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Hyperpipecolatemia: A New Metabolic Disorder Associated with Neuropathy and Hepatomegaly: A Case Study

P. D. GATFIELD, M.D., ELISABETH TALLER, R.T., G. G. HINTON, M.D., A. C. WALLACE, M.D., G. M. ABDELNOUR, M.D. and M. D. HAUST, M.D.,
London, Ont.

DURING the course of the determination of serum amino acid concentrations in a group of children with mental retardation and behaviour problems one child with a progressive generalized neurological disease was found to have a ninhydrin-positive compound not seen in sera of other patients. This was identified as pipecolic acid, a cyclic secondary amino acid that is involved in the metabolism of lysine,[1, 2, 3] and loading studies confirmed that there was a block in pipecolic acid metabolism. The progressive downhill course of the illness was unaffected by dietary measures to reduce the intake of pipecolic acid and its precursor, lysine, in spite of some reduction in serum pipecolate concentrations. This is a report of the clinical, biochemical and autopsy findings in what is believed to be the first such case studied.

With modifications of the paper chromatographic method used initially, pipecolic acid was detected in the sera of most retarded patients examined and in a control group. While the concentrations observed in all subjects were much lower than in the index case, the range of values was unusually wide. Other unusual problems were encountered in the assay. Stringent dietary precautions were found necessary to obtain reliable resting values. In addition, under the normal conditions of automatic ion-exchange column amino acid analysis, pipecolic acid gave so little colour that it was not detectable, even in the index case. It was found only when 2 ml. (five times the usual serum volume) was added to the column.

From the Children's Psychiatric Research Institute and the Departments of Biochemistry, Pediatrics and Pathology, University of Western Ontario, London, Ontario.
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Reprint requests to: Dr. P. D. Gatfield, The Children's Psychiatric Research Institute, P.O. Box 2460, London, Ontario.

Case Report

The patient, K.K., aged 18 months at the time of admission to this hospital, was delivered normally at term after an uncomplicated pregnancy, and weighed 3.5 kg. at birth. The parents, of Irish, Polish and French extraction, were unrelated. They were of average intelligence; there was a normal older sibling, and no family history of mental retardation or neurological disease. In the first few months of life the patient had a feeding problem attended with vomiting and loose stools, but by one year weighed 11.4 kg. and appeared well developed. He sat up at 10 months, started creeping at 14 months and could pull himself erect by 18 months but never stood unsupported or acquired speech.

At the age of 6 months he was admitted to another hospital for investigation of hepatomegaly, and was noted to be irritable and "floppy" in the lower extremities, although he could move them at will. A liver biopsy was reported to show normal tissue with normal amounts of glycogen. He was admitted to hospital again at the age of 16 months because progress in his motor development had continued to be slow. At that time horizontal nystagmus and general hypotonia were noted in addition to the hepatomegaly. Paper chromato-

graphy of the urine revealed a mild generalized increase in amino acid excretion.

On referral to this hospital at the age of 18 months he weighed 11.0 kg. (25th percentile) and measured 79 cm. (10th percentile) in length. He spoke no words but smiled readily and took an interest in toys. Head circumference was 48 cm. and chest 49 cm. There was no bruit in the head and the fontanelle was open 1 cm. There was good head control.

Tests of cranial nerve function disclosed no abnormality except for eye examination. The child held objects close to the eyes. The retina showed some clumping of pigment at the periphery, and generalized dusty pigmentary change, perhaps more marked around the macula. The discs were small and pale and the arteries were also small. There was spontaneous horizontal nystagmus and the pupils reacted slowly to light. No Kayser-Fleischer rings were seen. Intention tremor was marked, particularly of the head. Generalized hypotonia was found, but reflexes, though reduced, were obtainable symmetrically. The plantar responses were flexor and sensation to pin-prick was normal. The patient could sit alone and pull to standing, but his movements were all slow and weak. There was no startle reflex. On general physical examination the ears, nose and throat were normal. The chest was clear and heart sounds were normal; blood pressure was 90/65 mm. Hg. The liver was enlarged 4 cm. below the right costal margin and felt firm but was not tender. The spleen was not palpable. Genitalia were normal. The hands and feet were normal, without congenital stigmata.

The course in hospital was characterized by progressive deterioration during a six-month period, culminating in flaccid paralysis of all the extremities, absent reflexes, inability to suck and complete unresponsiveness. The patient was initially given a diet designed to be low in lysine in which most of the protein was derived from purified Zein,* a corn protein that is naturally low in lysine and tryptophan, and to which tryptophan was added. The protein intake was 3 g. per kg. per day, lysine intake was calculated at 0.8 g. per day and the calorie intake at 100 cal. per kg. per day. This diet was not taken well and when loose stools became a problem, mixed Pablum was substituted as a source of protein of relatively low lysine content. Diets containing 110 cal. per kg. with 2 g. and later 1 g. per kg. protein were tried for varying periods with regular diets in between. Although there was a moderate fall in serum pipecolic acid on the low lysine, low protein diets from 150 to 200 μmolar to 50 to 100 μmolar, there was no apparent effect on the steady deterioration in the patient's clinical condition. The apparent improvement in serum pipecolic acid values may have been due in part to fluid retention. Throughout the last six months of life the liver fluctuated markedly in size and there was a varying degree of periorbital and leg edema. No explanation for the fluid retention could be found. Serum proteins, sodium, potassium and chlorides remained within normal limits. The ECG did not suggest cardiac failure.

Laboratory tests, performed over the course of the three hospital admissions, included serum bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), thymol turbidity, alkaline phosphatase, prothrombin time, sedimentation rate, cephalin-cholesterol flocculation, serum glucose response to adrenaline and to a glucose load, serum copper, ceruloplasmin, protein-bound iodine, serum protein electrophoresis, 2-hour p.c. blood ammonia, serum uric acid, calcium, phosphorus and BUN, and an intramuscular pyelogram. They were all normal. Peripheral leukocyte culture showed a normal chromosome complement. The hemoglobin varied from 11.4 to 12.9 g. per 100 ml. with hematocrit from 34.9% to 38.2%, a normal leukocyte count and differential. Spinal fluid cells, protein and sugar were normal at age 16 months. At 22 months spinal fluid protein was 46 mg., at 24 months 38 mg., and at 27 months 50 mg. per 100 ml. (normal: 15 to 40 mg.). Cells and sugar were repeatedly normal. The EEG was mildly abnormal with excessive slow background activity. I.Q., estimated with the Kuhlman and Gesell tests shortly after admission, was 36.

BIOCHEMICAL STUDIES

Serum

For paper chromatographic studies of blood amino acids, overnight fasting serum was deproteinized by the addition of an equal volume of 0.6 M perchloric acid (PCA). Three millilitres of protein-free extract was added to an 0.8 by 8 cm. column of Dowex 50 -X2 (H^+) (50-100 mesh). After washing with 2 ml. water, amino acids were eluted with 5 N. NH_4OH. The eluate was taken to dryness in a stream of warm air, then made up to a volume sufficient for a 10-fold concentration of original sample. Initially, extracts equivalent to 0.4 ml. of serum were examined by two-way ascending paper chromatography in butanol-ethanol-acetic acid-water, 7-7-2-4 (solvent A), and Phenol*-ethanol-water-ammonia, 150-40-10-1 (solvent B), followed by staining in 0.2% ninhydrin in acetone. A chromatogram of the patient's serum (Fig. 1) showed an abnormal spot (arrow) that stained a deeper blue than the other amino acids with ninhydrin and fluoresced red on ultraviolet excitation.

