levels of activity of reuptake mechanisms or peptidases, overflow of the peptides to the body fluids from the CNS will occur. The key position of peptidases in the regulation of peptide levels is clearly indicated by the extensive feedback control of the peptidases which inactivate LHRH (Griffiths et al., 1975; Kuhl et al., 1978).

In congenital generalized lipodystrophy, a family of peptides bounded to carrier proteins was isolated from the urine. These peptides could induce an animal model of this disorder in mice and rabbits following repeated subcutaneous injections (Foss and Trygstad, 1975). A possible serotonergic hyperfunction seemed probable, due to increased 5-HIAA excretion in the urine from these patients. The abnormal peptide-carrier-protein complexes, and the symptoms of the disorders were normalized with fenfluramine treatment (Trygstad and Foss, 1977; Trygstad et al., 1977). In anorexia nervosa, different urinary peptide-carrier-protein patterns could be demonstrated (Trygstad et al., 1978), but from primary anorexia nervosa only a specific anorexigenic peptide could be isolated. The tripeptide pyroGlu-His-GlyOH was characterized and synthesized (Reichelt et al., 1978). The urinary peptide patterns from these patients also seemed to be of diagnostic value. In 1980, Trygstad presented preliminary evidence that autistic children may have specific urinary peptide patterns (Trygstad et al., 1980). At a later time, an extended investigation (Gillberg et al., 1982) was undertaken which compared patients having infantile autism with five other control groups. The first group of children had psychoses; the second, attentional deficit syndrome; the third, minimal brain dysfunction; the fourth, mental retardation with psychoses. The fifth group was made up of

normal controls. Of the autistic children, 54 percent showed a distinct pattern that was not seen in any other cases, except in 17 percent of the children with other psychoses. Only 8 percent of the autistic children showed a pattern that was seen in over 95 percent of the normal children and 93 percent of the mentally retarded children without psychoses. In a recent study, Gillberg (1984) examined 20 autistic children and found evidence of raised CSF levels of endorphin fraction II in those children who were self-destructive and appeared to have a relative insensitivity to pain. This finding appeared to support the theory that the fundamental problem of autism was hormonal rather than behavioral (Panksepp, 1979). It is then possible to hypothesize that urinary peptide patterns of autistic subjects may be of diagnostic value and a particular peptide(s) may be used as a marker for this syndrome.

CHAPTER II

OBJECTIVES

The purpose of this study was to investigate possible serum and/or urinary diagnostic biochemical markers for infantile autism. In the investigation, I intended to test the hypothesis that autism was distinguished by a unique set of biochemical markers which were significantly different from the control population.

To test this hypothesis, I proposed to determine if an elution pattern of a group of serum and/or urinary peptides could be diagnostic for autistic persons when compared to such patterns in an age and sex-matched controls.

To accomplish this end, I developed an isolation procedure based on that used for isolation of intestinal smooth muscle peptides (Reeve et al., 1982) which provided me with reproducible gel chromatography profiles. The fractions obtained from this gel chromatography were further characterized on high performance liquid chromatography (HPLC) with known peptide standards. Peaks from HPLC chromatograms, chosen as possible biochemical markers for this syndrome, were isolated by semipreparative HPLC and subjected to amino acid analysis for peptide confirmation.

Since elevated blood 5-HT concentrations occurred in 30% to 40% of autistic patients, I proposed to measure serotonin levels in order to define subgroups of the autistic population and determined their frequency in this

population. Further subgroup classification may be possible through determining of plasma catecholamines and serum analytes.

CHAPTER III

MATERIALS AND METHODS

Subjects

All subjects participated in the study were required to sign a consent form. Consent was obtained where possible from the subject who was studied, and if the person was not an adult or was unable to give consent, the permission was obtained from the parent or guardian. Consent was documented through a standard form prescribed by the Biomedical Sciences Review Committee on Research Involving Human Subjects, The Ohio State University, who approved this study involving autistic and normal control populations and the consent form utilized.

The ages of the autistic and age-matched control subjects ranged from 4 to 21 years old (Table 1). They were predominantly male since the syndrome incidence in the population was 4 to 1 male to female (Lotter, 1966). There was no particular ethnic enrichment in the sample of the population studied since epidemiological studies concerned with demographical information had established that little if any bias was present in the population (Wing, 1980).

Fourteen (14) autistic and 10 control subjects were recruited from Columbus Public Schools, through the Central Ohio Chapter of the National Society for Children and Adults with Autism (COC-NSAC), the Department of

TABLE 1

Age and Sex of Autistic Subjects and Normal Controls

Subjects	Age	Sex	Controls	Age	Sex
01	21	M	01	22	M
02	17	M	02	23	M
03	21	M			
04	12	M	03	12	M
05	13	M	04	12	M
06	20	M			
07	15	F	05	15	F
08	10	M	06	11	M
09	15	M	07	14	M
10	20	M			
11	14	F	08	14	F
12	4	F			
13	18	M	09	17	M
14	4	M	10	6	M

Human Services, College of Education, and the Department of Psychiatry, College of Medicine, The Ohio State University.

Clinical Diagnosis

Independent clinicians in psychiatry used two sets of criteria for entry of autistic subjects into the study. They were:

1) **Childhood Autism Rating Scale** (CARS, Schopler et al., 1980). This rating scale was based on behavioral observations. It made up of 15 rating scales. Each scale was scored on a continuum of 1 to 4 from normal to severely abnormal. It was important that the child's age must be considered in making each rating. The scores ranged from 15 to 60, and any total score below 30 was labeled nonautistic. Any child with a total score of over 36 and a rating of 3 or higher on at least 5 out of 15 scales was considered severely autistic. Other children who did not fit these two categories were called mild to moderate autistic.

2) **DSM-III diagnostic category of pervasive developmental disorders** (American Psychiatric Assoc., 1980). This procedure was multi-axial with five separated axes. Infantile autism was listed in axis I under the generic term pervasive developmental disorders. In order to be considered autistic, a child must exhibited lack of social response, language deficit, speech peculiarity, an absence of delusions/hallucinations, and an age of onset before 30 months. The scoring system for this procedure was given as 299.00 for full syndrome and 299.01 for those with residual autism.

Additionally Rimland E-2 diagnostic checklists, which comprised of 80 questions about the patient's history up to age of 5 years, were completed by

parents and evaluated by Rimland according to his criteria (Rimland, 1971). One plus score was given for each question characteristic of autism and one minus point was scored for a non-autistic characteristic. The child's extent of autism is the sum of scores. According to Rimland, any child with +20 a 80 question summation is diagnosed as having early infantile autism, and any child with the score below +20 is called an autistic-type child. All subjects met the CARS and DSM-III criteria, but only one subject met the Rimland E-2 criteria for early infantile autism. All the other "autistic" subjects were identified as autistic type children based on the Rimland criteria.

Medications

Although some subjects were on therapeutic agents at some point in their life, only three had any medications for the 6 months prior to entry into the study. At 2-3 years one subject was treated with methylphenidate and continued with thioridazine until the present. Another subject was administered phenobarbital, dextroamphetamine sulfate, and thioridazine and continued until the present. One subject was treated with metaproterenol sulfate for asthma at the time of the study. In 1977 another subject was on trifluoperazine and was switched to haloperidol which was discontinued in 1981. One subject was treated with methylphenidate and phenytoin which were switched to primadone and carbamazepine for about one year. At two years of age another subject was on dextroamphetamine sulfate for a period of time. In 1965 one subject was administered thioridazine for a short period and dextroamphetamine sulfate for 13 days. Another subject was

treated with phenobarbital for a short time. At five years of age one subject was on methylphenidate for 1 month.

Sample Collections

To standardize conditions of blood plasma and serum collection, all subjects (autistic and normal controls) sat for15 minutes; then blood was drawn in vacutainer tubes containing no anticoagulant, ethylenediamine tetraacetic acid (EDTA), or heparin. All blood samples were drawn at the Ohio State University Hospital Clinic by trained phlebotomists. The samples were centrifuged within one-half hour and plasma/serum separated, and a portion lyophilized (VIRTIS Research Equipment) immediately for peptide studies. Another aliquot was frozen for analysis of other analytes. A twenty-four hour urine sample was collected in a urine container with 1 g of thymol added. The parents or subjects were instructed to begin collection of urine samples between 3 PM to 3 PM of the next afternoon. Urine was either lyophilized as soon as the collection was completed, or frozen upon receipt.

Chemicals

Thymol (J.T. Baker Chemical Co., Phillipsburg, NJ). Acetonitrile UV (American Burdick and Jackson, Muskegon, MI). Triethylamine (TEA, Aldrich Chemical Co., Milwaukee, WN). Amberlite XAD-2, Sephadex G-25-80, ninhydrin, trifluoroacetic acid (99%), L-ascorbic acid, EDTA, and peptide standards were obtained from Sigma Chemical Company, St. Louis, MO.

Phenylisothiocyanate (PITC), Constant Boiling (6 M) HCl, Amino Acid Standard Kit 22, L-norleucine, PTH-Amino Acid Standard Kit, and pyridine were from Pierce Chemical Company, Rockford, IL. All other chemicals used were of reagent grade (A.R.).

Serum Analytes Determinations

The determinations of BUN, sodium (Na), potassium (K), chloride (Cl^-), carbon dioxide (CO_2), creatinine, glucose, aspartate transaminase (AST), and alanine transaminase (ALT) were performed on Beckman ASTRA 8 (Beckman Instrument Co.). Uric acid was determined on auto-analyser (Technicon Inc.).

Serotonin Assay

Whole blood 5-HT was determined by the method of Yuwiler et al. (1970) as follows: Six (6) mL of whole blood were collected in vacutainer tubes containing EDTA and stored at -40° C until assayed. Two (2) mL of whole blood of each subject were placed in a 50 mL glass centrifuge tube with 2 mL of 0.85 % NaCL, 1 mL of 3 % L-ascorbic acid saturated with KCl and EDTA, and 5 mL of 2 M K-phosphate buffer, adjusted to pH 10 with 6 M freshly prepared KOH and saturated with KCl. Twenty (20) mL of n-butanol saturated with deionized double distilled water were subsequently added to each glass centrifuged tube which was shaken (Eberbach shaker, Eberbach) for 5 minutes to extract 5-HT into the butanol layer. The mixture was then centrifuged (International Centrifuge, International Equipment Co.) at 400 x

g for 15 minutes to separate the organic from the aqueous layer. Fifteen (15) mL of the butanol layer (top) were transferred to another clean 50 mL glass centrifuge tube. Two (2) mL of 0.1 M HCl and 25 mL of cyclohexane were added to the butanol extract. The mixtures were shaken for 1 minute and centrifuged at 400 x g for 4 minutes to separate the two layers, 5-HT being extracted back into the aqueous layer (bottom) in the salt form. The butanol-cyclohexane layer was aspirated leaving approximately 2 mL of the acidic aqueous layer at the bottom of the tube. One (1) mL of this aqueous layer was added to 0.3 mL of 12 M HCl in a small culture tube, and the fluorescence was determined at 295 nm (activated) and 550 nm immediately by a Farrand spectrofluorometer MK2 (Farrand Optical Co., Inc.). Samples containing 2 mL of 0.85 % NaCl instead of wholeblood served as blanks. A standard curve was obtained for each run with duplicate standards containing 0.1, 0.2, 0.3, and 0.4 µg of 5-HT respectively. The 5-HT of each subject's blood was estimated from the standard curve and expressed as nanograms per deciliter of whole blood (Figure 1).

Preparation, Purification, and Isolation of Putative Neuropeptides from Urine Samples (Figure 2)

Protein Precipitation - Glacial acetic acid (4.5% (v/v), 2 mL/g lyophilized urine) was added to the urine sample and the mixture stirred for 4 minutes. Acetone (7 mL/g lyophilized urine) was then added and the mixture stirred for 4 hours in the cold room at 6° C. The resultant mixture was centrifuged (Sorvall Superspeed RC-2B, Ivan Sorvall, Inc.) for 30 minutes at 3,000 x g at 6° C and the precipitate discarded. Acetone was

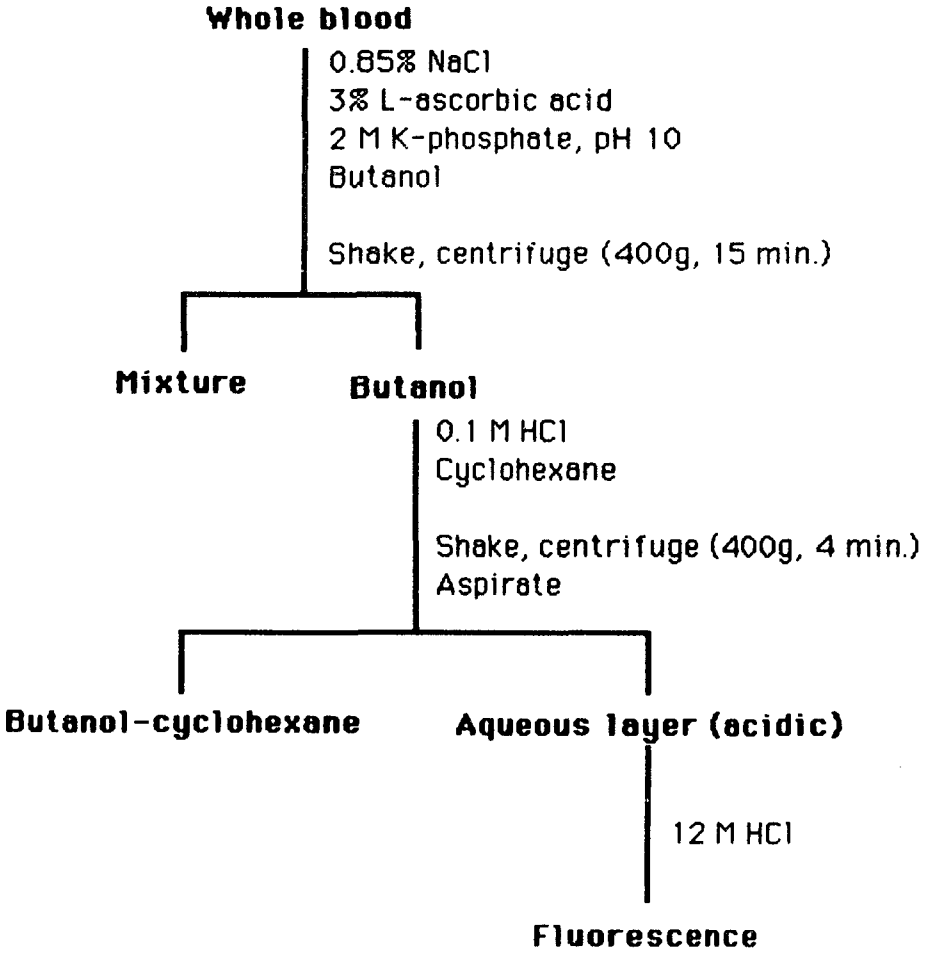

Figure 1. Outline of serotonin determination

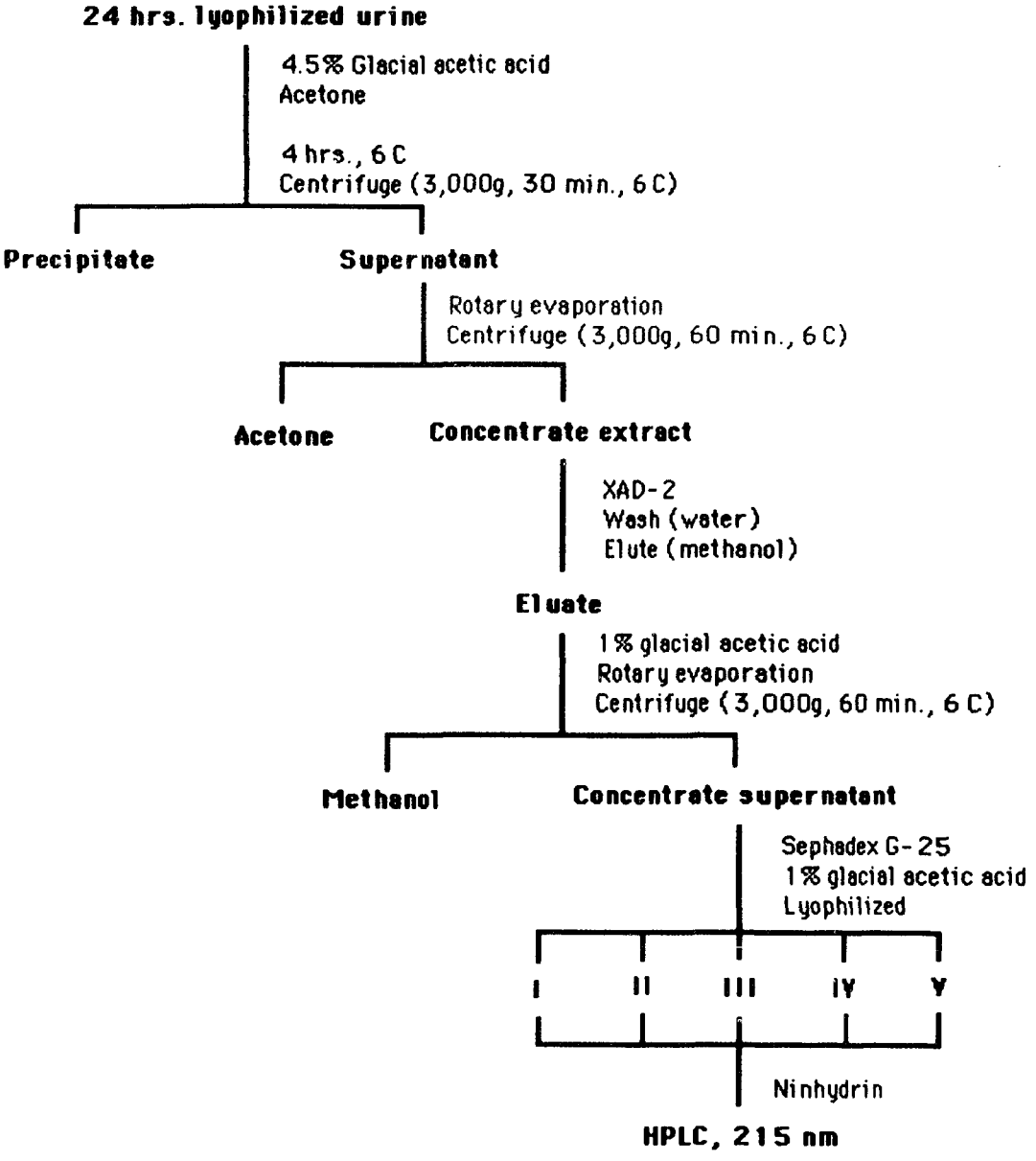

Figure 2. Outline of urinary peptide procedure

removed from the supernatant by rotary evaporation (Buchi Rotavapor-R, Brinkmann Instruments) and the remaining supernatant centrifuged for 1 hour at 3,000 x g at 6° C.

Amberlite XAD-2 Chromatography - The concentrated extract was loaded onto an Amberlite XAD-2 column (5 x 30 cm, Pharmacia Inc.) that had been washed with acetone, methanol, and equilibrated with water. After loading, the column was washed with water until the absorbance at 280 nm was less than 0.1 (Spectrophotometer 240, Gilford Instrument). The column was then eluted with 100% methanol until the eluate (approximately 500 mL) had reached an absorbance of less than 0.5 at 280 nm. Twenty-five (25) mL of 1% glacial acetic acid (v/v) was added to the methanol eluate and the resulting solution concentrated to 25 mL by rotary evaporation and centrifuged for 1 hour at 3,000 x g at 6° C.

Sephadex G-25 Chromatography - The resultant supernatant was loaded onto a Sephadex G-25 column (2.5 x 150 cm, Pharmacia Inc.) at 6° C which was standardized for molecular masses (Figure 3) and eluted with 1% glacial acetic acid (v/v). The eluted fractions (10 mL) were collected with a fraction collector (Fracto Meter 200, Buchler Co.) and monitored at 280 nm. The total collection was approximately 6,000 mL. Fractions from peaks having absorbance were pooled and lyophilized. Peptide material was detected by hydrolyzing aliquots of each pooled fraction with 2 M KOH, followed by neutralization and color development with ninhydrin (Rosen, 1957). To test the reproducibility and the recovery ability of the procedure, two twenty-four hours urine from the same individual were pooled and lyophilized. The dried sample was then divided into two equal aliquots and

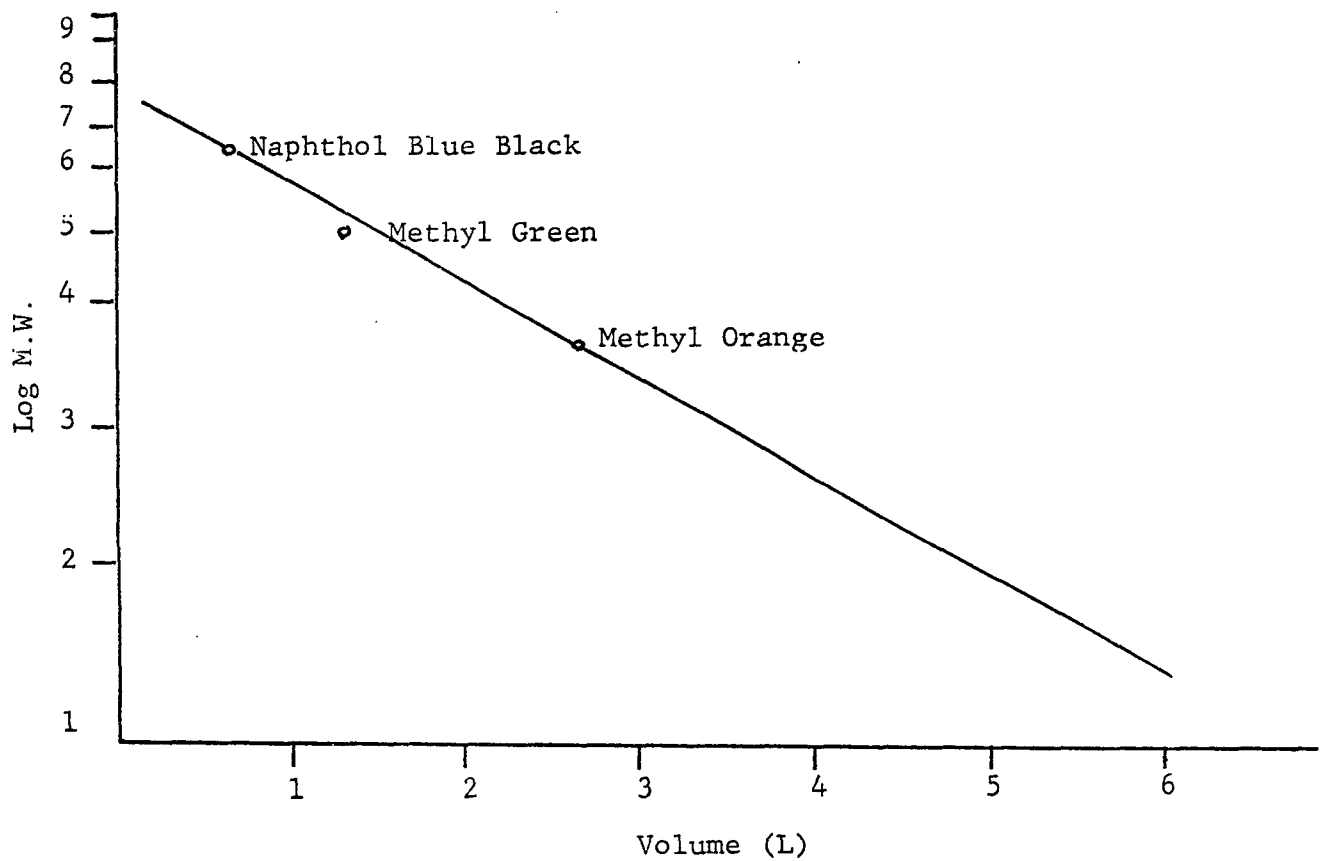

Figure 3. Sephadex G-25 column standardization for molecular masses

1 mg of substance-P was added to one aliquot. The samples were then analysed in the same manner as any other urine samples.

Reverse-Phase HPLC - Five (5) mg of peptide positive fractions were dissolved in 500 µL of 1% glacial acetic acid (v/v) and was centrifuged (International Centrifuge) at 400 x g for 10 minutes. The clear supernatant (20 µL) was separated by a Rainin HPLC system (Rainin Instrument Co., Inc.) equipped with a C-18 reverse-phase HPLC column (Supelcosil LC-18-DB, 150 x 4.6 mm, Supelco, Inc.) which was equilibrated in 10% acetonitrile (CH_3CN) and 90% trifluoroacetic acid (TFA), pH 2.05. The eluant was monitored at 215 nm (V^4 absorbance detector, Isco, Inc.) with a flow rate of 2.0 mL/min. After loading the samples, the putative peptide materials were eluted over 18 minutes with a CH_3CN stepped gradient (10-35%) in TFA as follows: CH_3CN was maintained at 10% for 2 minutes and was increased to 30% by 4 minutes. The gradient was then held at 30% CH_3CN until 6 minutes elapsed and was increased to 35% by 10 minutes. The run was isocratic at 35% until 16 minutes and the gradient was reduced to 10% CH_3CN by 18 minutes. Between runs, the column was allowed to equilibrate with 10% CH_3CN and 90% TFA for 10 minutes. At the start-up time and at the end of the day, the column was always washed with 100% CH_3CN for 10 minutes. With the first injection and after every 10 injections thereafter, a mixture of standards (delta sleep-inducing peptide (DSIP, 849 daltons), somatostatin (1638 daltons), met-enkephalin (574 daltons), substance-P (1348 daltons), neurotensin (1673 daltons), leu-endorphin (3466 daltons), glucagon (internal standard, 3482 daltons)) was run to check reproducibility of retention times.

HPLC Fraction Collections - Sephadex G-25 peptide positive fractions containing peaks of interest were dissolved in 1% glacial acetic acid (v/v)

and were separated by Rainin HPLC system equipped with a C-18 semipreparative reverse-phase HPLC column (Supelcosil LC-18-DB, 250 x 10 mm) which was equilibrated in 10% CH_3CN and 90% TFA, pH 2.05. The eluant was monitored at 215 nm with a flow rate of 6.0 mL/min. After loading 200 µL sample, the fraction was eluted over 30 minutes with a CH_3CN gradient (10-35%) in TFA. The gradient conditions were as follows: Isocratic at 10% CH_3CN for 4 minutes and increased to 30% by 8 minutes which remained constant until 12 minutes. The gradient was increased to 35% CH_3CN by 20 minutes and remained isocratic until 28 minutes. The gradient was then reduced to 10% CH_3CN by 30 minutes. The isolated peaks were collected using the fraction collector FRACT-100 (Pharmacia, Inc.). Peaks of the same retention time from multiple runs were pooled and lyophilized. In the case where peaks of interest had a similar retention time, a modified gradient program was employed. The gradient remained constant at 10% CH_3CN for 16 minutes. It was then increased to 30% by 30 minutes and was held constant at 30% until 50 minutes. The gradient was increased to 35% by 55 minutes and was reduced to 10% CH_3CN by 60 minutes. The purity of isolated peaks was checked through another modified gradient program employing an analytical reverse-phase column. The conditions for this modified program were as follows: The gradient was isocratic at 10% CH_3CN for 8 minutes. It was then increased to 30% by 15 minutes and remained constant until 25 minutes. The gradient was increased to 35% by 28 minutes and remained at this concentration until 32 minutes before it was reduced to 10% CH_3CN by 35 minutes. By prolonging the retention time, it was possible to check the purity of each isolated peak

for contamination. The contaminated samples were subjected to a repeat fractionation with an appropriate gradient program.

Preparation, Purification, and Isolation of Putative Neuropeptides from Serum Samples

Protein Precipitation - The precipitation of protein was done in the same manner as mentioned for the urine samples (Protein precipitation, page 27).

Amberlite XAD-2 Chromatography - The concentrated extract was loaded onto an Amberlite XAD-2 column (4 x 10 cm) that had been conditioned as mentioned before (Amberlite XAD-2 chromatography, page 30). After loading, the column was washed with 400 mL of water. The column was then eluted with 100% methanol to collect approximately 200 mL of eluate. Ten (10) mL of 1% glacial acetic acid (v/v) were added to the methanol eluate and the resulting solution was concentrated to 10 mL by rotary evaporation and centrifuged for 1 hour at 3,000 x g at 6° C. The eluate was then lyophilized and stored at -40° C until assayed or loaded onto a Sephadex G-25 column.

Sephadex G-25 Chromatography - Fractionation of serum samples was accomplished by loading concentrated eluate from Amberlite XAD-2 column onto a Sephadex G-25 column (1 x 45 cm). The samples were eluted in the same manner mentioned (page 30) and the eluted fractions (5 mL) were collected. The total collection was approximately 500 mL. Peptide materials were detected using ninhydrin.

Reverse-Phase HPLC - The dried samples were dissolved in 500 µL of 1% glacial acetic acid (v/v) and were centrifuged for 10 minutes at 400 x g. The resultant samples were separated by Rainin HPLC system by the urinary peptidic protocol (Reverse-phase HPLC, page 32).

HPLC Fraction Collections - The same HPLC conditions were the same as those for urine samples (HPLC fraction collection, page 32).

HPLC Data Collections and Calculations

The data collections and calculations were done through the CHROMATOCHART system (Interactive Microware, Inc.). CHROMATOCHART used ADALAB's Digital to Analog (D/A) converter to produce a varying output voltage. Twenty times each second, the program read the A/D converter value for the active channel, averaged and summed the value with previous values if necessary and stored the data in extended RAM. By using internal standard, the amount of unknown was calculated as area of peak x correction factor - where correction factor = amount of internal standard/area of internal standard in unknown.

Statistical Analysis

The results are expressed as Mean ± SD (standard deviation). Statistical significance was evaluated by using Student's 't' test as well as Mann-Whitney U test and 'p' values less than 0.05 were considered to be significant.

Amino Acid Analysis

Peptide materials were hydrolyzed under vacuum for 20 hours at 110° C in 6 M HCl and the acid was removed by lyophilization. The samples were dissolved in 100 µL of coupling buffer (CH_3CN:pyridine:triethylamine:water, 10:5:2:3 by volume). The solutions were dried under vacuum at room temperature and the samples were dissolved once more in 100 µL of coupling buffer followed by an addition of 5 µL of PITC. After a 5 minutes incubation period at room temperature, the solutions were evaporated to dryness under vacuum. The resulting phenylthiocarbamyl (PTC) derivatives were converted to phenylthiohydantoin (PTH) derivatives by an addition of 50 µL of 1 M HCl in methanol and incubated at 65° C for 10 minutes. The PTH-amino acids obtained after vacuum drying were then dissolved in 500 µL water:CH_3CN (7:2 by volume). Twenty (20) µL were analyzed by HPLC as described below (Figure 4).

Reverse-Phase HPLC Separation of PTH-Amino Acids

PTH-amino acids were separated by a Rainin HPLC system equipped with C-18 reverse-phase column (NOVA PAK C, 150 x 4.6 mm, Waters Associates) which was maintained at 39° C by a HPLC column temperature controller (Rainin Instrument Co., Inc.). The eluant was monitored at 254 nm with a flow rate of 1 mL/min. The solvent systems consisted of (A) 0.03 M sodium acetate, pH 5.0 - CH_3CN (5:1 by volume) and (B) isopropyl alcohol - water (3:2 by volume). The gradient conditions were as follows: Isocratic at 0% B for 1 minute and increased to 40% B by 4.5 minutes and remained constant

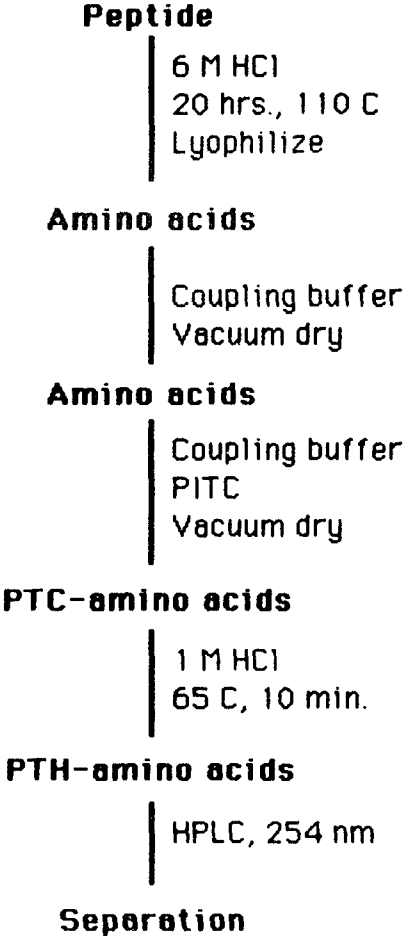

Figure 4. Outline of amino acid analysis

until 15 minutes. The gradient was then reduced to 0% B by 20 minutes. The column was allowed to reequilibrate for 10 minutes between runs.

CHAPTER IV

RESULTS

Serum Analytes Determination

BUN, Na, K, Cl^-, CO_2 , creatinine, uric acid, glucose, AST, and ALT were analyzed in 13 autistic subjects and 10 age-matched controls. Sodium and CO_2 were significantly lower ($p<0.005$, $p<0.05$ respectively) in autistic subjects compared to controls whereas uric acid and glucose ($p<0.05$) were significantly higher in autistic subjects (Table 2). The significance of these analytes using the Student's 't' test, excluding CO_2, was confirmed by a non-parametric Mann-Whitney U test which did not require any assumptions about the populations.

Neurochemical Determinations

Plasma catecholamines (L-dopa, dopamine, norepinephrine and epinephrine) were analyzed in 13 autistic subjects and 10 age-matched controls by Dr. Hussein Abou-Issa's laboratory. The method employed and the results are found in the appendix. Whole blood serotonin, done on the same subjects, was found to be significantly higher ($p<0.05$) in autistic subjects (Table 2), and the frequency was 35% as expected. The significance of this analyte was also confirmed by the Mann-Whitney U test.

TABLE 2

Whole Blood Serotonin, and Serum Analytes in Autistic Subjects and Normal Controls[a]

	Autistic (n = 13)	Control (n = 10)
BUN (mg/dL)	11.8 ± 3.19	11.6 ± 3.03
Na (mEq/L)	140 ± 1.68[b]	143 ± 2.51
K (mEq/L)	4.2 ± 0.20	4.3 ± 0.38
Cl^- (mEq/L)	105 ± 3.20	105 ± 2.20
CO_2 (mmol/L)	26.1 ± 2.43[c,d]	28.0 ± 2.47
Creatinine (mg/dL)	0.8 ± 0.24	0.7 ± 0.20
Uric Acid (mg/dL)	5.4 ± 1.46[c]	4.2 ± 1.23
Glucose (mg/dL)	91.6 ± 12.0[c]	88.9 ± 7.78
AST (U/mL)	26.8 ± 9.11	33.0 ± 8.88
ALT (U/mL)	21.5 ± 15.0	22.0 ± 12.7
Serotonin (ng/dL)	4.51 ± 0.91[c]	3.33 ± 1.63

[a]Data are represented as the Mean ± SD.
[b]$p < 0.005$
[c]$p < 0.05$
[d]N.S. with Mann-Whitney U non-parametric test.

Urinary Putative Neuropeptides

Lyophilized Urine - The freeze-dried urinary samples of all autistic subjects, compared to controls, showed a striking increase ($p<0.005$) in the precipitable materials (W/V) (Table 3).

Sephadex G-25 Chromatography - The results of the reproducibility study were shown in Figure 5. It can be seen that with one pooled urine specimen the procedure developed was reproducible. The recovery of substance-P was found to be approximately 70% after the different Sephadex G-25 fractions were analyzed on HPLC.

Representative patterns of G-25 chromatography of the autistic and control subjects (Figure 6) had five major fractions in autistic subjects as compared to four major ones and one minor fraction in the control subjects. When the patterns were compared, there were no differences in Fractions I, III, and IV between both groups. Fraction II of normal controls was found to be higher ($p<0.01$) as compared to autistic subjects. Fraction V of autistic subjects was significantly higher ($p<0.05$) than that from control subject urines (Table 3).