Identification of Unknown

The unknown was identified as piperidine-2-carboxylic acid (pipecolic acid) on the basis of

*Nutritional Biochemicals, Cleveland, Ohio.

*Fisher Scientific Co. Ltd., Toronto, Ont.

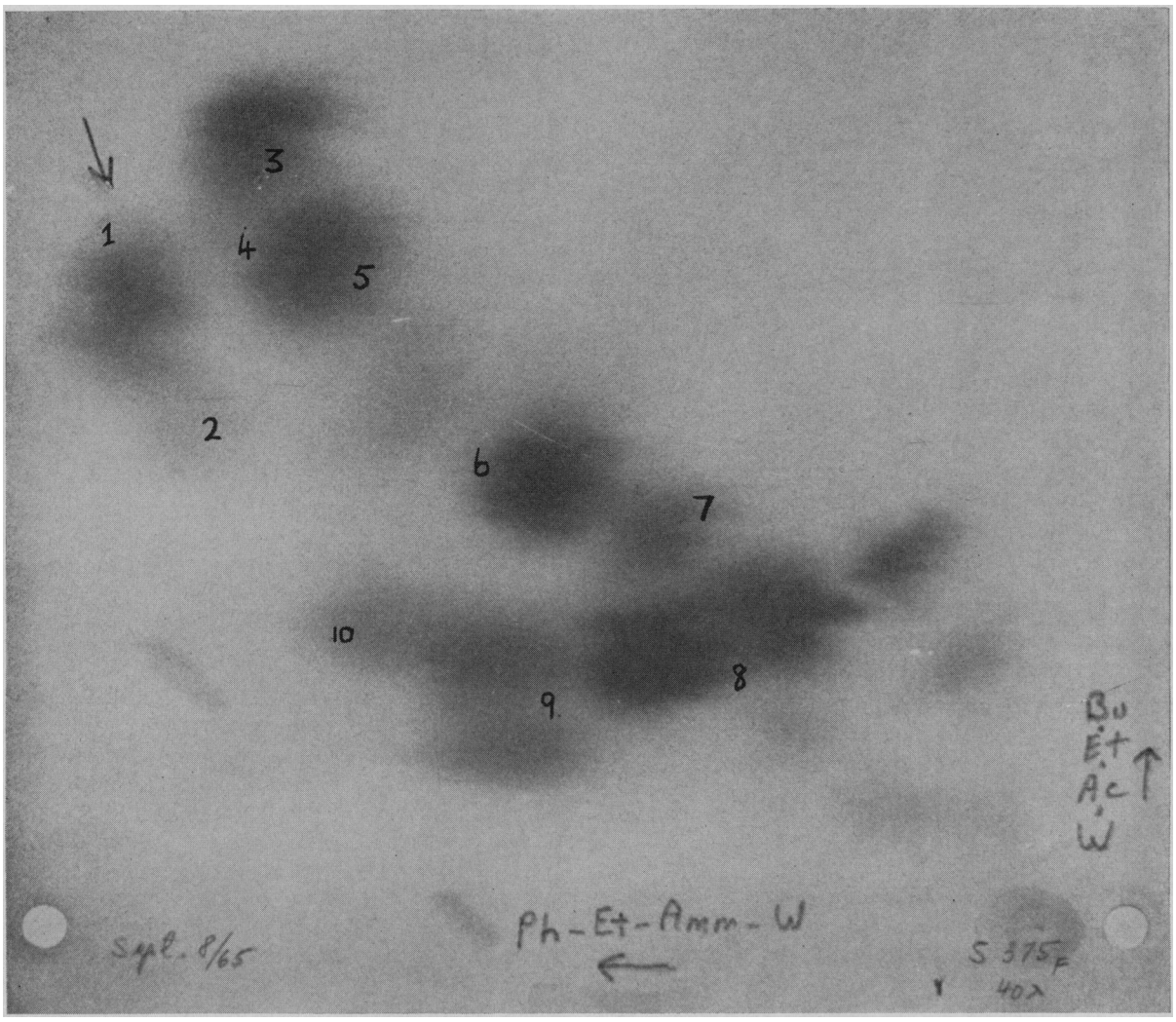


Fig. 1.—8 x 8 in. chromatogram of extract of 0.4 ml. serum stained with 0.2% ninhydrin in acetone containing 20% glacial acetic acid. 1 Unknown (+ Ethanolamine), 2 Proline, 3 Leucine + Isoleucine, 4 Phenylalanine, 5 Valine, 6 Alanine, 7 Threonine, 8 Glutamine, 9 Lysine + Histidine, 10 Arginine.

its characteristic blue-violet colour with ninhydrin, green colour with isatin and brilliant red fluorescence on ultraviolet excitation of the ninhydrin-stained paper chromatogram.[4]

The ninhydrin-stained spot, when cut out and eluted in glacial acetic acid, had an absorption maximum at 562 μ, similar to that of authentic pipecolic acid.*

Sera overspotted with authentic DL-pipecolic acid were run in solvent A, butanol-pyridine-water, 1-1-1, or methanol-water-pyridine, 20-5-1, as first solvents and solvent B as second solvent. The unknown co-chromatographed with authentic pipecolate in all three two-way systems.

The compound was eluted from the Beckman amino-acid analyzer column between cystine and the buffer change in the same position as authentic pipecolate. The ratio of absorption at 440 mμ to 570 mμ was unusually high at .53 for both unknown and pipecolate. Since the molar extinction for authentic pipecolic acid was only about 3% of that of leucine, it was necessary to use 2 ml. of plasma, five times the normal volume, to obtain a recognizable peak in the pipecolic acid region. Integration of the peak gave a value of 324 μmolar, which is in fair agreement with that of 283 μmolar, determined by eluting the stained paper chromatogram of the same serum sample (see below). With the Technicon automatic column system operated under conditions recommended by Piez and Morris[5] both unknown and pipecolic acid were eluted between valine and cystine with equivalent 440 mμ and 570 mμ absorption.[25]

*Sigma Chemical Co., St. Louis, Mo., U.S.A.

For gas-liquid chromatography 2 ml. serum was deproteinized and desalted on Dowex 50 as for paper chromatography. The amino acids in the dried eluate were converted to the trifluoracetyl derivatives of their methyl esters by the method outlined by Karmen and Saroff.[6] Samples were run on an F and M Model 402 with 4 foot by 1/8 inch i.d. columns under the following conditions: Column A was packed with 3% SE 30 on Gas Chrom Q*; flash heater temp. 204° C.; detector temp. 230° C.; nitrogen gas flow 33.0 ml. per minute; initial temperature 80° C. for 16 minutes, programmed at 10° per minute to 180° and held. Column B was packed with 3% Hi EFF 3 BP on Gas Chrom. Q*; flash heater temp. 204° C., detector temp. 230° C.; nitrogen gas flow 33.0 ml. per minute; initial temp. 10° for 10 min., then programmed at 3° per min. to 180° and held.