Gradient HPLC - DSIP, met-enkephalin, neurotensin, substance-P, somatostatin, leu-endorphin, and glucagon standards were separated cleanly by gradient HPLC within a 18 minute period (Figure 7). Since all putative peptides from both control and autistic subjects chromatograph within 12 minutes, glucagon was used as an internal standard. Identification of peaks found in the chromatogram of fractions from autistic and control subjects

TABLE 3

Weight of Solute in Lyophilized 24 hour Urine Sample and Peak Area of Sephadex G-25 Chromatograms of Autistic Subjects and Normal Controls[a]

			Peak Area (cm^2)				
	N	Solute Mass (g/dL)	I	II	III	IV	V
Autistic Subjects	13	4.60 ± 1.59[b]	1.74 ± 1.38	1.24 ± 0.52[c]	1.43 ± 0.85	0.77 ± 0.36	1.59 ± 1.53[d]
Control Subjects	5	2.60 ± 1.16	2.76 ± 2.11	5.81 ± 5.12	0.82 ± 0.39	0.87 ± 0.80	0.13 ± 0.07

[a]Data are represented as the Mean ± SD.
[b]$p < 0.005$
[c]$p < 0.01$
[d]$p < 0.05$

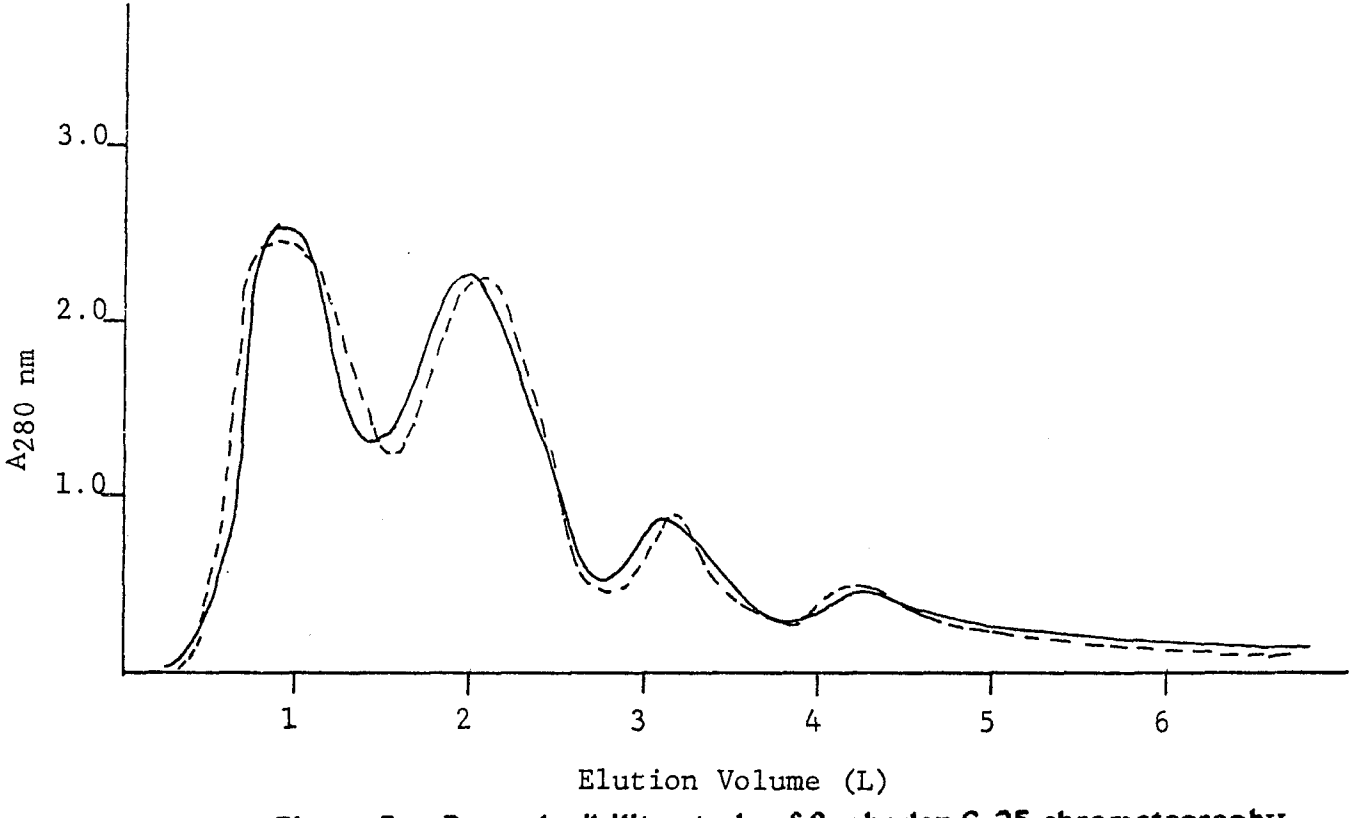

Figure 5. Reproducibility study of Sephadex G-25 chromatography

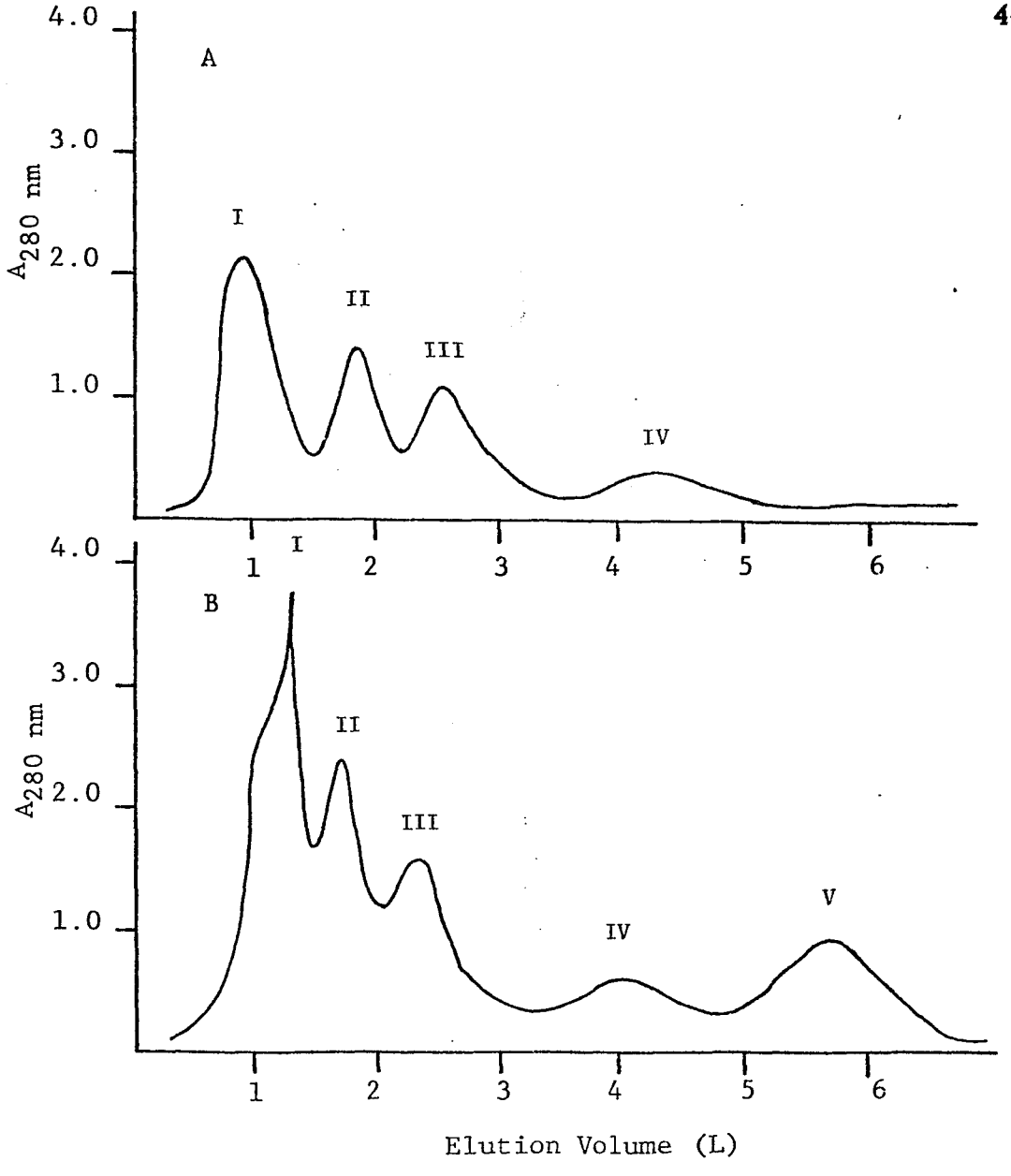

Figure 6. Representative Sephadex G-25 chromatogram of urine sample from autistic subjects (B) and normal controls (A)

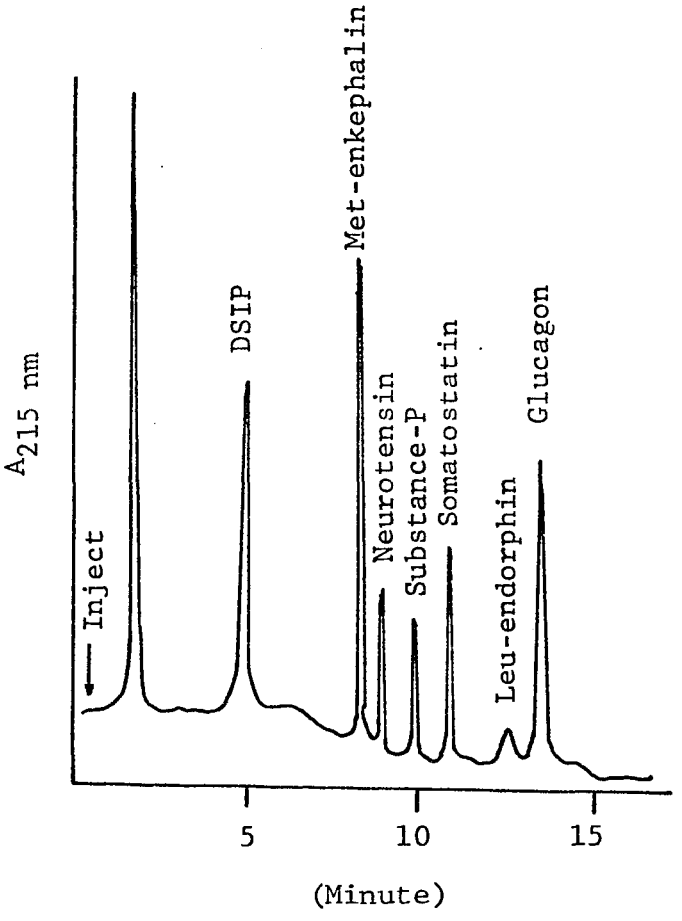

Figure 7. Chromatogram of standards

was achieved by matching the retention times of peaks with those of the standard compounds. Further confirmation was achieved by addition of a known quantity of each standard to samples from both autistic and control subjects. The chromatograms of autistic subjects' urinary substances from Sephadex G-25 Fraction I showed the presence of a peak, co-chromatographing with met-enkephalin, which was not found in controls (Figure 8). Tables 4 and 5 represented percent area of materials absorbed at 215 nm found in Sephadex G-25 Fraction I of autistic and control subjects with known peptides that matched the retention time of standards. Table 5 showed percent area of a peak with retention time that matched met-enkephalin's retention time and none was found in control subjects. This table also showed three other common peaks among most autistic subjects with retention times of 5.41, 6.37 and 6.73 minutes respectively. It also can be seen that a peak which co-chromatographed with neurotensin was common to both autistic subjects and normal controls. Figure 9 and Table 6 showed the differences in chromatographic patterns of Sephadex G-25 Fraction V between autistic and control subjects and percent area of materials found in Sephadex G-25 Fraction V of autistic subjects, with a peak and percent area of retention time of 7.6 minutes being common to all autistic subjects. No detectable amounts of materials from Sephadex G-25 Fraction V of normal controls was found in the time frame of those obtained for autistic subjects. Table 7 represented the absolute amounts of various peptides identified by standards from Sephadex G-25 Fractions I and V of autistic subjects and normal controls. Although these peptides matched the retention times of known standards, caution must be exercise in assuming that they are the same peptides. In the present investigation I only studied

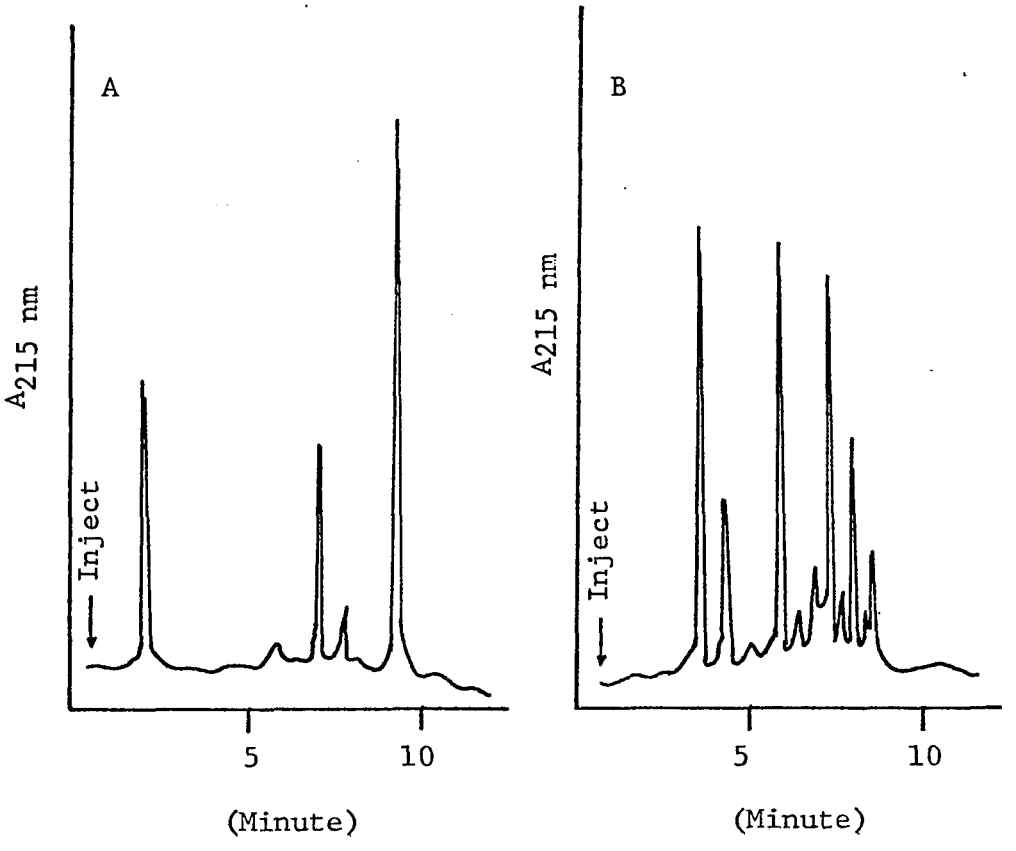

Figure 8. Representative urinary chromatographic patterns from Fraction I of autistic subjects (B) and normal controls (A)

TABLE 4

% Area of Urinary Peptides Found in Fraction I of Autistic Subjects

	Time (min.)													
	1.90	2.27	2.90	3.04	3.57	3.74	4.01	4.27 DSIP	4.48	4.83	5.11	5.25	5.41	5.60
Subjects														
01	1.37	-[a]	-	-	-	2.64	-	-	-	29.4	4.46	1.79	4.45	1.93
02	-	-	-	-	7.13	-	-	2.21	9.30	1.86	-	-	21.2	0.99
03	-	-	-	-	3.63	-	-	5.67	1.96	1.88	-	-	9.73	0.77
04	-	-	-	-	-	-	-	-	62.2	2.38	-	-	3.45	1.91
05	-	-	-	-	-	-	10.7	-	-	0.83	-	-	5.25	-
06	-	-	-	-	-	1.54	-	-	3.96	-	-	-	27.3	-
07	4.60	11.0	4.09	23.1	1.94	-	1.42	-	-	-	-	-	25.6	5.61
08	-	-	-	-	1.80	11.5	1.96	-	2.76	2.62	-	-	23.3	-
09	-	-	3.07	-	5.35	2.17	1.08	-	0.53	16.51	3.2	-	22.1	-
10	-	-	-	2.15	11.0	2.28	-	-	-	1.78	-	-	32.1	-
11	-	-	-	-	-	-	10.7	-	-	-	-	-	5.32	-
12	-	-	-	-	-	-	-	-	-	27.5	4.21	-	-	-
13	-	-	-	-	1.38	-	-	-	83.6	-	-	-	3.89	3.30
Mean	0.46	0.85	0.55	1.94	2.48	1.55	1.99	0.61	12.6	6.51	1.68	0.14	14.1	1.12
± SD	1.30	3.19	1.42	6.37	3.45	3.16	4.09	1.64	27.2	10.6	3.81	0.50	11.2	1.71

[a]Undetectable amount

TABLE 4 (Cont.)

% Area of Urinary Peptides Found in Fraction I of Autistic Subjects
Time (min.)

	5.90	6.37	6.54	6.73	7.05	7.21	7.52[a]	7.70	8.20[b]	8.42	9.21[c]	9.61	10.8
Subjects													
01	3.73	1.37	-[d]	-	2.82	-	2.24	-	39.4	-	2.04	-	-
02	14.3	7.19	5.32	16.2	0.65	2.97	6.06	0.76	2.87	1.02	-	-	-
03	8.77	22.5	6.21	15.1	0.74	3.18	4.08	-	11.4	-	-	-	-
04	11.6	5.94	2.36	-	-	-	2.41	-	-	-	-	-	-
05	1.38	9.62	8.55	5.25	11.8	7.87	1.95	4.49	2.13	-	14.4	-	6.16
06	9.60	4.65	5.40	15.8	-	-	3.24	-	1.90	-	-	-	-
07	2.73	4.08	3.52	-	1.64	-	4.52	-	1.56	1.15	2.46	-	1.07
08	5.99	1.99	9.48	10.6	20.8	-	1.84	-	3.05	-	-	2.36	-
09	-	10.6	3.75	4.88	-	9.06	0.67	2.63	1.28	-	-	-	-
10	3.20	6.14	4.09	16.2	-	5.91	6.36	-	5.61	1.42	-	-	-
11	-	11.6	9.68	6.06	13.9	11.2	9.67	5.14	-	-	11.0	-	5.81
12	2.27	-	-	2.74	-	-	4.46	-	58.8	-	-	-	-
13	-	-	-	3.59	-	-	4.24	-	-	-	-	-	-
Mean	4.89	6.59	4.49	7.41	4.03	3.09	3.98	1.00	9.84	0.28	2.30	0.18	1.00
± SD	5.00	6.12	3.41	6.50	6.87	4.07	2.39	1.85	18.1	0.53	4.74	0.65	2.23

[a]Met-enkephalin, [b]Neurotensin, [c]Substance-P, [d]Undetectable amount

TABLE 5

% Area of Urinary Peptides Found in Fraction I of Normal Controls

	Time (min.)									
	2.81	3.70	4.41	4.82	5.04	5.48	5.68	5.85	6.00	6.46
Subjects										
01	3.63	7.28	1.65	-[a]	-	23.6	-	-	13.6	2.60
02	-	-	-	5.00	-	-	-	17.6	-	8.71
03	3.79	-	45.7	-	5.78	26.1	3.93	-	6.93	-
04	-	2.33	-	3.57	11.6	1.64	-	14.7	4.32	1.81
05	-	-	-	-	-	-	-	18.8	-	8.43
06	-	3.71	-	7.95	-	3.26	-	38.4	8.45	4.41
Mean	1.24	2.22	7.88	2.75	2.90	9.10	0.66	14.9	5.55	4.33
± SD	1.92	2.92	18.5	3.33	4.86	12.2	1.79	14.3	5.25	3.58

[a]Undetectable amount

TABLE 5 (Cont.)

% Area of Urinary Peptides Found in Fraction I of Normal Controls

	Time (min.)									
	6.66	6.82	7.16	7.34	7.72	8.14[a]	8.25	8.46	9.62	10.6
Subjects										
01	15.2	20.9	1.20	2.14	1.86	-[b]	3.30	-	1.65	1.48
02	-	-	-	-	-	68.7	-	-	-	-
03	2.27	-	-	-	-	1.99	-	-	-	-
04	-	2.36	6.17	5.37	9.34	32.9	-	1.63	-	-
05	-	-	-	-	-	3.97	-	7.04	-	54.1
06	-	-	3.48	-	-	26.5	-	-	-	-
Mean	2.91	3.87	1.81	1.25	1.87	22.3	0.55	1.44	0.28	9.27
± SD	6.08	8.38	2.53	2.19	3.74	26.6	1.35	2.82	0.67	22.0

[a]Neurotensin
[b]Undetectable amount

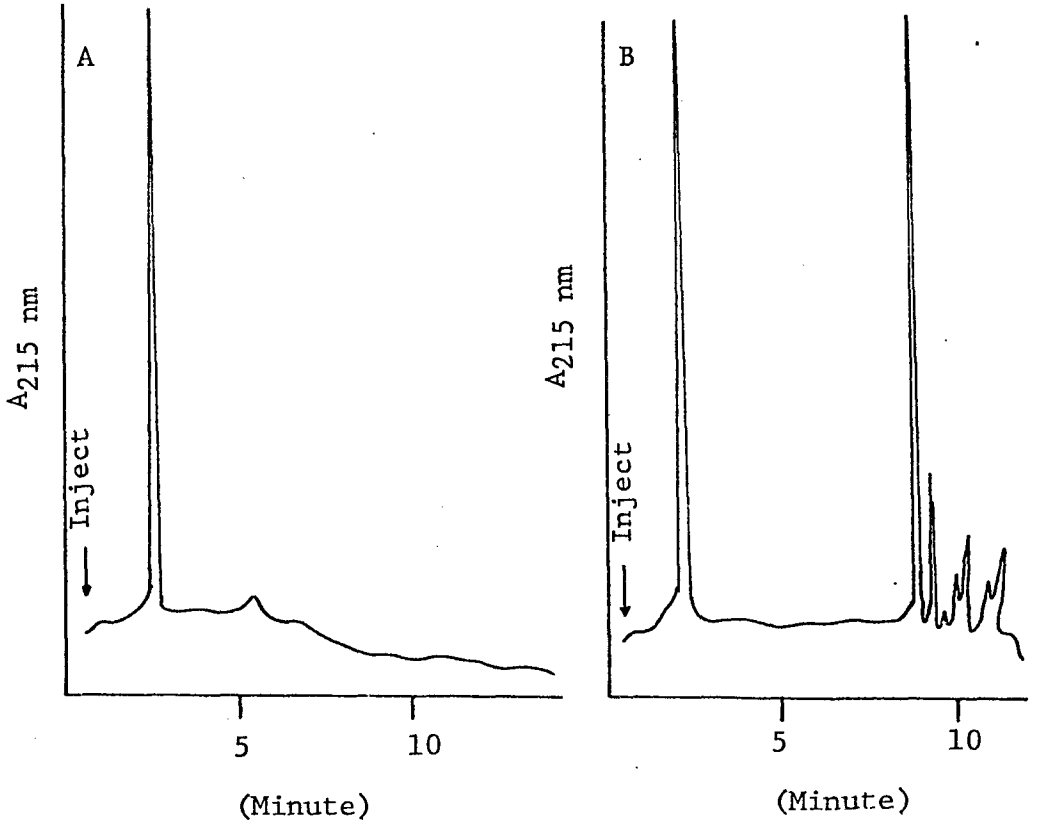

Figure 9. Representative urinary chromatographic patterns from Fraction V of autistic subjects (B) and normal controls (A)

TABLE 6

% Area of Urinary Peptides Found in Fraction V of Autistic Subjects

	Time (min.)									
	6.45	6.61	7.21	7.56[a]	8.16[b]	8.81	9.08[c]	9.24	9.93	10.10[d]
Subjects										
01	-[e]	-	-	50.9	18.9	-	8.03	-	3.94	18.2
02	-	-	-	77.5	6.44	4.15	5.74	-	-	6.17
03	-	-	-	70.0	2.56	-	5.24	-	8.16	13.0
04	-	5.61	-	63.3	16.1	-	-	10.8	-	4.22
05	38.3	-	7.61	42.6	-	-	-	5.35	-	6.21
06	-	-	-	78.8	10.2	-	5.56	-	5.44	-
07	-	-	-	65.3	8.92	5.58	7.98	-	3.28	8.94
08	4.14	-	-	74.6	8.73	-	5.88	-	-	6.64
09	-	-	-	92.1	4.42	-	-	-	-	3.49
10	-	-	-	68.1	22.8	-	9.10	-	-	-
Mean	4.24	0.56	0.76	68.3	9.91	0.97	4.75	1.62	2.08	6.69
± SD	12.0	1.77	2.41	14.2	7.76	2.08	3.51	3.65	2.96	5.62

[a]Met-enkephalin?
[b]Neurotensin?
[c]Substance-P?
[d]Somatostatin?
[e]Undetectable amount

TABLE 7

Known Peptides which Co-chromatographed with Known Standards Found in the Urine of Autistic Subjects and Normal Controls

	N	*Neurotensin* (μg)	*N*	*Met-enkephalin* (μg)	*N*	*Substance-P* (μg)	*N*	*Somatostatin* (μg)	*N*	*DSIP* (μg)
Autistic Subjects										
Fraction I	5	69.0 ± 18.1[a]	5	37.5 ± 31.4	4	24.4 ± 26.1	1	0.52	1	145
Fraction V	1	1.27	4	2.86 ± 1.65	1	1.12	1	0.52	-	-
Normal Controls										
Fraction I	4	28.5 ± 8.83	-	-	-	-	-	-	-	-

[a]Data are represented as the Mean ± SD.

Sephadex G-25 Fractions I and V due to the discovery of met-enkephalin in Fraction I and common putative peptide in Fraction V.

Fractionation by HPLC - Figure 10 showed a peak with a retention time of 8.18 minutes isolated from pooled Sephadex G-25 Fraction I of four twenty-four hour urine samples from one autistic subject. From the same pooled samples a peak with a retention time of 7.6 minutes was recovered from Sephadex G-25 Fraction V (Figure 11). The purity of these peaks were checked by the modified gradient program as mentioned in the Methods section where the retention time was prolonged (Figure 12).

Serum Putative Neuropeptides

Gradient HPLC - Representative separation of sera that had been purified on Amberlite XAD-2 from both autistic and control subjects are found in Figure 13 and 14. Since all putative peptides chromatograph within 13 minutes, glucagon was again used as an internal standard. Identification of peaks found in chromatograms from autistic and control subjects was achieved in the same manner as that with the urinary samples. Tables 8 and 9 represented percent area of materials absorbed at 215 nm found in autistic and control subjects with known peptides that matched the retention time of standards. From Figures 13 and 14 and Tables 8 and 9, it can be seen that a peak with a retention time of 5.56 minutes was common in both autistic and control subjects. Statistical analysis showed no significant differences in the amount of this unknown peptide among the two groups. Another unknown peptide with a retention time of 6.75 minutes seemed to be common in all control subjects. In contrast, only three autistic subjects

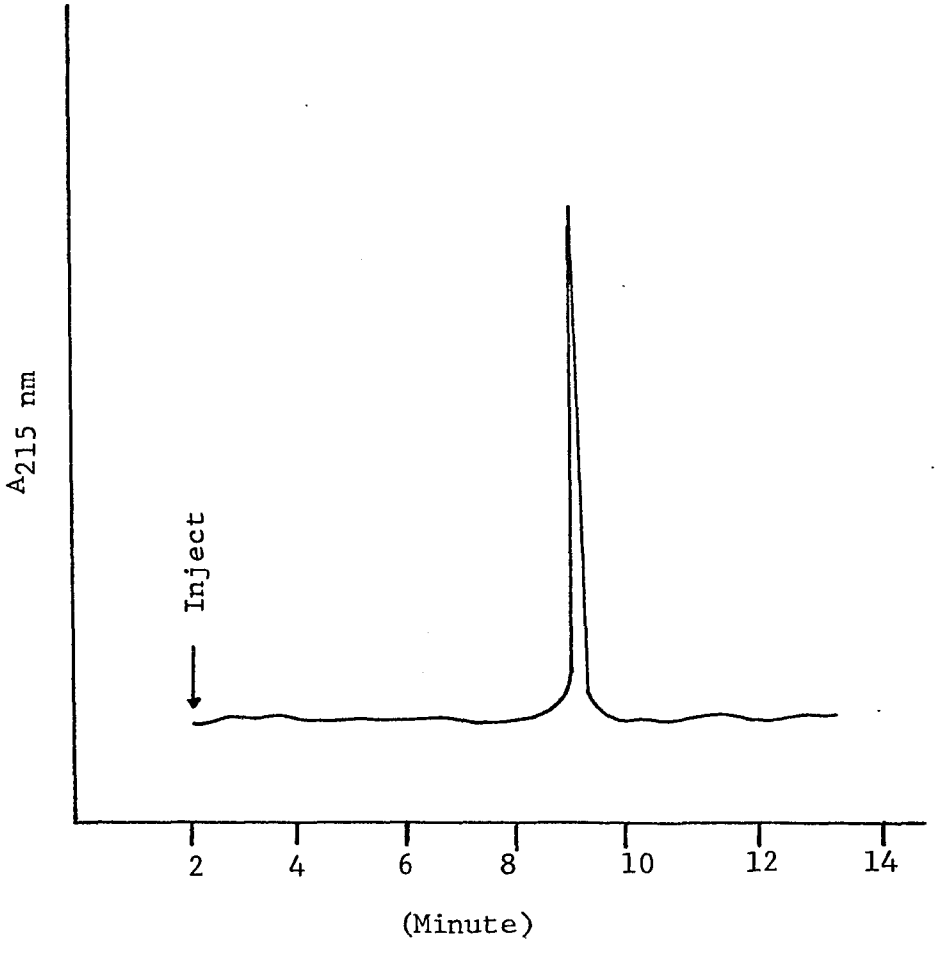

Figure 10. HPLC chromatogram of a peak with a retention time of 8.18 minutes isolated from urinary Fraction I of an autistic subject

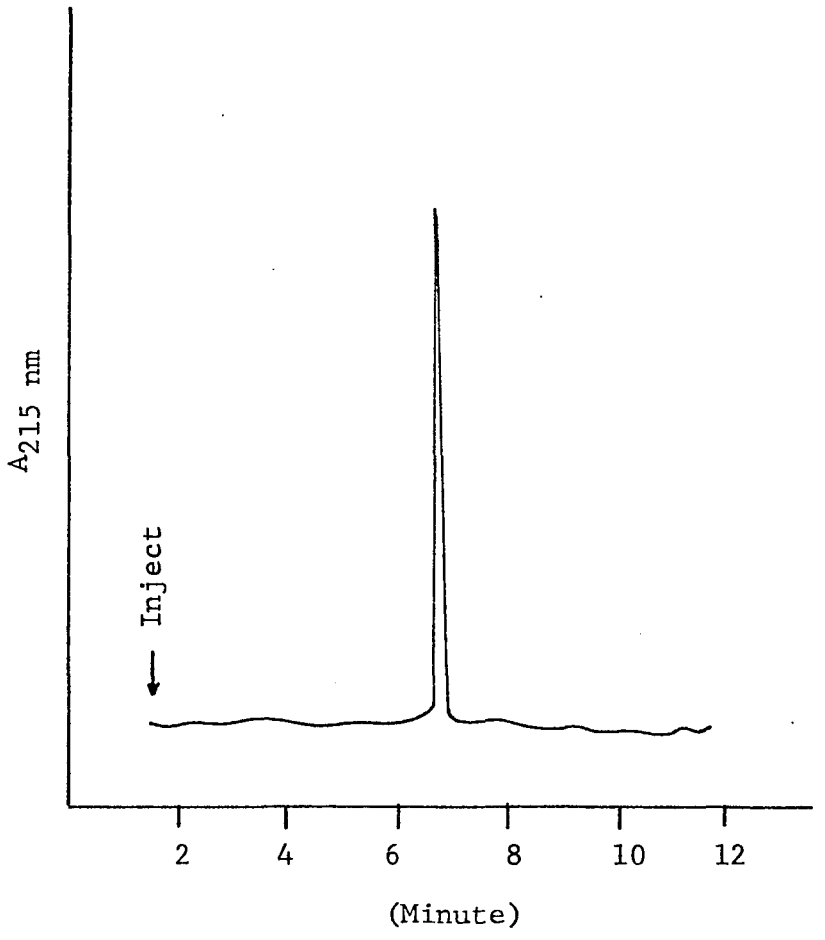

Figure 11. HPLC chromatogram of a peak with a retention time of 7.6 minutes isolated from urinary Fraction V of an autistic subject

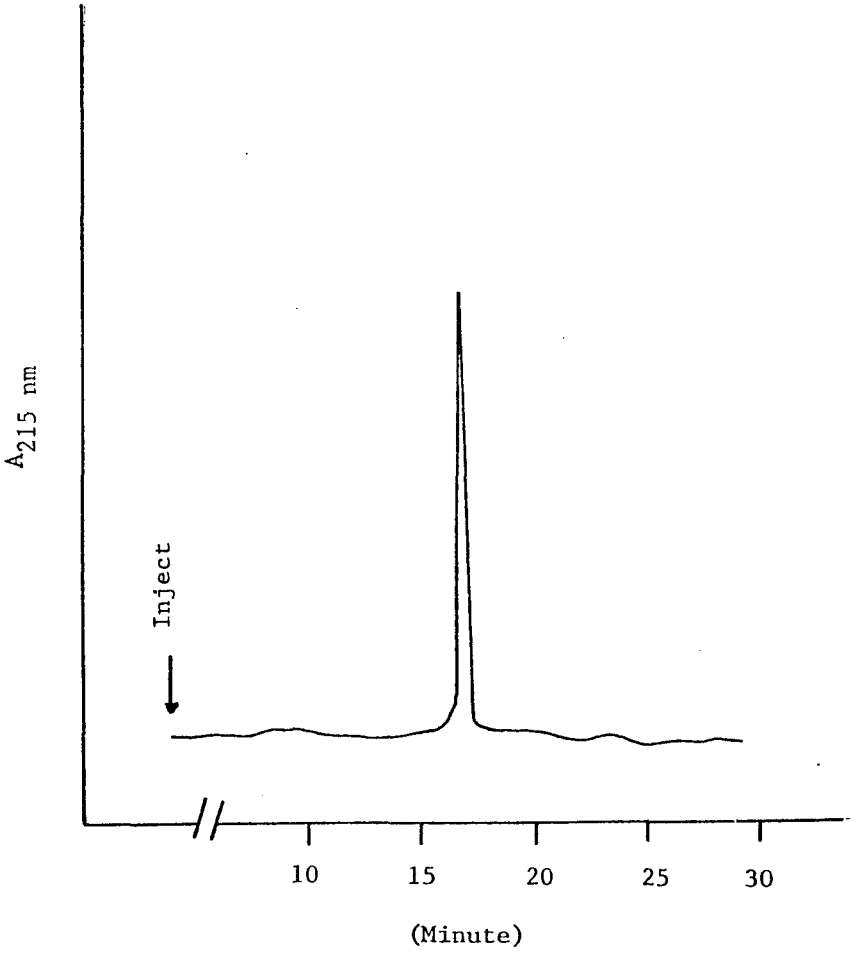

Figure 12. HPLC chromatogram of a peak with a prolonged retention time from urinary Fraction V of an autistic subject