With both columns there was a large peak in the patient's serum that was not detectable in two controls or in a pooled sample from several controls. This peak coincided with that found when authentic pipecolate was added to serum and when the TFA pipecolic acid methyl ester was added in the injection syringe. The retention times were: for column A, 14.0 minutes or 3.21 x that of 1-octanol; for column B, 25.7 minutes or .68 x that of myristol.

Pipecolate Assay

After identification of the unknown as pipecolic acid, staining of the chromatograms was altered slightly. Since acetic acid has been reported to enhance the colour produced by cyclic secondary amino acids with ninhydrin,[7] varying amounts were added to the ninhydrin solution for colour development; maximal intensification of the ninhydrin colour and fluorescence (3- to 4-fold) was produced with a solution of ninhydrin (0.2% w/v) in glacial acetic acid-acetone (20% v/v). The solvent pair used initially was selected to separate as many amino acids as possible. At normal serum concentrations of each ethanolamine obscured pipecolic acid. Addition of pipecolic acid to mixtures of amino acids calculated to simulate serum showed that the characteristic blue colour of pipecolic acid was visible at serum concentrations above 5 μmoles in the presence of normal amounts of ethanolamine. For those patients found to have serum pipecolate values over 5 μmoles and for both the control series, 90% phenol was used as the second solvent to retard the movement of ethanolamine. This procedure usually permitted the detection and semiquantitative estimation of pipecolic acid in paper chromatograms of 0.8 ml. of serum. Standards equivalent to serum concentrations of 0.5, 1.5 and 3.0 μmolar were carried through the deproteinizing and desalting steps.

Serum concentrations from 0.5 to 3 μmoles could be read by visual comparison with standards. In all cases where higher values were found, samples were re-chromatographed with pipecolic acid standards sufficient to bracket the samples. The stained spots were cut out and eluted in a tube with 0.6 ml. glacial acetic acid. The absorption at 562 mμ of samples and standards was read after one hour against a blank obtained by eluting an unoccupied part of the paper chromatogram. About 3 x 10^{-9} moles of pipecolate are required for an optical density reading of .100, so that with serum levels below 3-4 μmoles the spectrophotometric values are not reliable.

Normal Serum Concentrations

The series of 84 patients in which the index case was detected were consecutive admissions (excluding mongoloids) to a hospital for assessment and short-term care of children with mental retardation and/or behaviour problems. Subsequently, to determine normal serum pipecolate concentrations, two groups were used. Forty-one subjects, aged 2 to 15 years, were patients at a general children's hospital who had been fasted for at least 8 and often 12 hours before a surgical or diagnostic procedure. Fourteen of these had tonsillectomies, 13 had urinary tract disorders and the remainder had a wide variety of illnesses. Fourteen healthy controls, aged 4 to 14 years, were children of staff members. They were given a low pipecolic acid diet for two days (see Diet Restrictions) and fasted overnight. The values found in 55 control subjects and in the four retarded patients with values over 5 μmoles are shown in Fig. 2. Eighty patients whose serum concentrations were under 5 μmolar but not precisely known are omitted. In both patients and control groups there is a small proportion of subjects with values 5 to 10 times the common range of 0.5 to 3.0 μmolar, but these are still well below those found in the index case.

After this part of the study had been completed, Woody, Ong and Pupene[8] reported Rfs for N^{ϵ}-acetyllysine that were similar to those for pipecolic acid in the solvents we used. Authentic N^{ϵ}-acetyllysine* and pipecolic acid were then

*Applied Science Labs, Box 440, State College, Pa., U.S.A.

*Cyclo Chemical Corporation, Los Angeles, Calif., U.S.A.

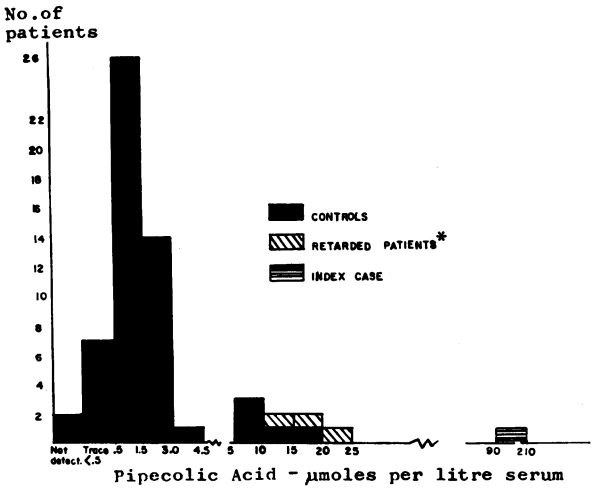


Fig. 2.—Serum extracts were chromatographed two ways and stained with ninhydrin (see text). Over 3 μmolar the stained spot was eluted and absorption at 562 mμ was compared with standards. Under 3 μmolar concentrations were estimated by visual comparison with standards. The 0.5 μmolar standard was just barely detectable. Samples which were not clearly greater than that standard are recorded as "Trace <.5 μmoles". *Eighty mentally retarded patients with values under 5 μmolar whose concentrations are not precisely known are omitted.

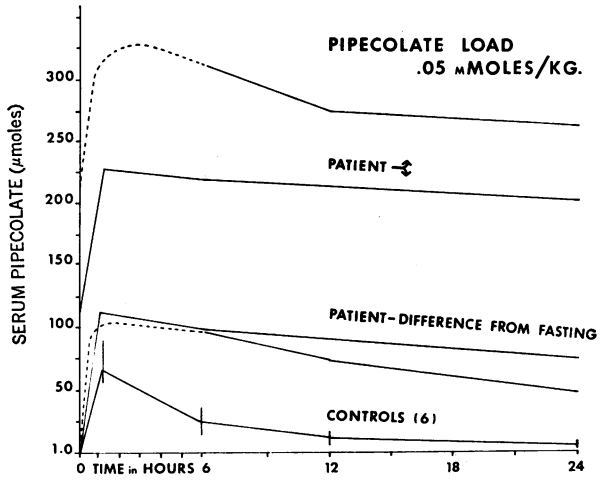


Fig. 3.—Subjects were given commercial bean juice containing pipecolic acid orally and fasted for six hours. From 6 to 24 hours, foods previously shown not to affect serum pipecolate were given. Controls were 2 to 12 years old.

found to occupy the same position, but the different ninhydrin colours of the two compounds permit their distinction. When mixtures of the two compounds were run with increasing amounts of N^{ϵ}-acetyllysine, it was found that the blue colour produced by 2 x 10^{-9} moles pipecolate (equivalent to 2 μmolar in serum) is easily detected in the presence of up to 6 x 10^{-9} moles N^{ϵ}-acetyllysine, at which point the spot begins to look mauve. As the amount of N^{ϵ}-acetyllysine increases, the colour of the spot changes before the size of spot and intensity of colour increase. Since this spot was always blue, it seems safe to conclude all the colour was due to pipecolate.