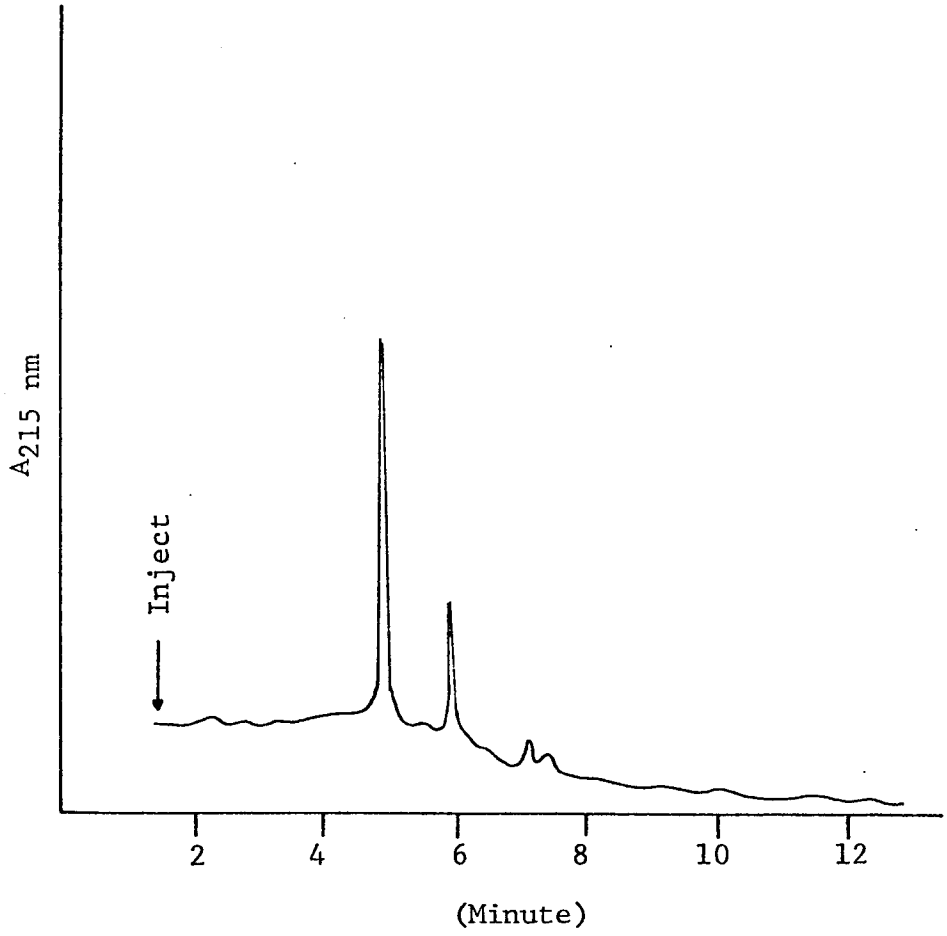

Figure 13. Chromatographic pattern of Amberlite XAD-2 prepurified sera from autistic subjects

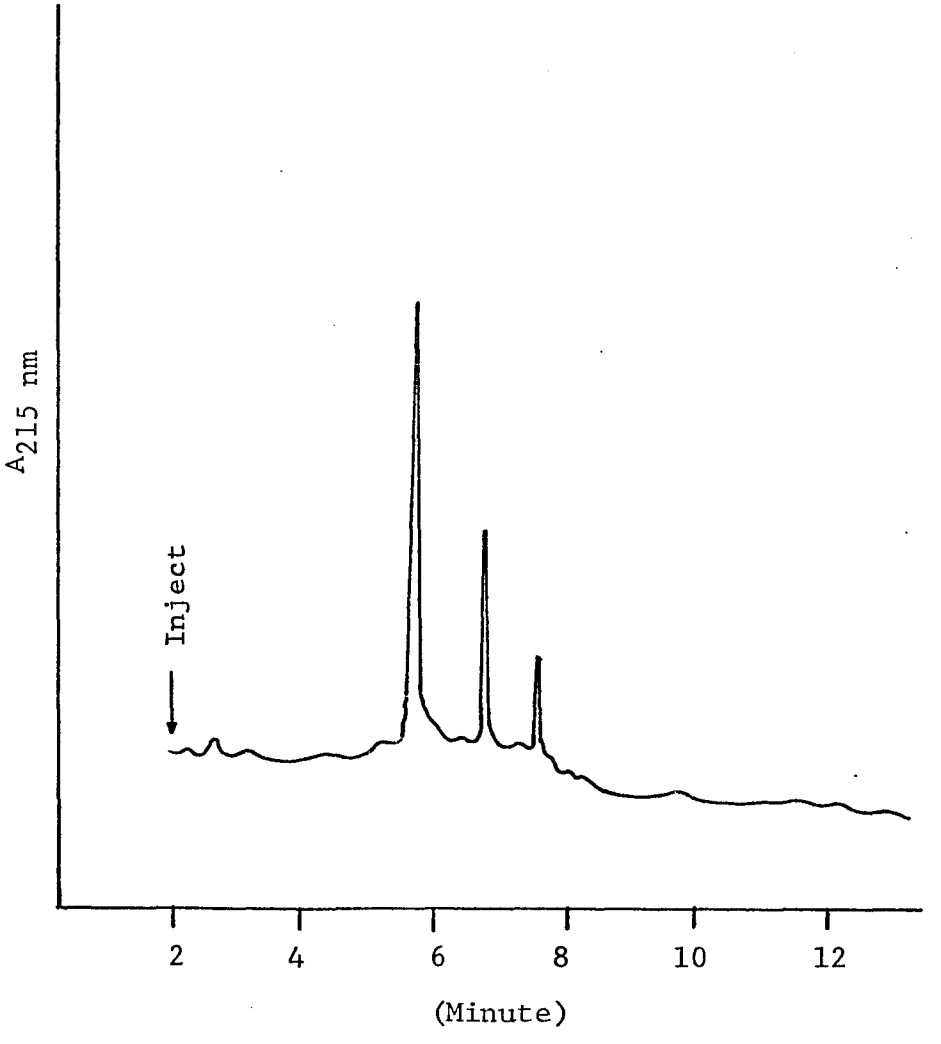

Figure 14. Chromatographic pattern of Amberlite XAD-2 prepurified sera from normal controls

TABLE 8

% Area of Serum Peptides Found in Fraction II of Autistic Subjects

	Time (min.)								
	1.96	2.64	3.68	4.93	5.56	6.18	6.48	6.78	7.33
Subjects									
01	-[a]	-	-	7.12	63.5	4.39	12.3	-	-
02	-	-	-	-	92.6	-	-	7.38	-
03	11.5	-	2.70	-	74.2	-	-	-	-
04	16.7	-	-	-	52.8	4.47	3.70	3.56	-
05	-	3.16	-	-	90.0	-	6.90	-	-
06	-	4.91	-	-	52.9	3.61	9.76	3.28	-
07	-	-	-	-	48.7	-	7.35	-	-
08	2.82	-	-	-	64.8	1.47	1.48	-	3.91
09	-	-	-	-	82.8	-	17.2	-	-
Mean	3.44	0.90	0.3	0.79	69.2	1.55	6.52	1.58	0.43
± SD	6.24	1.83	0.90	2.37	16.6	2.03	5.89	2.63	1.30

[a]Undetectable amount

TABLE 8 (Cont.)

% Area of Serum Peptides Found in Fraction II of Autistic Subjects

	Time (min.)							
	7.91	8.09	8.20[a]	8.34	9.10[b]	10.9	12.6[c]	14.1
Subjects								
01	3.94	-[d]	-	4.50	-	-	-	-
02	-	-	-	-	-	-	-	-
03	4.28	-	-	-	-	-	9.66	-
04	9.05	-	-	-	-	-	-	-
05	-	-	-	-	-	-	-	-
06	3.83	10.5	5.25	3.27	-	-	-	2.63
07	4.33	20.3	-	3.79	-	15.6	-	-
08	1.84	1.61	-	-	1.59	12.4	1.09	2.97
09	-	-	-	-	-	-	-	-
Mean	3.03	3.60	0.58	1.28	0.18	3.41	1.71	0.62
± SD	2.96	7.14	1.75	1.95	0.53	6.12	3.35	1.24

[a]Neurotensin,[b]Substance-P,[c]Leu-endorphin,[d]Undetectable amount

TABLE 9

% Area of Serum Peptides Found in Fraction II of Normal Controls

	Time (min.)										
	1.59	2.03	2.18	2.74	4.03	5.00	5.57	6.04	6.34	6.62	6.75
Subjects											
01	-[a]	-	4.20	-	-	2.42	52.7	-	-	-	23.3
02	4.18	-	-	4.29	-	-	76.4	-	-	-	11.4
03	-	-	-	2.54	-	-	43.1	2.80	3.12	6.02	8.42
04	-	-	5.69	-	-	-	49.1	-	-	-	24.9
05	-	-	-	-	-	-	81.4	-	-	-	14.5
06	-	-	-	-	4.13	-	50.8	-	-	-	23.3
07	-	-	3.05	1.43	0.87	3.53	32.7	1.67	5.10	1.43	18.3
08	-	-	2.78	-	2.28	1.93	33.4	-	6.33	-	12.7
09	-	14.2	-	-	-	-	64.8	-	-	-	8.86
10	-	-	-	-	-	-	81.4	-	-	-	14.8
Mean	0.42	1.42	1.57	0.83	0.73	0.79	56.6	0.45	1.46	0.75	16.1
± SD	1.32	4.50	2.17	1.49	1.49	1.33	18.5	0.98	2.46	1.91	6.09

[a]Undetectable amount

TABLE 9 (Cont.)

% Area of Serum Peptides Found in Fraction II of Normal Controls

	Time (min.)										
	6.89	7.17	7.30	7.38	7.56[a]	7.87	8.19[b]	8.44	9.92	10.2	12.7
Subjects											
01	3.81	-[c]	-	-	11.2	-	-	2.42	-	-	-
02	-	-	3.72	-	-	-	-	-	-	-	-
03	3.30	5.94	8.23	2.68	4.92	4.21	4.68	-	-		-
04	3.70	-	-	-	14.8	1.91	-	-	-	-	-
05	-	-	-	-	3.72	-	-	-	-	-	4.12
06	4.91	-	-	-	9.39	-	-	4.08	3.42	-	-
07	4.28	2.26	2.45	1.35	11.9	2.48	1.61	2.06	0.86	1.42	1.25
08	4.51	2.34	3.24	-	12.2	12.6	3.21	2.36	-	-	-
09	-	-	-	-	12.1	-	-	-	-	-	-
10	-	-	3.74	-	10.5	-	-	-	-	-	-
Mean	2.45	1.05	2.14	0.40	9.06	2.16	0.95	1.09	0.43	0.14	0.54
± SD	2.15	1.97	2.72	0.91	4.64	3.99	1.69	1.51	1.09	0.45	1.32

[a]Met-enkephalin, [b]Neurotensin, [c]Undetectable amount

from the total of nine showed the presence of this peak. A peak with a retention time of 6.48 minutes was found in seven out of nine autistic subjects and none in the control subjects. All the control subjects, except one, showed a peak which match the retention time of met-enkephalin and none was found in autistic subjects. Two autistic subjects showed a peak which had the same retention time as leu-endorphin and this was confirmed by addition of a known quantity of leu-endorphin standard to the samples.

Fractionation by HPLC - The purity of the peak with a retention time of 5.56 minutes from pooled sera of autistic and control subjects was shown in Figure 15. This was confirmed by the modified gradient method where the retention time was extended as described in the Methods section.

Sephadex G-25 Chromatography and Gradient HPLC - Sera Sephadex G-25 chromatography elution patterns from autistic and control subjects are presented in Figures 16 and 17. The patterns from both groups were very similar and contained three fractions. Since only one sample from each group had been studied, it was difficult to be sure whether the second fraction from autistic subject was of any significance. Gradient HPLC of Sephadex G-25 Fraction I from both autistic and control subjects showed no materials with absorbance at 215 nm (Figure 18). Although these chromagens absorbed light at 280 nm, they were not peptide materials and was confirmed with ninhydrin. Similar results were obtained with Sephadex G-25 Fraction III from both groups (Figure 19). Figures 20 and 21 showed the HPLC patterns from Sephadex G-25 Fraction II of autistic and control subjects. In autistic subjects, the peaks with retention times of 5.56 and 6.74 minutes were the same as that found in samples chromatographed on HPLC directly after Amberlite XAD-2 column. the peak with a retention time

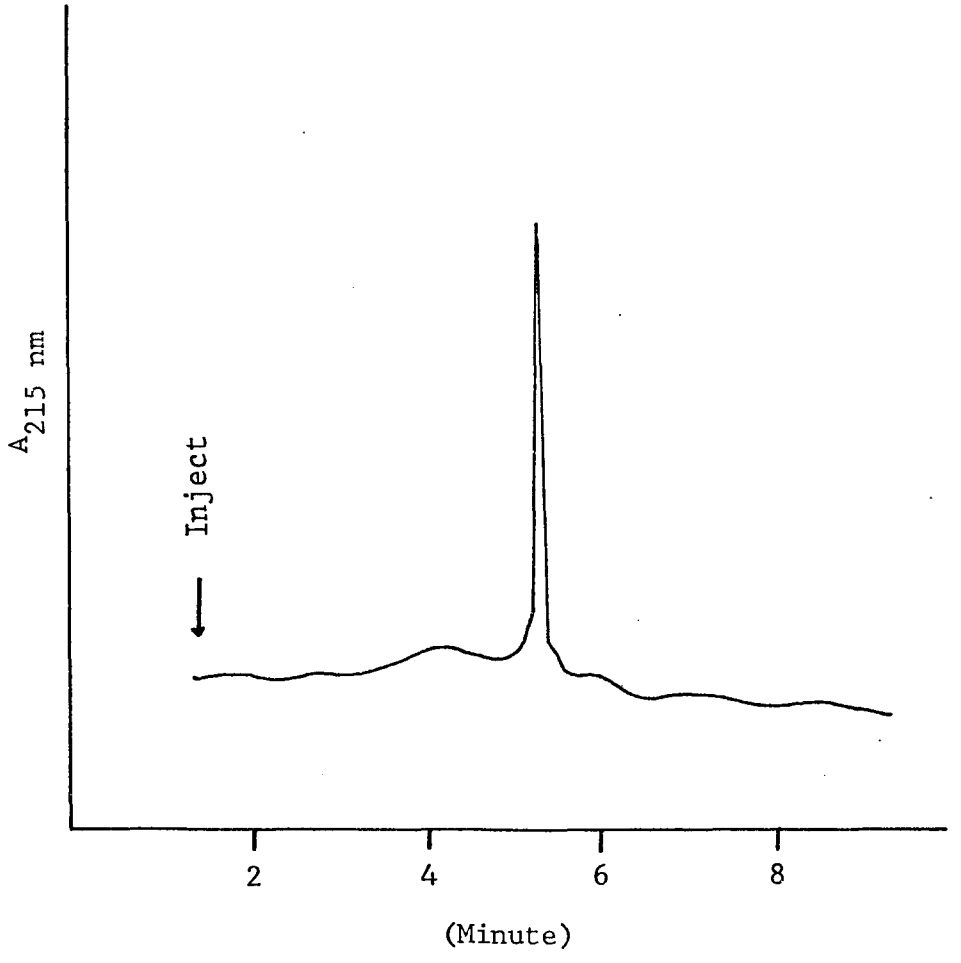

Figure 15. Chromatogram of a peak with a retention time of 5.56 minutes fractionated from pooled prepurified sera of normal controls

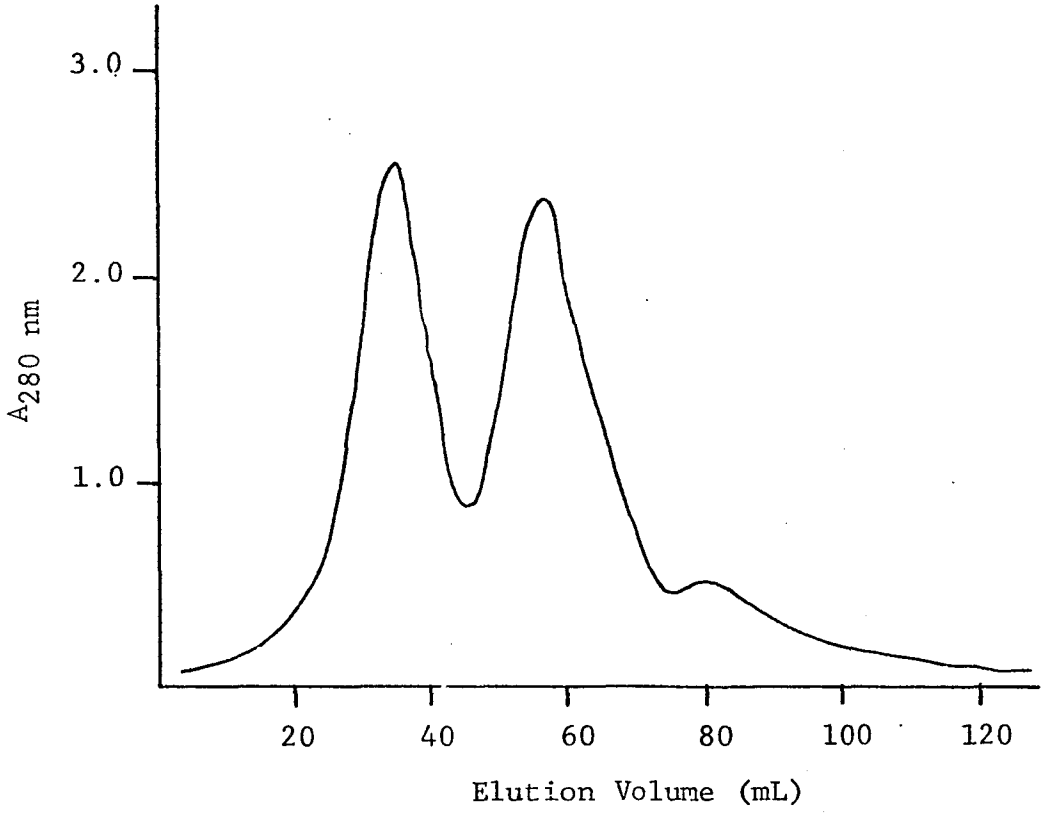

Figure 16. Sephadex G-25 chromatogram of serum sample from an autistic subject

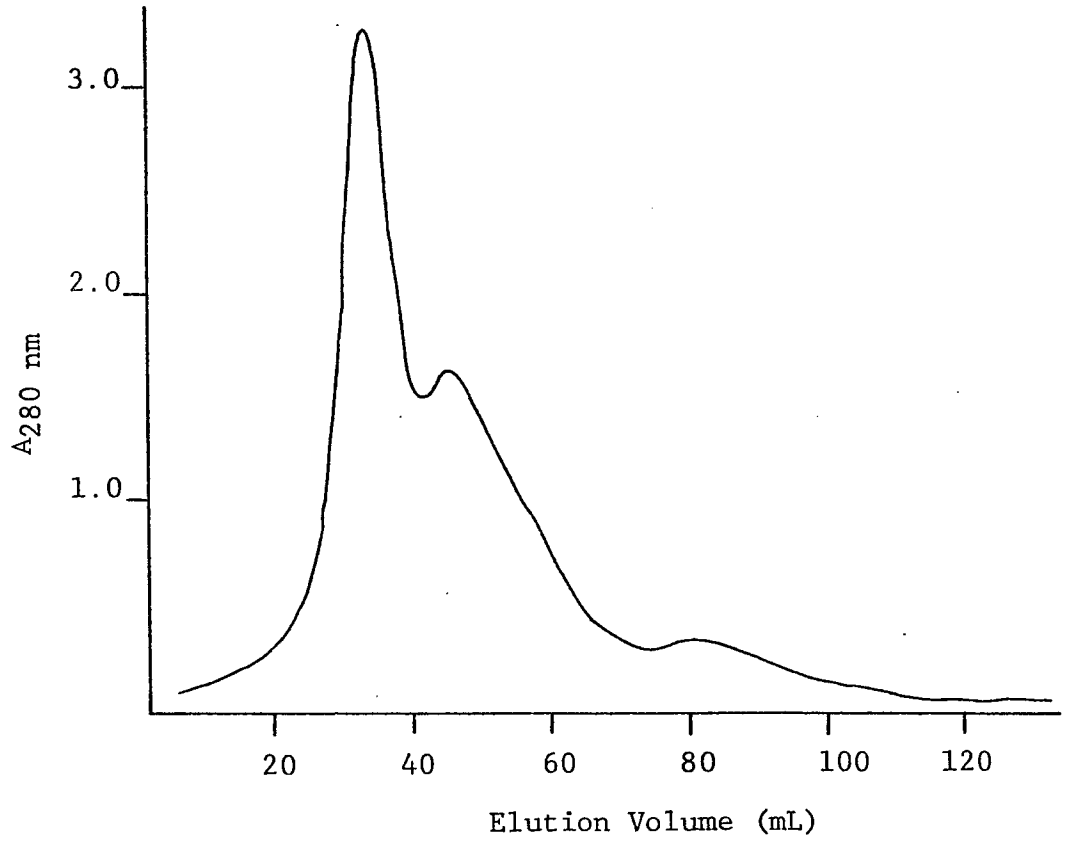

Figure 17. Sephadex G-25 chromatogram of serum sample from a normal control

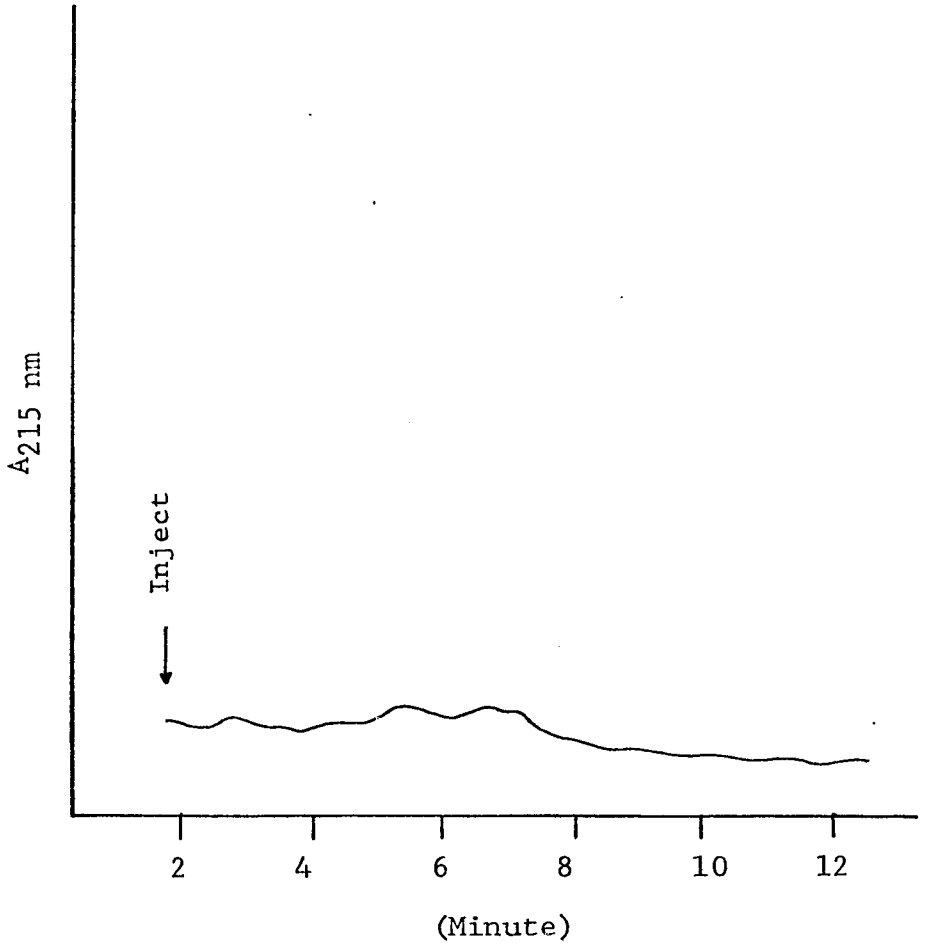

Figure 18. HPLC chromatographic pattern from serum Fraction I of an autistic subject and a normal control

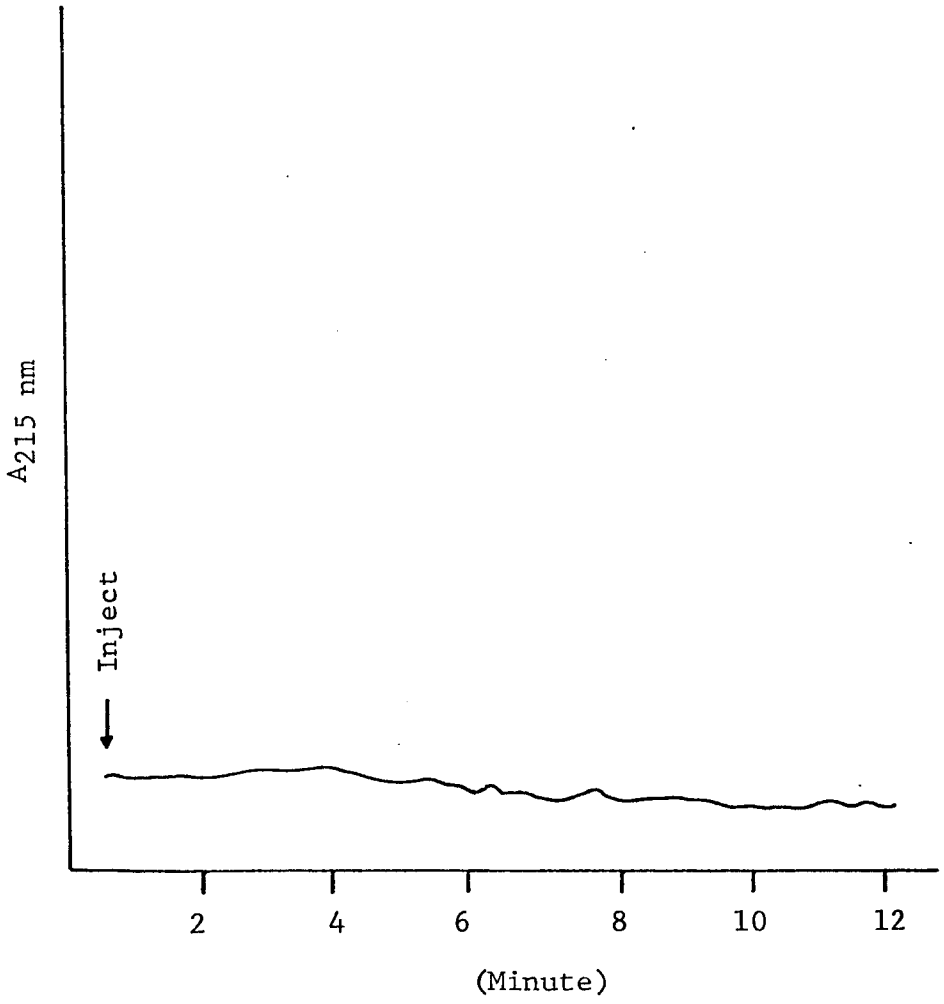

Figure 19. HPLC chromatographic pattern from serum Fraction III of an autistic subject and a normal control

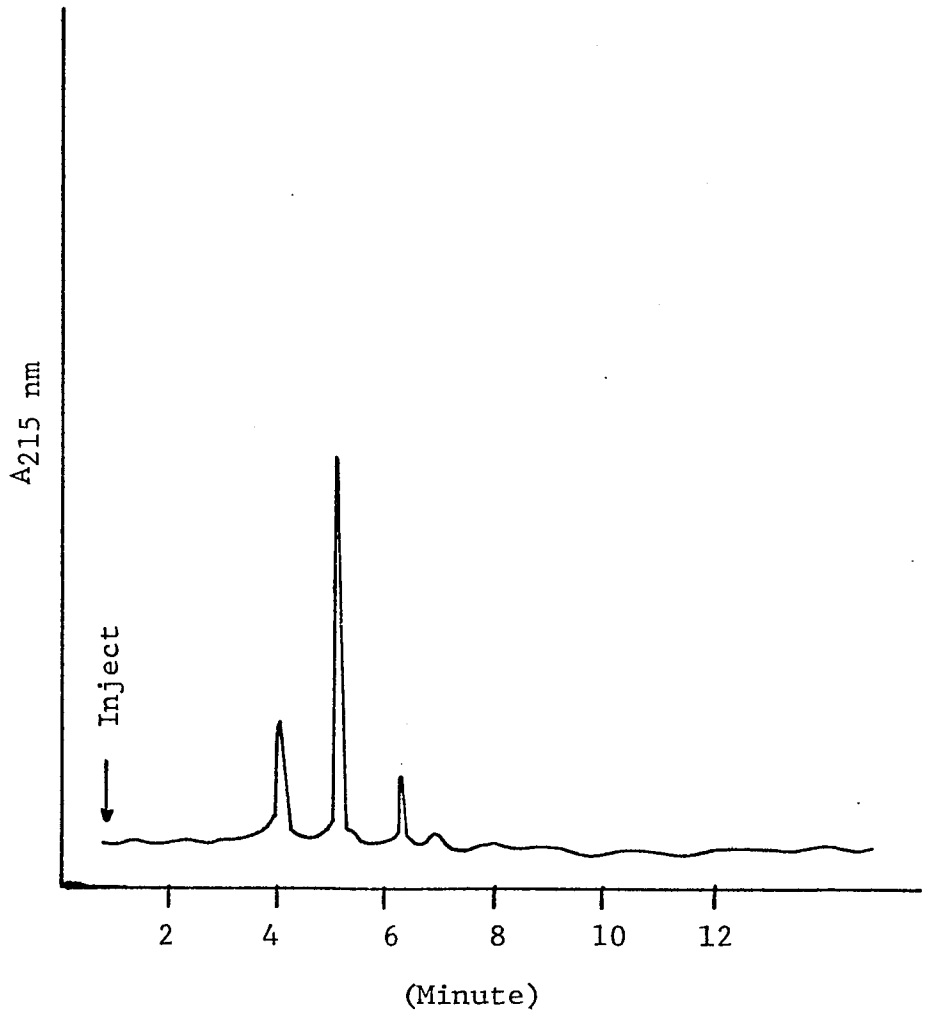

Figure 20. HPLC chromatographic pattern from serum Fraction II of an autistic subject

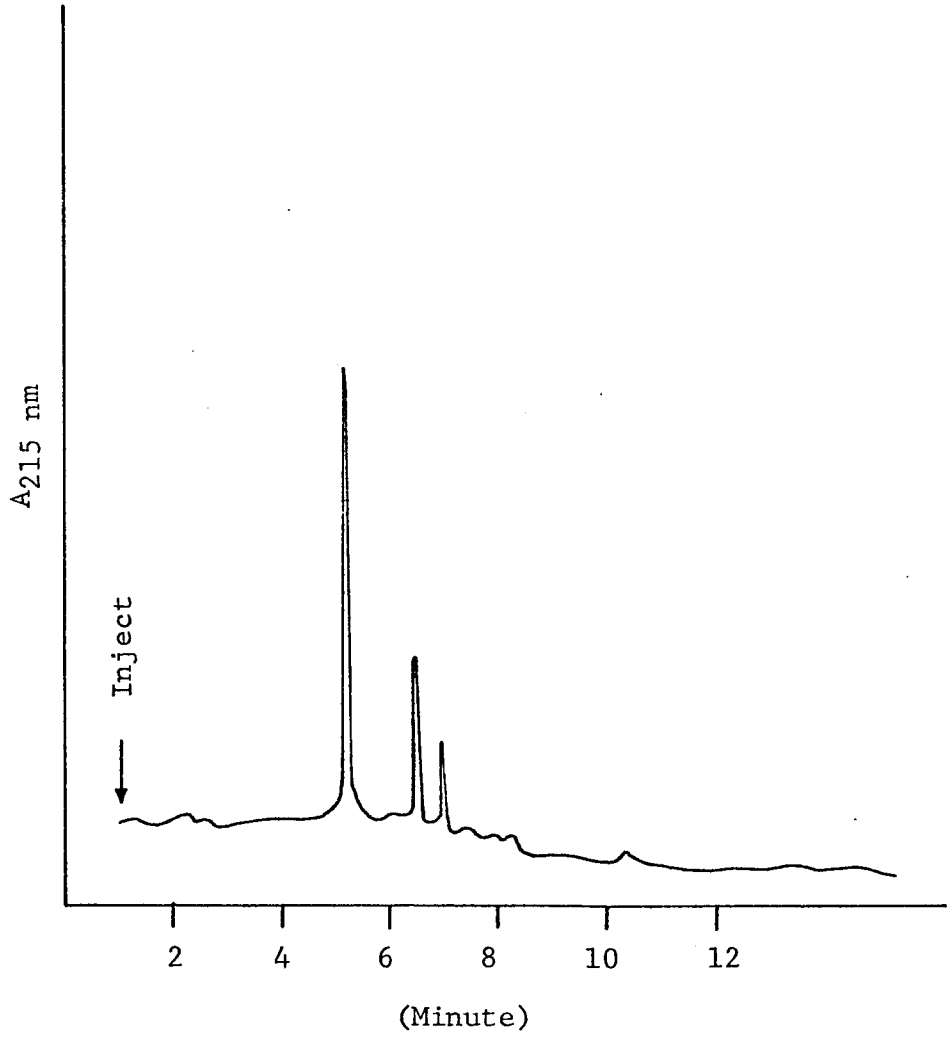

Figure 21. HPLC chromatographic pattern from serum Fraction II of a normal control

of 4.50 minutes was uncommon and could be significant. HPLC chromatogram of control subject was the same as that derived from Amberlite XAD-2 prepurified samples having the three major peaks of retention times of 5.56, 6.72, and 7.56 minutes.

Amino Acid Analysis

All common PTH amino acids were cleanly separated by HPLC within 15 minutes by the procedure described as can be seen in Figures 22 and 23. By individual HPLC of each PTH amino acid, these retention times were confirmed. Linearity studies of glycine (5.25 minutes) and tryptophan (11.45 minutes) calculated as a ratio of the compound to that of internal standard norleucine and plotted against the amount in nmole were found to be linear (Figures 24 and 25). The blank amino acid (HCl only) analysis showed two peaks where one was probably contaminated acid (10.58 minutes, Figure 26). Acid hydrolysis of standard met-enkephalin yielded glycine, tyrosine, methionine, and phenylalanine with the expected ratio of 2:1:1:1 (Figure 27). Amino acid analysis of the peak which co-chromatographed with neurotensin from Sephadex G-25 Fraction I of autistic and control subjects seemed to have only alanine (Figure 28). Amino acid analysis of the common peak from Sephadex G-25 Fraction V of autistic subjects was found to contain tyrosine, methionine, and an unidentified amino acid with the ratio of 3:1:1 (Figure 29).