Diet Restrictions

During the study children were occasionally found whose serum gave high values initially but on retesting the pipecolic acid was normal. On learning of the wide distribution of this amino acid at significant concentrations in many edible plants, particularly beans, peas, cabbage and apples,[9] it was suspected that the dietary restrictions (8 to 12 hours' fasting) might be inadequate. The juice from commercial canned cut wax and green beans was administered orally to six patients, aged 2 to 12 years, who had normal fasting concentrations of pipecolate. The volume of juice, adjusted to provide 0.05 millimoles of pipecolic acid per kg., varied from 135 to 260 ml. For the subsequent 24 hours, intake was limited to those foods previously demonstrated to have no effect on serum pipecolate, i.e., corn flakes, Pablum, bacon, milk and toast. Serum estimations were made at 0, 1, 6, 12 and 24 hours. Since it was found that pipecolic acid values were still elevated after 24 hours (see controls, Fig. 3), all patients in whom values over 5 μmolar were found were retested after being on a diet free of green vegetables, soup and apples for two days and fasted overnight.

CSF and Urine Pipecolate

Spinal fluid was deproteinized and desalted as for serum. Aliquots of 24-hour urine collections were acidified with HCl, centrifuged and then desalted as for serum. Extracts equivalent to 0.6 ml. spinal fluid and urine containing 0.02 or 0.03 mg. creatinine were examined by two-way chromatography in solvent A as first solvent and solvent B or phenol alone as second solvent.

Pipecolic acid was just detectable in paper chromatograms of CSF (1-2 μmolar) from the patient and was not found in five controls (< 1 μmolar). It was not detected in urine samples from the patient until the solvent was changed to separate pipecolate from ethanolamine. Even then it was barely visible.

Automatic Column Analysis for Other Amino Acids

Amino acids in serum, urine and spinal fluid were examined with the Beckman 120 C automatic analyzer with the conditions for physiological fluids recommended by the manufacturer.[10] Serum and CSF were deproteinized by the addition of sufficient 3 M. PCA to make a

TABLE I.—URINE AMINO ACIDS
(μmoles/mg. creatinine)

	Controls - 9		Patient
	Mean	Range	Mean of 5 samples
Taurine	.79	.11 - 1.99	.58
Threonine	.24	.09 - .52	.55
Serine	.55	.21 - 1.11	1.82
Asparagine-glutamine	.85	.18 - 2.51	1.91
Glutamate	.02	.01 - .03	.06
Glycine	1.45	.71 - 2.34	1.99
Alanine	.53	.20 - 1.07	.95
αAmino butyrate	.09	.02 - .15	.13
Tyrosine	.22	.09 - .42	.42
Phenylalanine	.11	.05 - .16	.30
β-amino isobutyrate	.16	.07 - .42	.30
Ethanolamine	.72	.64 - .97	.95
Lysine	.23	.07 - .58	.17
1-Methylhistidine	.31	.14 - .56	.31
Histidine	1.59	.77 - 3.71	2.70
3-Methylhistidine	.33	.27 - .45	.33
Arginine	.02	.01 - .04	.06
Pipecolate		Not detectable	.04

0.4 M. concentration. After centrifugation 2 M. potassium citrate was added to bring the supernate to about pH 2.4. This precipitated most of the perchlorate and the supernate was taken to the column. For urine, the manufacturer's recommended procedure was followed except that for removal of ammonia the urine was taken to dryness in about 30 minutes under vacuum.

The fasting serum amino acids apart from pipecolate were normal. The patient's urine amino acid excretion was at the upper limit of that of our control group (Table I). The major CSF amino acids were normal except homocarnosine (see Brain, Liver and Kidney, below). In the single specimen examined its concentration (1.1 μmolar) was at the lower limit of the control group (mean 3.7 μmolar, range 0.7-6.5 μmolar).

Loading Studies

For pipecolic acid loads with the patient, commercial bean juice was used as in "Diet Restrictions". Urine was collected for periods of 0 to 6 and 6 to 24 hours. Oral administration of pipecolic acid gave a prompt rise in serum pipecolate (Fig. 3). The rise is greater and more prolonged in the patient than in controls.

For lysine loads 0.2 g. per kg. L-lysine was given by stomach tube and for casein loads 2 g. per kg. Casilan* in water was given by tube. For the patient loads, serum was extracted and desalted on the Dowex columns. A portion was examined by paper chromatography. The remainder of the eluate was stored at −20° C. and

*Glaxo-Allenburys Ltd., Toronto, Ont.

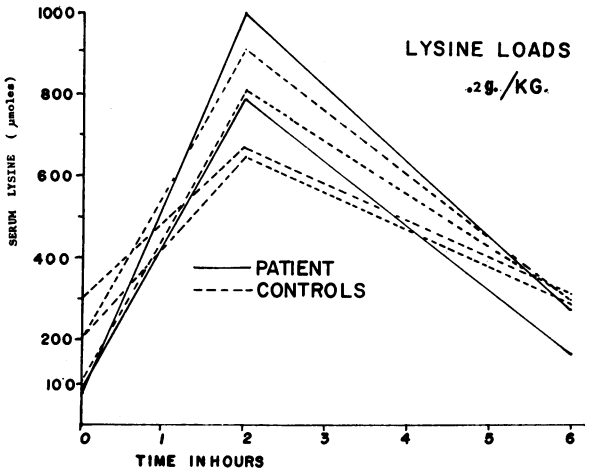


Fig. 4.—Patient and four controls, aged 3 to 7 years, were fasted overnight and for six hours after oral L-lysine.

examined a year later when the automatic column became available. Comparison of lysine concentrations in a few samples where sufficient stored serum remained for analysis gave good agreement for lysine between eluate and serum. The patient was fasted for six hours for lysine and nine hours for casein except that 50 g. glucose was given after six hours in the case of casein. Urine was collected for 0 to 6 hours. Serum amino acids were measured with the automatic column in controls. Serum pipecolic acid was measured in all loads with the paper chromatographic procedure. The patient's serum lysine response to a lysine load (Fig. 4) did not differ from controls. The effects of lysine and casein loads (or rather the lack of effects) on serum pipecolate are shown in Fig. 5. These data indicated that there was a block in pipecolic acid metabolism but no block in lysine metabolism. Furthermore, lysine and casein appeared not to contribute to pipecolic acid for-

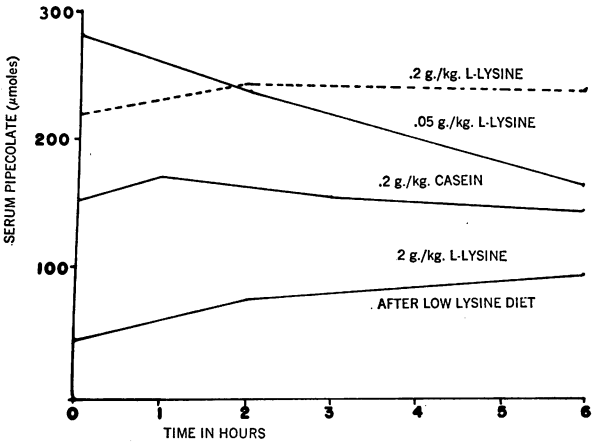


Fig. 5.—The patient's serum pipecolic acid was determined at one, three and six hours after oral casein and two and six hours after three oral lysine loads.

TABLE II.—ORGAN PIPECOLIC ACID (μmoles/kg.)