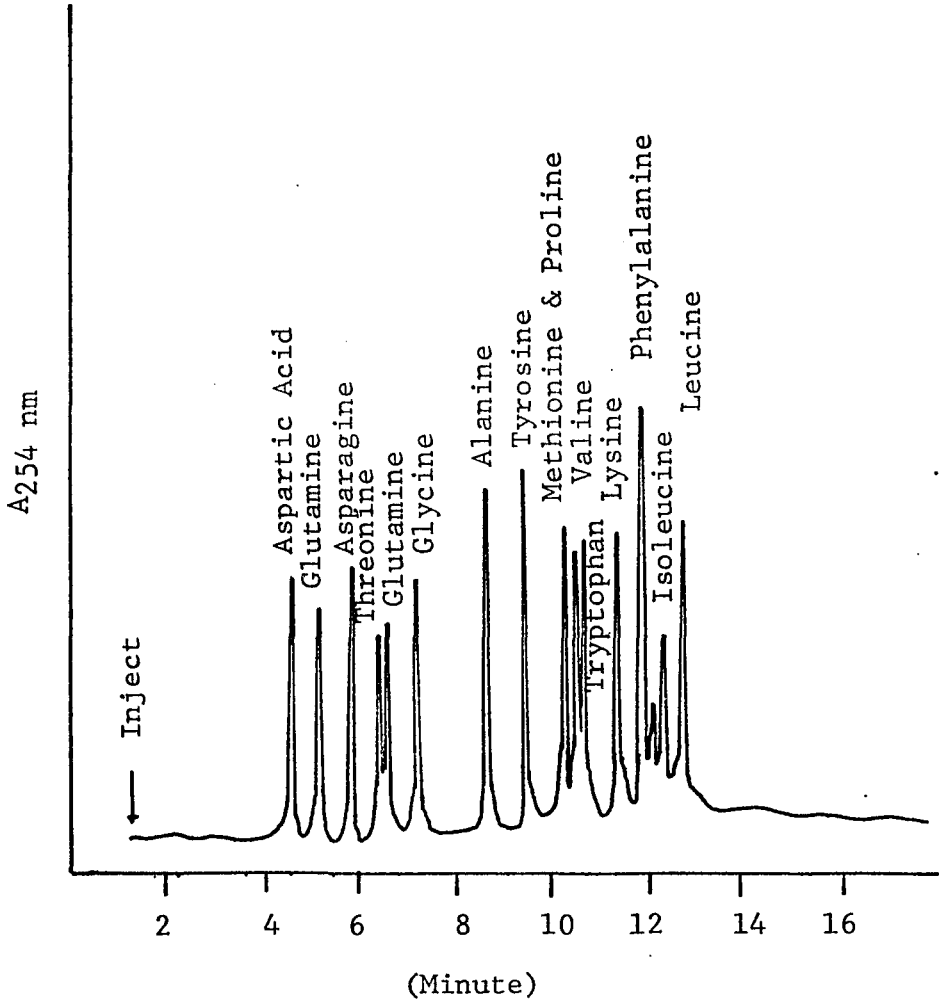

Figure 22. HPLC chromatogram of PTH-amino acid standards

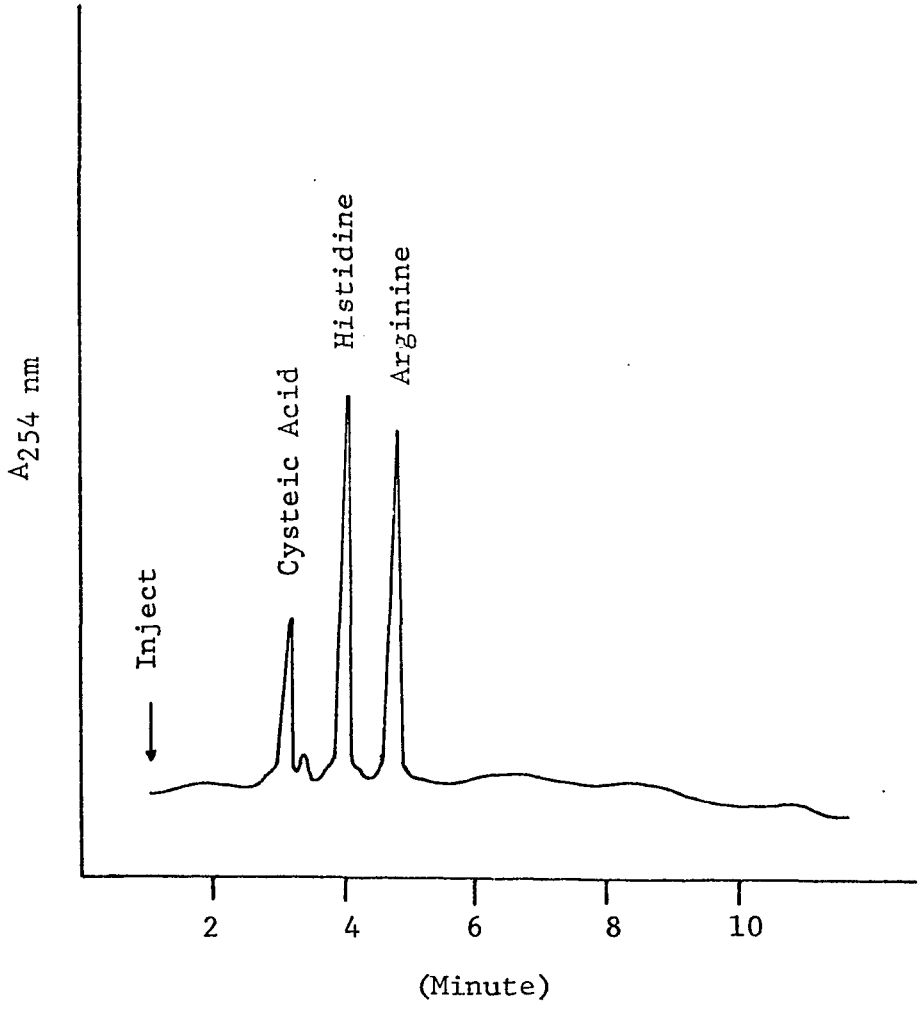

Figure 23. HPLC chromatogram of PTH-amino acid standards

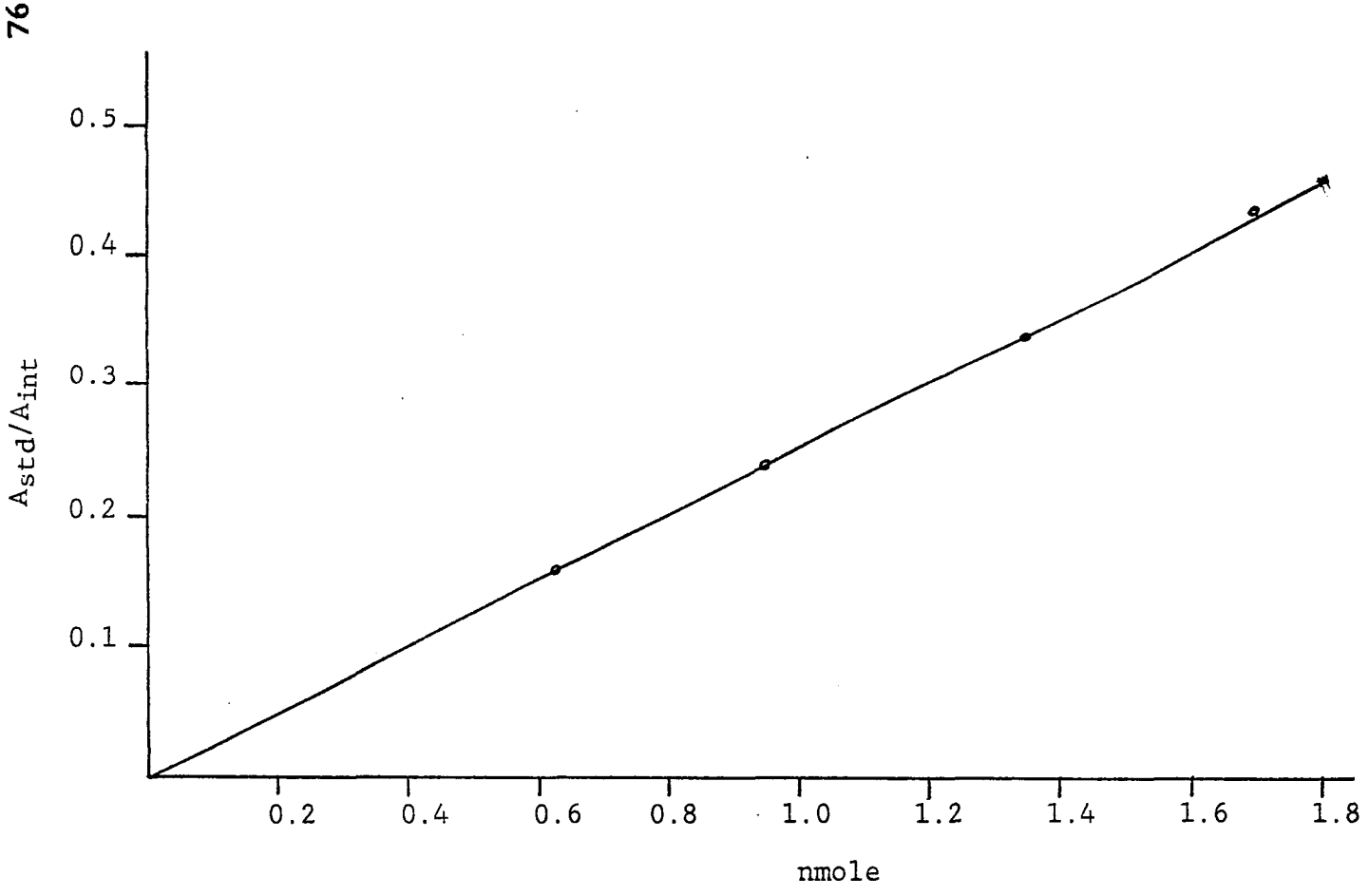

Figure 24. Linearity study of PTH-glycine with increasing concentration

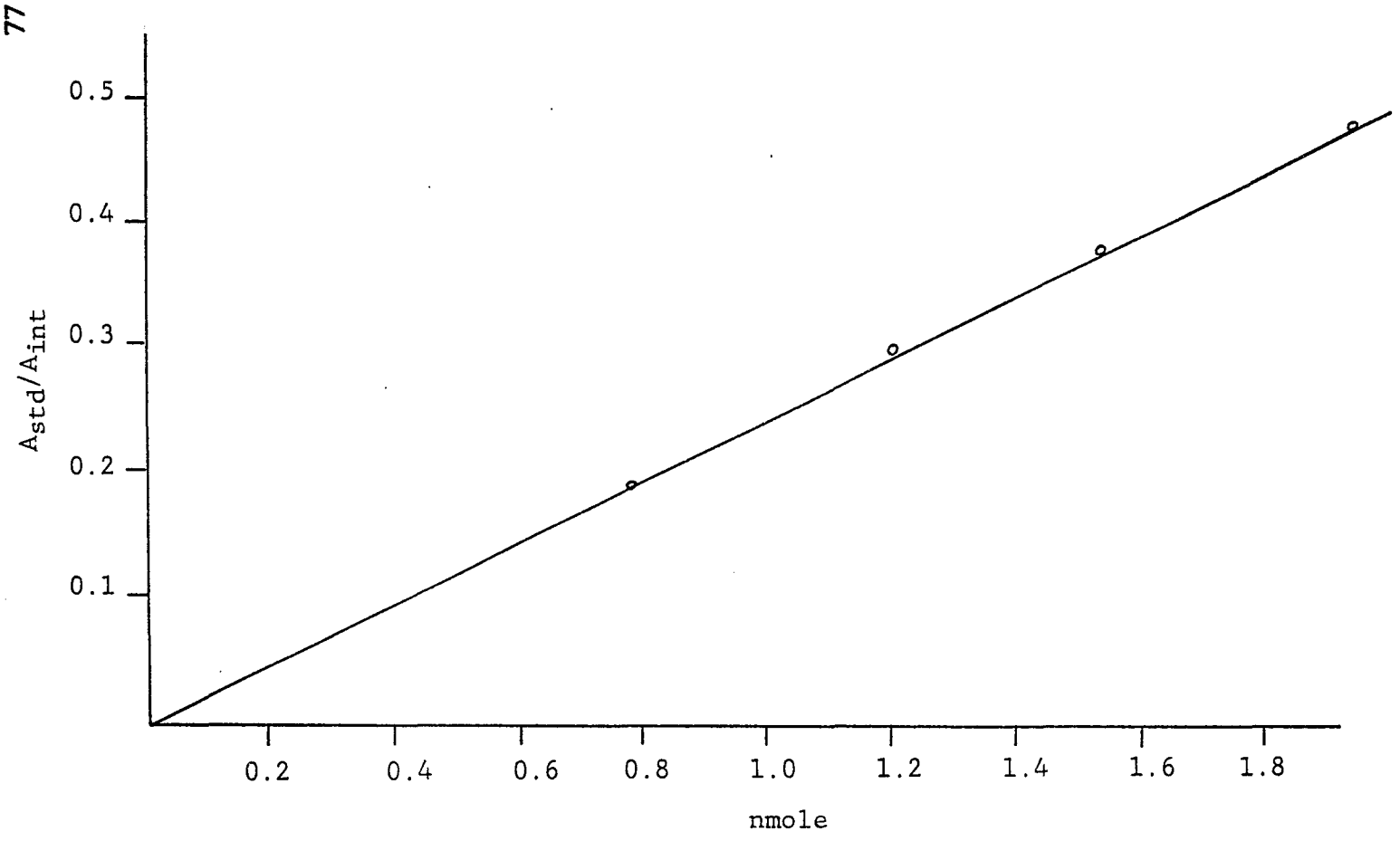

Figure 25. Linearity study of PTH-tryptophan with increasing concentration

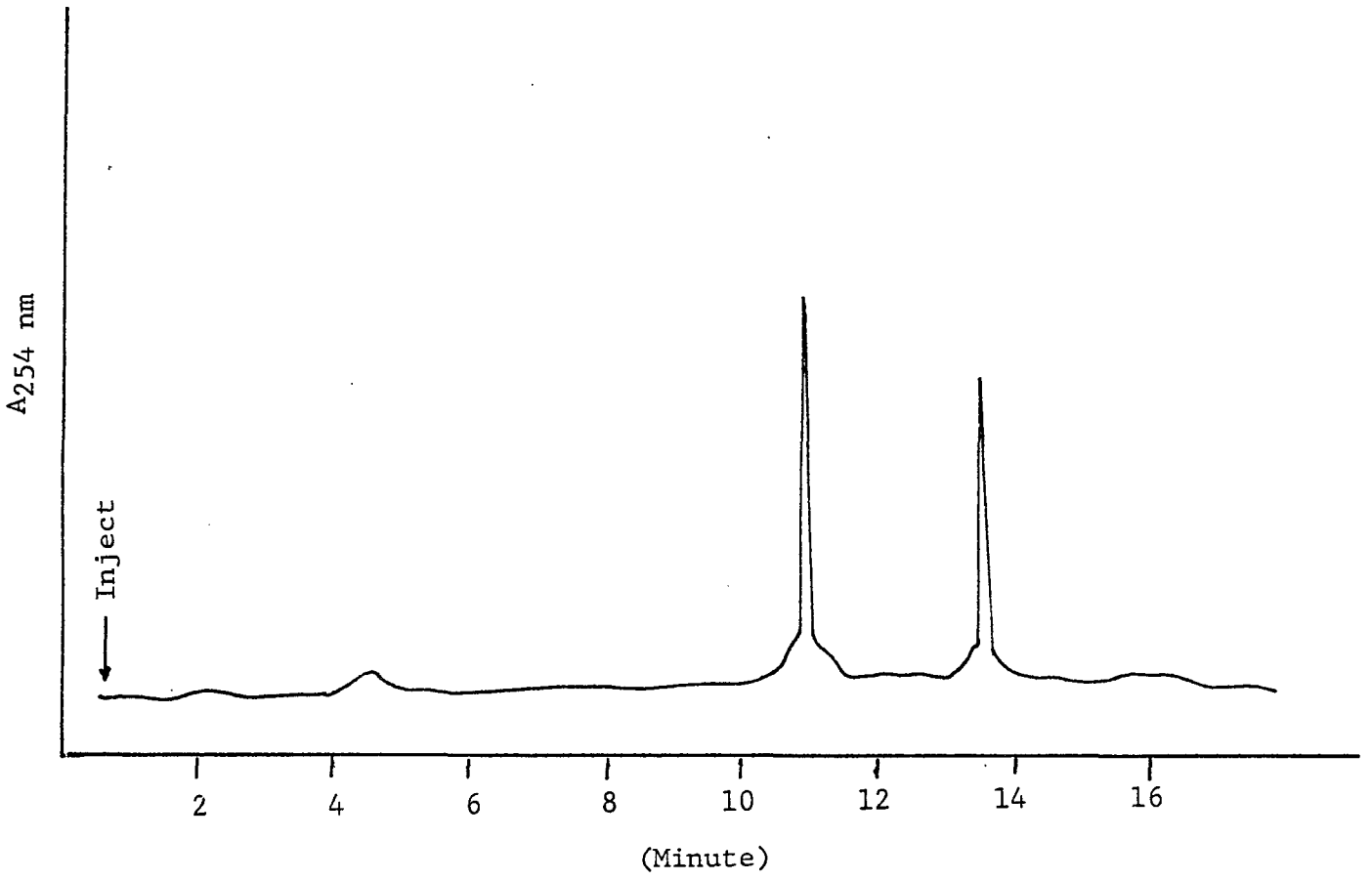

Figure 26. Blank amino acid HPLC chromatographic pattern

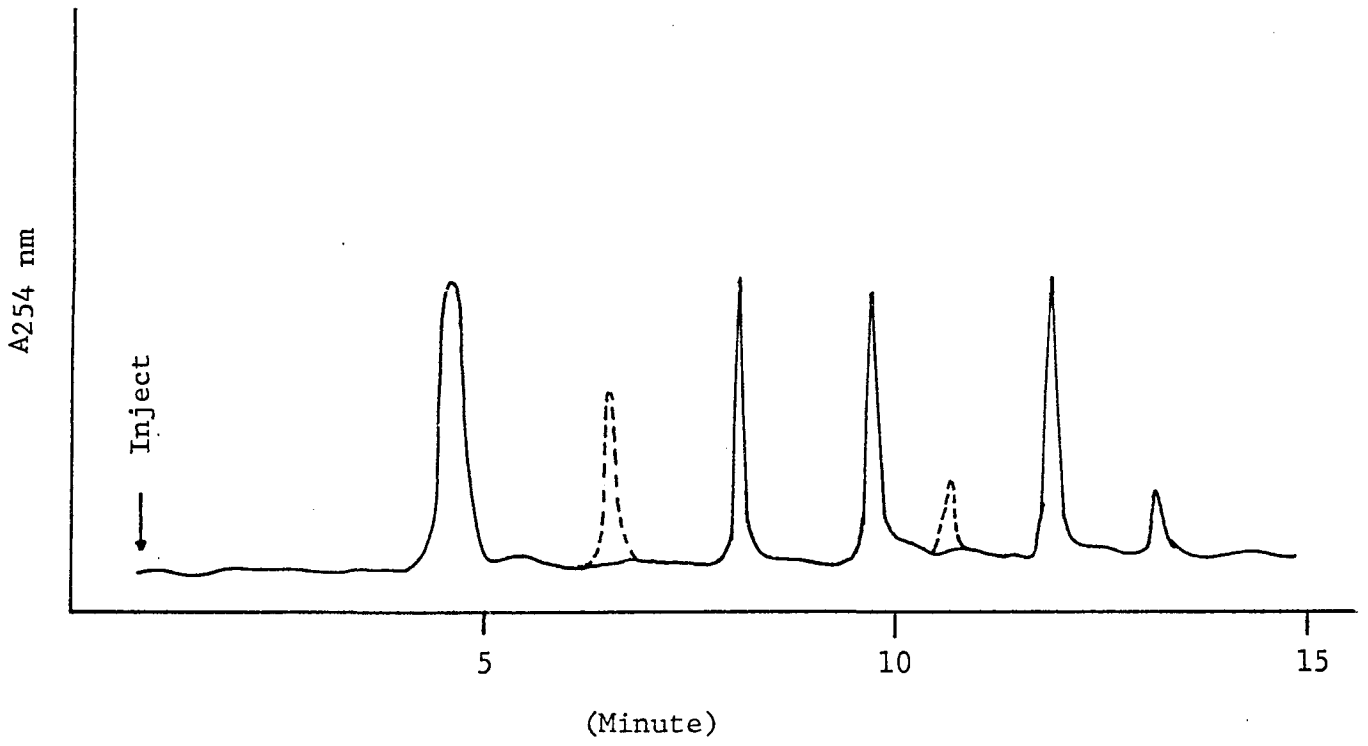

Figure 27. Standard met-enkephalin amino acid analysis HPLC chromatographic pattern

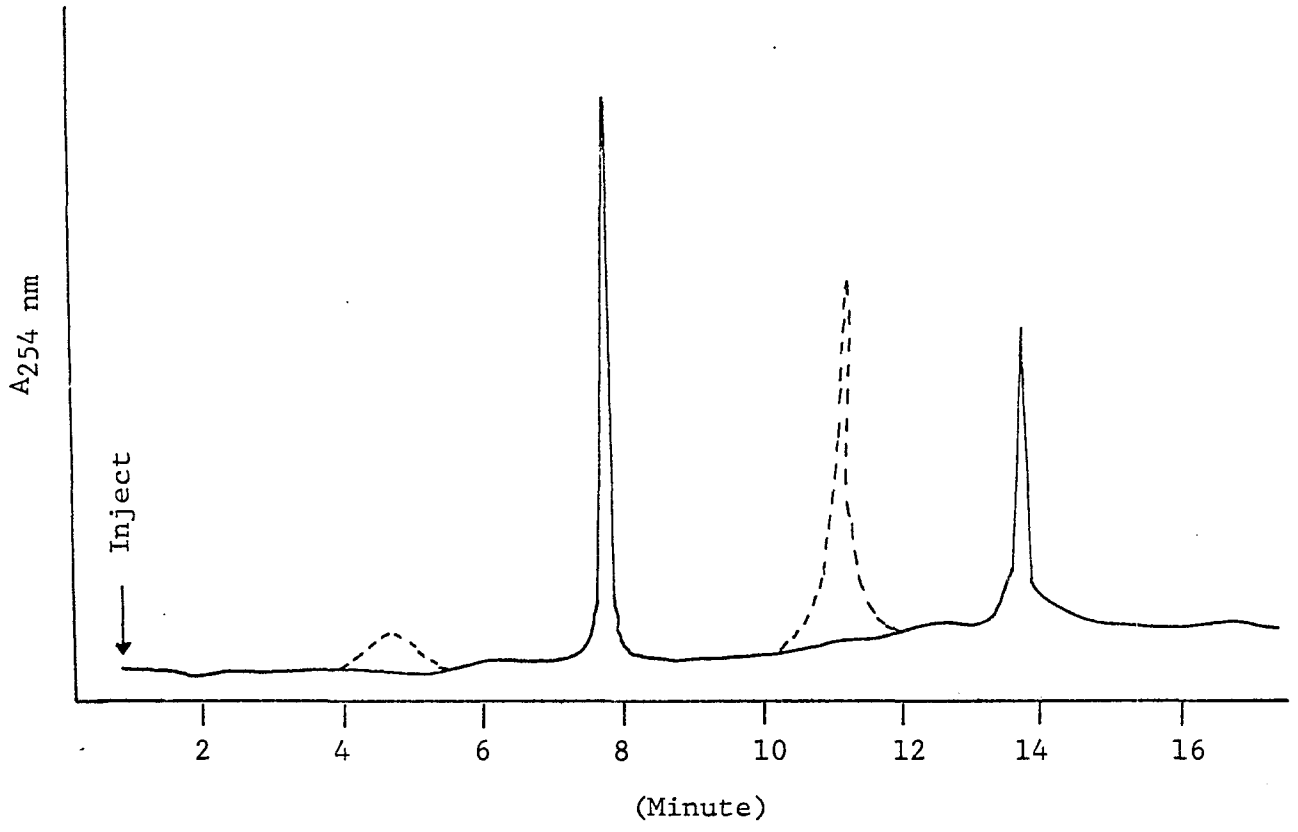

Figure 28. Amino acid HPLC chromatographic pattern of the peak which co-chromatographed with neurotensin from Fraction I of an autistic subject

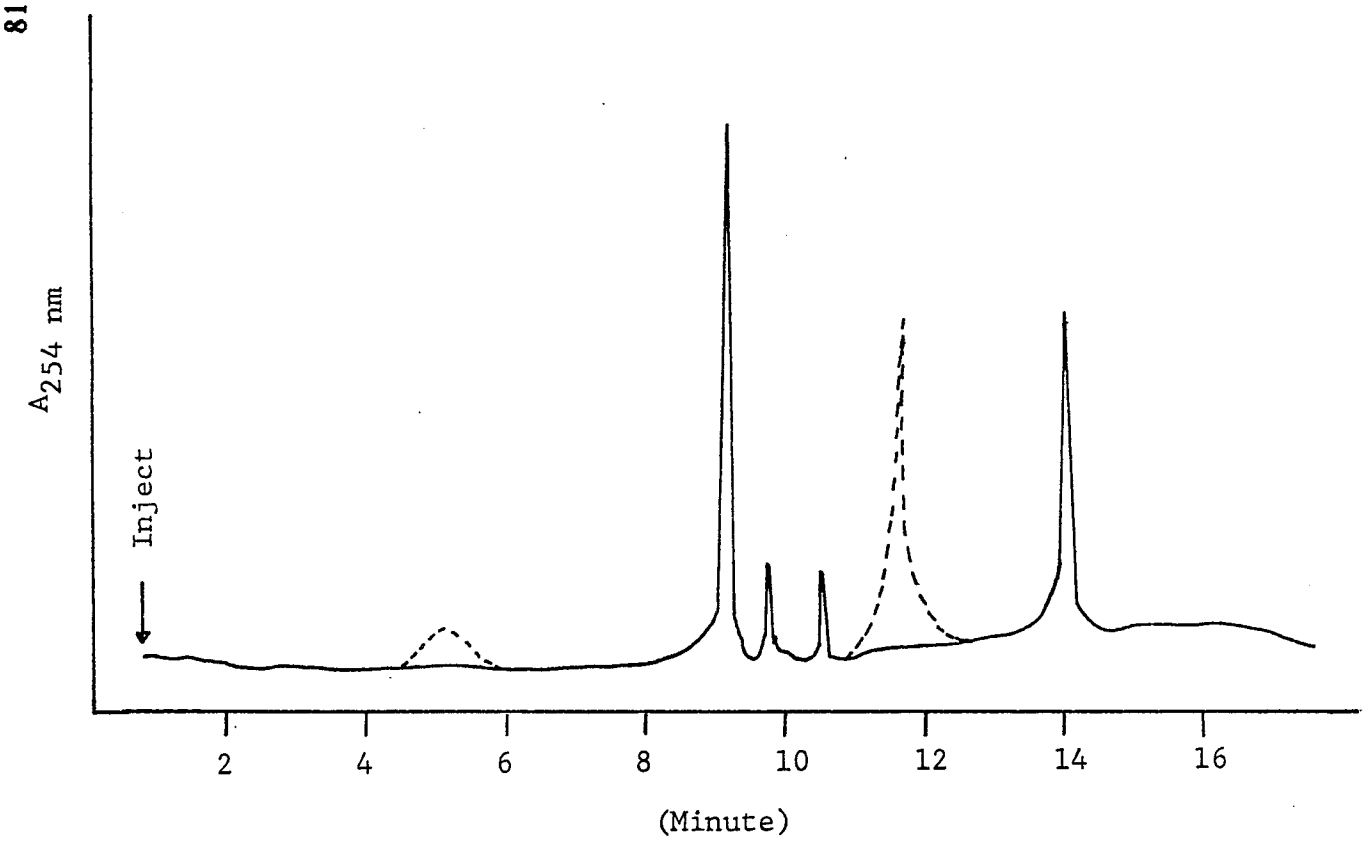

Figure 29. Amino acid HPLC chromatographic pattern of the peak with a retention time of 7.6 minutes from Fraction V of an autistic subject

CHAPTER V

DISCUSSION

Urinary and Serum Peptides

All of the autistic subjects in the study showed abnormal patterns of urinary peptide compared to controls. I observed only one Sephadex G-25 peptide elution pattern among all of the subjects studied. In contrast Gillberg et al. (1982) found two types of peptidic patterns, A (a majority, similar to the current finding) and B. They also reported that their typical pattern (A) seemed to be among the more severe autistic subjects. These groups compose of those that exhibit mental retardation, no useful speech and derangement in behaviors. This is in contrast to the finding in my study because only one pattern was observed no matter whether the subjects were severely handicapped or not. The population I studied ranged from near-normal to severely autistic. Trygstad et al. (1980) also reported that typical peptidic patterns helped to differentiate primary from secondary anorexia nervosa and to differentiate schizophrenia from other psychoses. Since the peptidic pattern of the autistic subjects in this study is distinct from that of controls, particularly Fraction V, it is possible that the pattern found may be useful in distinguishing autism from other childhood psychoses.

Further separation of Fraction I and V on HPLC showed a number of peaks which eluted as one peak from Sephadex G-25 chromatography.

Although several peaks were found in Fraction V of all autistic subjects, only one peak with a retention time of 7.6 minutes was found to be common to all subjects. Although this peak has a similar retention time as that of met-enkephalin, it proved not to be the same compound. According to the molecular mass, this peptide is 300 daltons or less compared to met-enkephalin which is approximately 570 daltons. This peptide did not compare in amino acid composition with met-enkephalin since it was composed of tyrosine, methionine and an unidentified amino acid. However, the sequence of amino acids of this peptide has not yet been established. Since it is common to all autistic subject urines and its absence in a control, this peptide may ultimately serve as a specific marker for autism. The peak from Fraction I which co-chromatographed with met-enkephalin but was not found in the controls also deserved further investigation as another marker of this syndrome. Although this peak has the same retention time as that of met-enkephalin, it also may not be the same compound. Co-chromatography is not a definitive criterion for identification since a substance in Fraction I co-chromatographing with neurotensin was composed of only alanine residue after amino acid analysis.

The results of HPLC separation of serum constituents prepurified on XAD-2 column showed the converse of urine since a peak which co-chromatographed with met-enkephalin was common in all but one of the control subjects and in none of the autistic subjects. This serum peptide may also prove to be a valuable diagnostic marker. Serum peptide assays are more efficient than urinary determinations since results can be obtained within 2-3 days as compared to two weeks. Although there is a difference in the peptide HPLC pattern of Fraction II eluting from Sephadex G-25, the

significance has not been determined since only one serum sample from each group has been processed.

Although the origin of the peptides in this study remains obscure, other studies have showed that peptides in the urine and serum are from the CNS. TRH activity was found in the urinary precipitate from congenital generalized lipodystrophy (Foss and Trygstad, 1975). Protein fractions in human serum with the ability to induce symptoms reminiscent of schizophrenia in rats had also been found (Heath and Krupp, 1968; Sardesai et al., 1977). Furthermore, peptides isolated from the urine of some human subjects with anorexia nervosa have been demonstrated partially to induce the symptoms of this disorder in rats (Reichelt et al., 1978). Whatever their origin and basic mode of action, the peptides responsible for the chromatographic pattern seen in this study are likely to reflect some underlying biological dysfunction. The pattern and peptides found might prove to be important not only in diagnosis but also as a base for further investigation.

Neuroanalytes

Serotonergic mechanisms are involved in a broad range of adult and childhood disorders (Bouillon, 1978). Serotonergic activity in the brain is believed to exert an effect on many neuronal systems as well as affecting the following physiological functions and behaviors: sleep, body temperature, pain, sensory perception, sexual behavior, motor function, neuroendocrine regulation, appetite, learning and memory, and an immune response. The finding of hyperserotonemia in the autistic subjects is in agreement with

many previous studies (Ritvo et al., 1970; Campbell et al., 1974; Hanley et al., 1977). Although a majority of the autistic subjects in the study showed higher than normal levels of serotonin, there were those who had the levels below their age-matched controls. Hyperserotonemia in these subjects could be due to a greater number of blood platelets. This statement is supported by an early study which showed that autistic children have a higher blood platelet count than controls (Ritvo et al., 1970). Since the measurement of blood serotonin levels does not reflect the levels in the brain, its significance in this disorder is unclear. The most important point of all is that the studies of serotonin levels in autism so far have not led to any substantial hypotheses for treatment of this syndrome. For better understanding of high levels of serotonin in the blood, the relation of hyperserotonemia to clinical picture will have to be clarified through the comparison of high and low blood serotonin groups. There is also a general need for a better biological and behavioral characterizations of hyperserotonemic subjects. Clinical features accompanying hyperserotonemia in autism and other diagnostic groups need to be compared and the genetic contribution established. Since serotonergic activity is involved in physiological functions as well as behaviors, measurements of blood serotonin in conjunction with CSF 5-HIAA may be more helpful. 5-HIAA, the principle serotonin metabolite, is a more direct index of brain indoleamine metabolism. It was found that levels of CSF 5-HIAA were lower in autistic patients when compared with less disturbed children with other types of early-onset childhood psychosis (Cohen et al., 1974; 1977). It has also been shown that lower CSF 5-HIAA levels were related to greater impairment in attentional functioning and social relatedness (Cohen et al., 1974; 1977). So the simultaneous study

to establish the relation between blood serotonin and CSF 5-HIAA levels may be important not only in understanding the clinical features of this syndrome but could lead to the treatment.

Since both brain dopaminergic and adrenergic systems are thought to affect a variety of physiological functions and behaviors, including motor function, cognition, brain-stimulation reward mechanism, eating and drinking behaviors, sexual behavior, selective attention, arousal, memory and learning (Calne et al., 1975; Costa and Gessa, 1977; Amaral and Sinnamon, 1977; Roberts et al., 1978; Moore and Bloom, 1979), it is important that these catecholamines are studied in connection with autism. The finding of high levels of dopamine may not reflect any abnormality in connection with this syndrome since plasma dopamine levels have no known relation to brain dopaminergic function. High concentrations of plasma norepinephrine in the subjects indicate that DBH activity cannot be taken as an index of sympathetic activity, since it has been found earlier that the activity of DBH was lower in autistic subjects as compared to controls (Lake et al., 1977). From the observation, I can only speculate that the high levels of norepinephrine may be due to a response to stress of venipuncture or on a chronic basis as a "predisposition to some forms of childhood psychosis" (Belmaker et al., 1978). This peripheral abnormality of norepinephrine may reflect a central disorder. It has also been proposed that in autistic subjects there is a dysfunction in the mesocortical ring and neostriatum (Damasio and Murer, 1978). Since this region is the target area for dopaminergic neurones originating in the brainstem, it is possible that modifications in catecholamine metabolism in this region could lead to abnormal behaviors seen in these subjects. There is evidence from several studies that in autistic

patients the brainstem has dysfuntional sections (Bonvallet and Allen, 1963; Rimland, 1964; Hutt and Hutt, 1970; Simon, 1975).

Suggestions for Further Work

Although the urinary peptidic patterns of autistic subjects in this study are unique when compared to those of normal controls, it is still difficult to be certain of their usefulness as diagnostic tools since the number of samples is quite small. I feel that a further study with a larger number of subjects with this syndrome is essential so as to be confident of the pattern generated. It is also important that a study be carried out in connection with other behavioral disorders. This may include schizophrenia, other childhood psychoses, epilepsy, mental retardation, minimal brain dysfunction, and attention deficit disorder. By generating patterns from these different disorders, the uniqueness of autistic pattern can be tested and compared. If by comparison the pattern is still unique to autism, it is possible then that the pattern can be used as a differential diagnostic tool for this syndrome. With certain common pathology among these different disorders, the generated patterns may also help in shedding some light to the underlying problems of autism, since more are known of other disorders. Serum peptidic patterns also deserve further study, and it probably can be carried out in conjunction with the urine study. Its usefulness as a diagnostic tool has to wait until a more optimize methodology is developed.

The different fractions, particularly I and V, from Sephadex G-25 also deserve further study. One experiment that can be carry out is the effect of these fractions on platelet serotonin uptake and efflux. As mentioned earlier

that platelet may be used as a partial model for neuron-containing 5-HT, so effect exerts by any of these fractions on the platelet may point to the derangement of the serotonergic system itself. It is also important that individually purified peak be tested so the identity of the particular peptide that affect the platelet-serotonin mechanism can be elucidated. Another study that can be carried out is the effect of these purified peptides on any behavioral changes in the animal models. Characterization of these peptides by performing amino acid sequencing may help in determining whether these peptides are of CNS origin or not.

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APPENDIX

The Procedure for Catecholamine Determinations

Plasma catecholamines were assayed by a modification of HPLC method of Causon et al. (1981) using electrochemical detection as follows: Blood was collected by direct venipuncture into heparin-coated tubes. Plasma was separated and store at -20° C until assayed. Catecholamines were extracted on acid washed alumina (50 mg/1.0 mL plasma). Catecholamines were bound to alumina by adding 0.5 mL of 1.5 M Tris-HCl, pH 8.6, containing EDTA (0.02 M) to plasma. After vortexing and shaking for 15 minutes, the alumina was allowed to settle and the supernatant was carefully aspirated. The precipitated alumina was then washed 4 times each with 5 mL of deionized double distilled water. The catecholamines were then eluted from the washed alumina with 0.1 M HCl (0.25 mL) and centrifuged. To 0.2 mL of the supernatant was added 0.2 mL of the mobile phase. From this, 0.25 mL was injected. Catecholamine analysis was done on an Altex C-18 ultrasphere silica column (0.5 x 25 mm).

Standard catecholamines were prepared in the mobile phase (0.1 M HCl (1:1)) and used for the preparation of the standard curves. Dihydroxy-benzylamine (DHBA) was used as an internal standard. Values of catecholamines were determined by measuring the peak heights and interpolated from the standard curve for each catecholamine. Calibration

was achieved by comparison with the ratio of catecholamine peak height/DHBA peak height obtained from extracted standards.

Results

Plasma Catecholamines in Autistic Subjects and Normal Controls[a]

	Autistic (n = 13)	Control (n = 10)
L-Dopa (pg/mL)	221 ± 205	168 ± 129
Dopamine (pg/mL)	730 ± 444[b]	249 ± 128
Norepinephrine (pg/mL)	2399 ± 1943[c]	675 ± 850
Epinephrine (pg/mL)	961 ± 977	355 ± 635

[a]Data are represented as the Mean ± SD.

[b]$p<0.01$

[c]$p<0.05$