Patient		Liver 543, 600	Cerebral cortex 236, 233	Kidney 326
Controls	*Cause of death*			
A. 5 yrs.	Traumatic rupture of vena cava	14, 8	Not detectable	
B. 33 mos.	Neuroblastoma	18, 14		
C. 14 mos.	Aspiration pneumonia, cardiac arrest	18, 14	Not detectable	Not detectable
D. 9 yrs.	Down's syndrome		Not detectable	
E. 5 yrs.	Trisomy 17-18, pneumonia	36, 48	Not detectable	Not detectable
F. 9 yrs.	Traumatic compression of cervical cord	12, 6		
G. 9 yrs.	Osteogenesis imperfecta, mental retardation, pneumonia	19, 25		Not detectable

mation. Measurement with the automatic column of serum amino acids after casein failed to show an unusual rise in any ninhydrin-positive compound and all returned to normal by nine hours, i.e. no alternate source of serum pipecolate was suggested. Similarly, measurement of urine amino acids after pipecolic acid and lysine loads failed to reveal consistent differences between patient and controls that might indicate alternate routes of metabolism that were being used in our patient.

Brain, Liver and Kidney

Brain, liver and kidney from the patient were frozen about two hours after death and from seven controls, aged 14 months to 9 years, 4 to 12 hours after death. One-gram samples were homogenized in five volumes of 0.4 M. PCA in a Ten Broeck-type glass homogenizer in ice and spun at 10,000 G for 10 minutes at 4° C. For pipecolic acid analysis 3.5 ml. of supernate was desalted on the Dowex 50 columns as for serum. Extracts equivalent to 0.025 to 0.3 g. tissue were spotted, depending on the separation and size of the pipecolate spot found in trial runs. For other amino acids the 10,000 G supernate was treated with potassium citrate as for serum, and supernate equivalent to 0.1 to 0.2 g. tissue was taken to the column.

Pipecolate was found in all control livers examined but not in brain or kidney (Table II). The patient's liver concentrations at autopsy were elevated 30-fold and were about twice those found in his own serum, brain and kidney. The automatic column failed to show any abnormalities in other amino acids in liver and kidney.

In the brain it was found that a peak emerging from the automatic column at 252 minutes in the basic run in controls was much reduced in the patient. For identification purposes a PCA extract of two portions of frontal pole from a control was run and the column eluate collected in 2-ml. fractions. Return of part of the 252-minute fraction to the column gave a single peak at 252′ and hydrolysis in 2 N. HCl for 20 hours at 100° C. yielded two major peaks when run on the column. They coincided exactly in position and in relative 440/570 absorption with gamma amino butyric acid (GABA) and histidine. Using the colour coefficient of commercial gamma amino butyryl histidine (homocarnosine), the calculated yield of GABA and histidine was 113% and 121%, respectively. Addition of homocarnosine to the control-brain extract yielded a symmetrical peak. Addition of carnosine gave a shoulder on the leading edge of the peak. The authentic compounds gave similar symmetrical and asymmetrical peaks respectively when added to control CSF.

In addition, citrate was removed from part of the fraction containing the unknown with the Dowex 50 column as in desalting serum. The unknown was then found to yield a single spot when two-way paper chromatograms were developed in five different solvents, both with and without authentic homocarnosine. In solvent A the unknown ran with homocarnosine and ahead of carnosine.

Pipecolic acid accumulated in all parts of the patient's brain examined (Table III), particu-

TABLE III.—BRAIN HOMOCARNOSINE AND PIPECOLIC ACID (μmoles/kg.)

	Cortical grey	Subcortical white	Cerebellum
Homocarnosine			
Control A	294		
Control B	129		
C	258	300	440
C		403	335
D	196	518	460
D		436	521
E	89		149
E			108
F	164	556	1100
F		590	744
Mean ± S.D.	205 ± 56	467 ± 100	483 ± 301
Patient	22	13	28
	20	22	34
			13
*Pipecolic acid**			
Patient	203	296	703
			982
			800

*No pipecolic acid was detected (<5 μmoles per kg.) in samples of mixed cerebrum or cerebellum from controls.

larly in the cerebellum. Homocarnosine was uniformly low. The latter has previously been found in slightly higher concentrations in the cerebellum in man[11] and our data tend to confirm this observation. Accentuation of the biochemical defect in the cerebellum is interesting because nystagmus and intention tremor were two of the most prominent early signs in our patient.

AUTOPSY FINDINGS

The autopsy was performed two hours after death. Tissues were chilled for biochemical determinations, and fixed in formalin for light microscopic studies and in glutaraldehyde for electron microscopy.

The body length and weight were within normal limits and there were no external gross malformations except for a high-arched palate. The pleural cavities were free from fluid and adhesions. The right lung weighed 70 g., the left 65 g., and both lungs were dry and appeared well aerated. Microscopically, sections from both sides showed a slight to moderate increase in interstitial connective tissue. There were small areas of necrosis in the bronchiolar and alveolar ducts, and numerous lipophages indicating focal lipoid pneumonia. Special stains failed to reveal any other material in the cells of the bronchioles and alveoli.

The heart weighed 60 g. and was unremarkable on gross inspection. Microscopically, there was interstitial edema with a slight increase in connective tissue. The individual myocardial fibres were small; vacuoles and small proteinaceous granules were present in the sarcoplasm. Bundles of peripheral nerves in the pericardium showed a moderate degree of fibrosis, and the Schwann's cells were separated from each other by increased connective tissues. Neither heart nor lungs showed lesions to suggest cardiac failure as a major feature.

The liver was markedly enlarged and weighed 630 g. The capsule was quite thick and the organ cut with increased resistance, revealing a yellow-brown surface which was, however, not nodular. The gallbladder and biliary ducts showed no abnormalities. On microscopic examination, there was a slight but definite increase in dense connective tissue around central veins; in addition, "young" elements of fibrous tissue could be seen in the interlobular spaces and traversing the lobules (Fig. 6). A few foci of necrosis of liver cells with regeneration were observed. Many parenchymal cells in the portal areas contained vacuolated nuclei (Fig. 7). With special stains, only traces of glycogen could be demonstrated in the ballooned nuclei. The walls of arteries and arterioles had a smudgy appearance.

The gastrointestinal tract was grossly normal. Microscopically, the myenteric plexus showed scattered neuronal necrosis, and some ganglion cells were abnormally large, but no storage material was found in these cells. The peripheral nerve bundles showed some degeneration and fibrosis (Fig. 8).

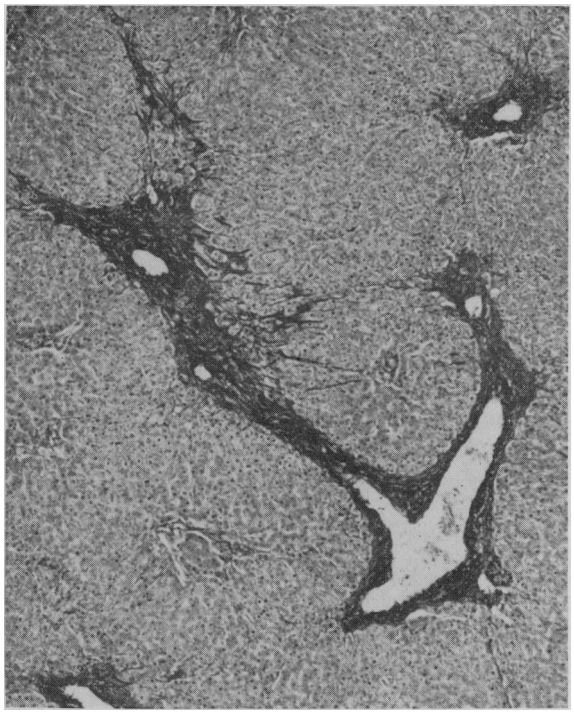


Fig. 6.—Trichrome stain of liver. Collagen is increased in the portal areas and proliferating young fibrous tissue infiltrates the liver cords from these foci. (× 40.)

The spleen weighed 35 g., was grossly normal and had a dark purple cut surface. There was a con-

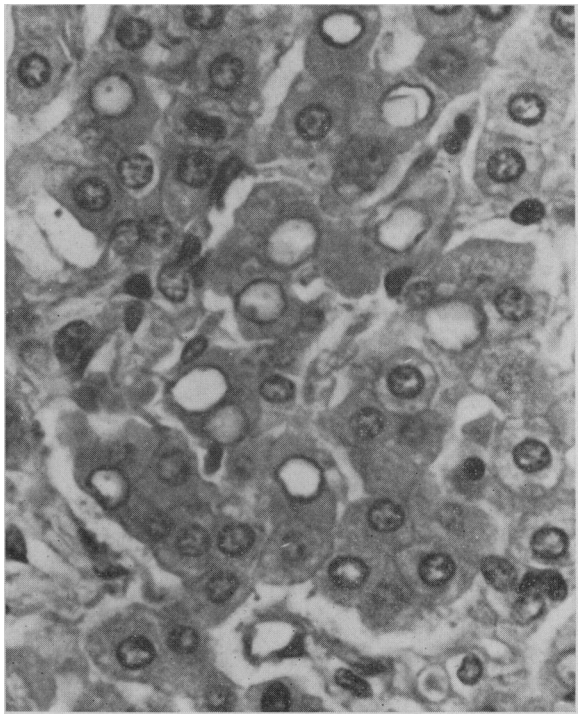


Fig. 7.—High-power view of liver cells showing vacuolated nuclei. Only small traces of glycogen could be demonstrated in them, although stainable glycogen was fairly abundant elsewhere in the liver cells. (Hematoxylin and eosin stains, × 325.)

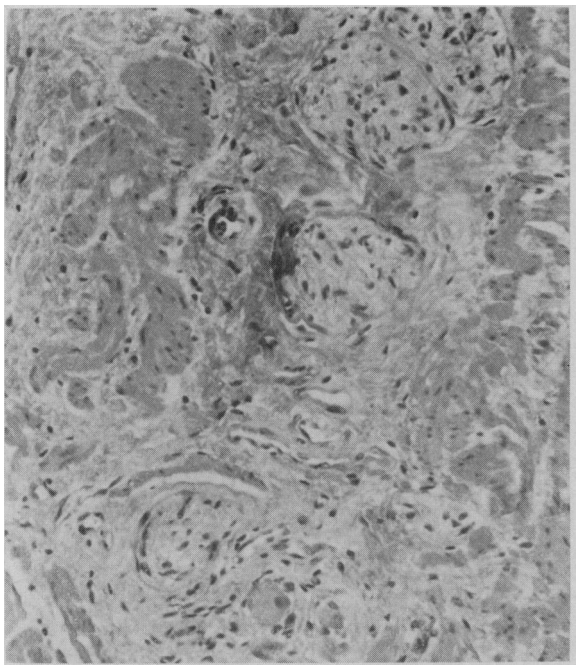


Fig. 8.—Section of small intestinal wall showing fibrosis of the nerve bundles. There is some degeneration of the nerve fibres. (Hemalum-phloxine-saffron stains, × 130.)

siderable decrease in the lymphoid tissue microscopically, and an increase in fibrous tissue involving vessel walls, perivascular areas and germinal centres of the follicles. Sinusoidal lining cells were prominent.

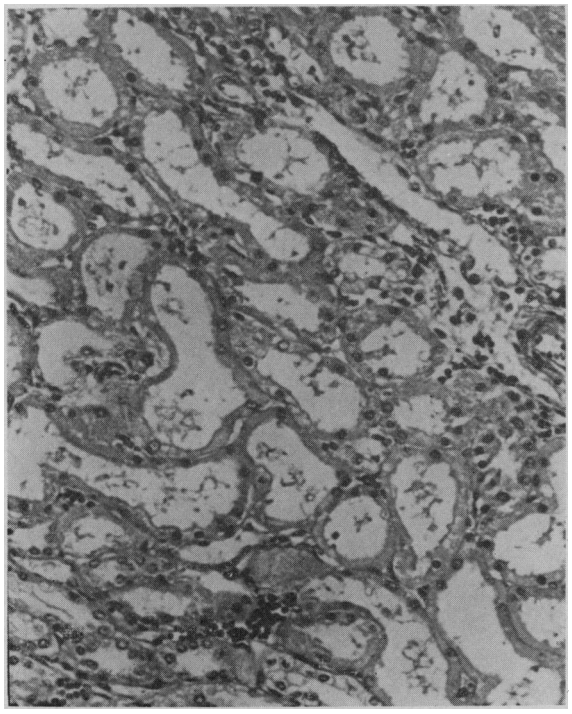


Fig. 9.—Section of kidney near cortico-medullary junction. The tubules are dilated, but no crystalline material is present and the epithelium appears normal. (Hematoxylin and eosin stains, × 130.)

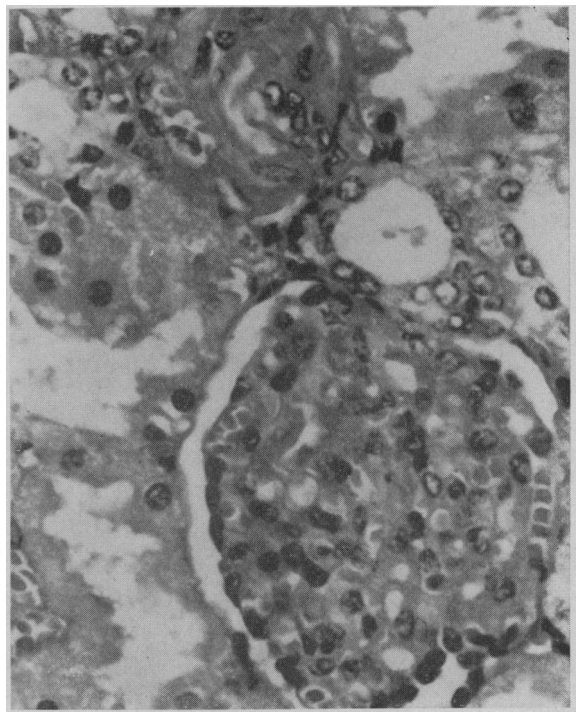


Fig. 10.—View of renal cortex showing a fibrosed glomerulus. The other glomerulus shows slight thickening of the capillary basement membranes. (Hematoxylin and eosin stains, × 325.)

The thymus was likewise depleted of its lymphoid component and very atrophic. There was replacement fibrosis and the Hassall's corpuscles were small. Approximately 70 to 75% of the remaining cells present were similar to plasma cells but were larger and lacked the perinuclear halo. Large histiocytes were observed in the interstitium and capsular connective tissue.

The right kidney weighed 55 g. and the left 60 g. Both kidneys were grossly normal, with a smooth cortex, normal cortical thickness and distinct corticomedullary junction. Microscopic examination, however, showed a moderate diffuse tubular dila-

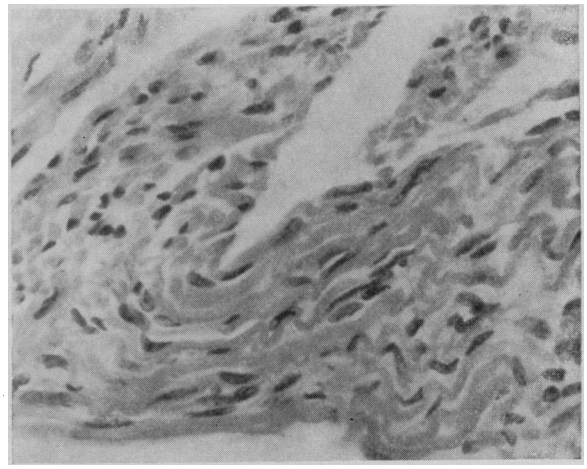


Fig. 11.—Striated muscle from diaphragm. There is severe atrophy. (Hematoxylin and eosin stains, × 130.)

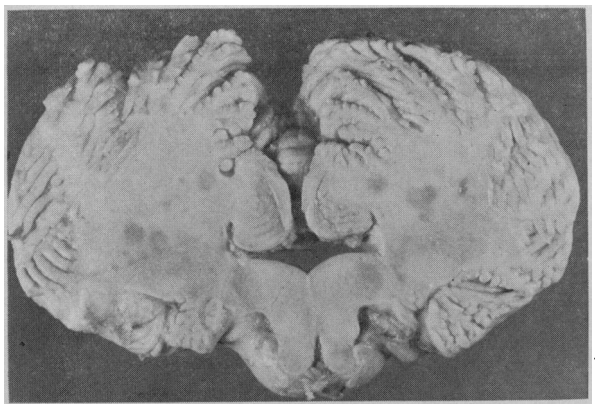


Fig. 12.—Gross section of cerebellum and pons following formalin fixation. Roughly circular grey areas of obvious demyelination can be seen. The entire white matter was firm and rubbery, especially in the cerebellar folia. (Enlarged approximately, × 1¾.)

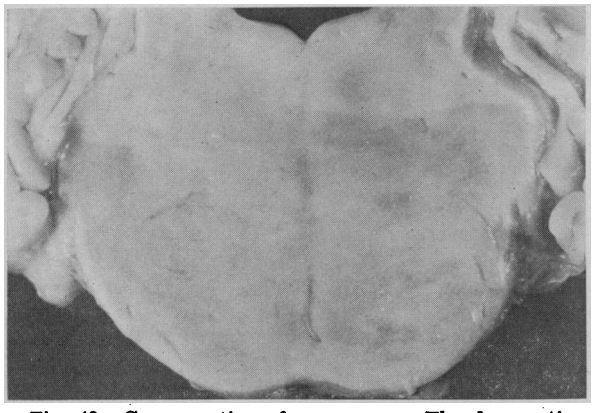


Fig. 13.—Gross section of upper pons. The decussation of the pyramids is partially obliterated by foci of demyelination and gliosis. (Enlarged approximately × 4¼.)

tation at the corticomedullary junction (Fig. 9). There was a definite reduction in the number of normal glomeruli in the subcapsular zone; a moderate number of partially or completely obliterated and hyalinized glomeruli and a few immature glomeruli were present in this zone. Slight but definite thickening of the capillary basement membrane was present in some of the remaining, otherwise unaltered glomeruli (Fig. 10). The ureters, urinary bladder, prostate and adrenals were not remarkable.

Sections of the eyes showed a few foci of pigmented cells in the inner nuclear layer of the retina, but the number of these did not appear to be excessive.

Representative sections of striated muscle showed severe atrophy of fibres with prominent central nucleoli and proliferation of sarcolemmal nuclei. There was an increase in intermysial connective tissue with fibrosis (Fig. 11). Bundles of peripheral nerve showed a variable degree of fibrosis.

The brain weighed 1130 g., which is within normal limits. The cerebral and cerebellar hemispheres were symmetrical and no abnormalities were observed on external examination. Serial slices, however, revealed scattered sunken, soft yellow-grey

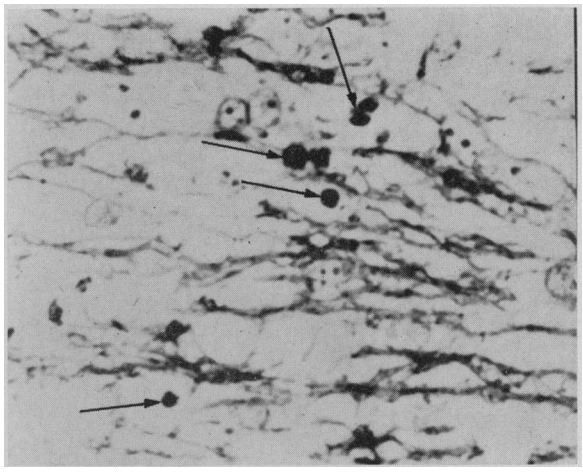


Fig. 14.—Myelin stain of white matter in midbrain showing destruction of myelin fibres with abnormal "balls" of degenerated myelin, which are round and darkly stained (arrows). (× 130).

areas throughout the white matter at all levels. They were readily seen in the internal capsule in the region of the basal ganglia, but were especially prominent in the pons, medulla and cerebellum (Figs. 12 and 13). Accompanying gliosis resulted in firmness of some portions of the cerebellum. The appearance of these lesions by light microscopy was essentially the same wherever they occurred. The changes included demyelination, gliosis and the accumulation of abnormal substances in microglia and astrocytes. Neuronal degeneration was present, but only to a moderate degree, and was interpreted, where present, to be a secondary feature. Demyelination was extreme in pons and cerebellum, and was accompanied by proliferation of astrocytes and microglia. There were abnormal "balls" of degenerated myelin in the affected areas (Fig. 14). Many of the microglia were filled with spherical droplets that stained with Sudan 4 and Sudan black (Figs. 15 and 16). Other microglial cells contained linear crystalloid material which often distorted the cell and its nucleus. Some of these

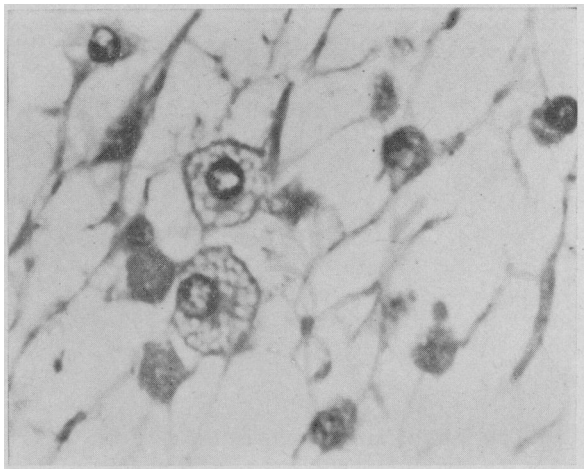


Fig. 15.—Microglia in area of demyelination containing spherical droplets unstained in paraffin sections. (× 325.)

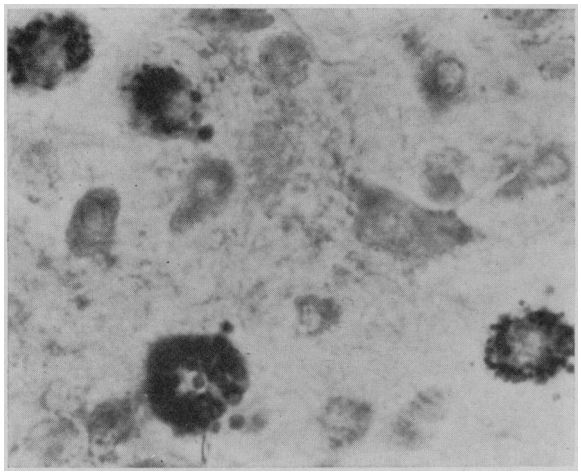


Fig. 16.—Sudan black stain of cells seen in Fig. 15. Droplets stain intensely for fat. They were also stained with Sudan IV. (× 325.)

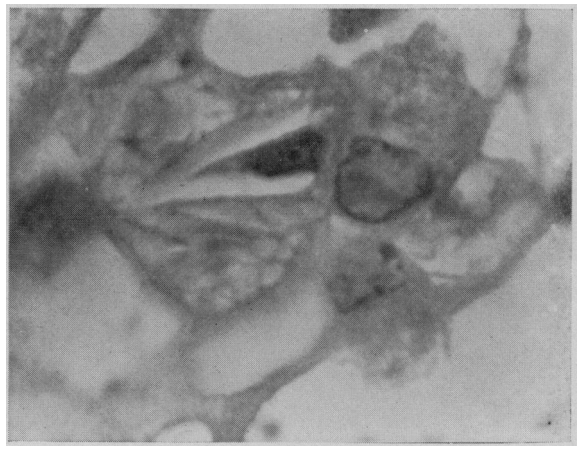


Fig. 18.—Detail of crystalline material as seen in Fig. 17. The material is intracytoplasmic and distorts the nucleus. Some crystals appeared to be PAS-positive. (× 810.)

structures were PAS-positive, while the lipoid droplets were PAS-negative (Figs. 17 and 18).

Sections one micron in thickness stained with toluidine blue showed globular masses of lipoid material associated with gliosis. These were especially prominent in the molecular layer of the cerebellum. The lipoid masses were largely intracellular, and although it was difficult to identify the cell types they appeared to be microglial rather than astrocytic (Figs. 19, 20 and 21). Electron-microscopic examination showed distortion of the myelin sheaths in the areas of demyelination. The distortion and irregularities of these membranes were caused by smudginess, loss of lamellar arrangement and thus the typical periodicity, and focal formation of clumps of electron-opaque substance, obviously derived from degenerated myelin (Fig. 22). The intracellular, round lipoid accumulations observed particularly at the level between the cerebellar inner granular and molecular layers and within the latter, had a peculiar appearance. The borders of these were smooth or serrated and the electron-opaque contents showed invariably effects of cut-

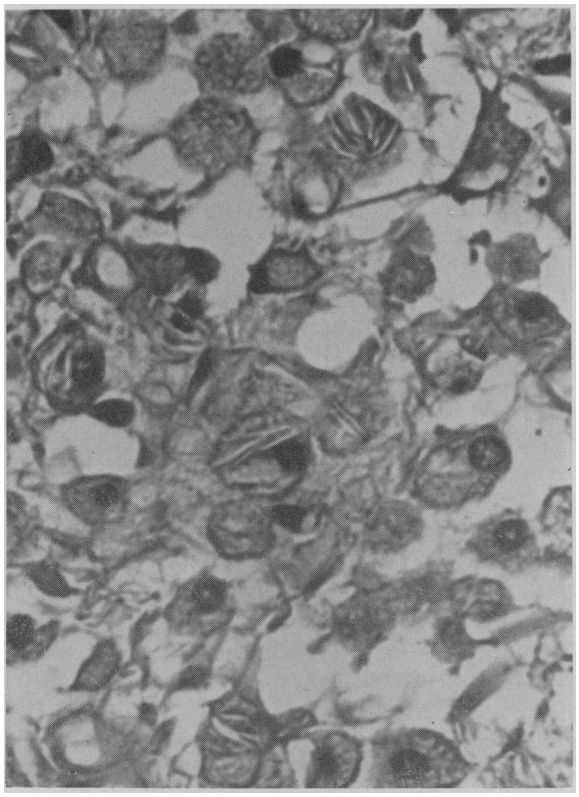


Fig. 17.—Cells in area of demyelination containing crystalline material. A few cells contain droplets similar to those illustrated in Fig. 15. (× 325.)

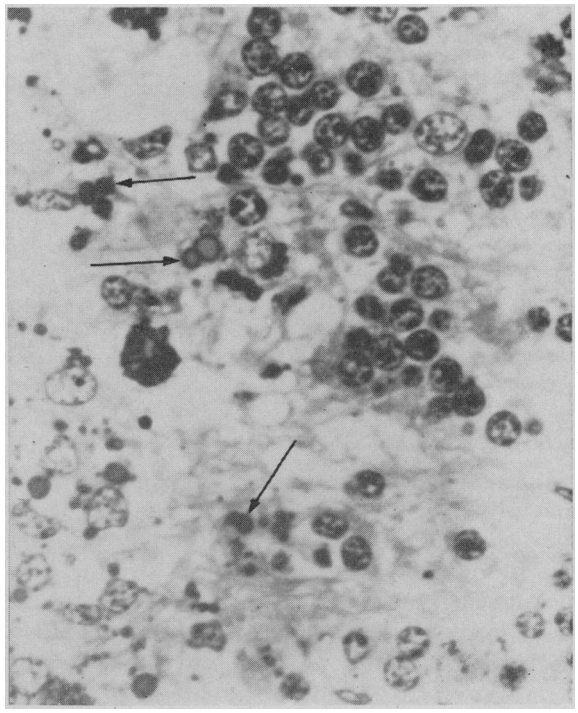


Fig. 19.—Junction of molecular and granular layer of cerebellum; plastic-embedded, one-micron-thick section stained with toluidine blue. Dark masses of lipoid material (arrow) are present in cytoplasm of some cells; other masses are extracellular. (× 325.)

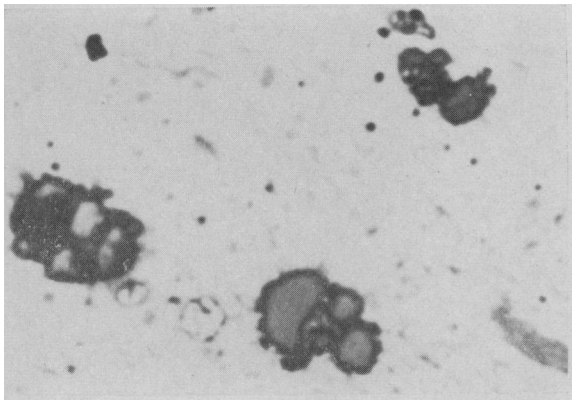


Fig. 20.—Molecular layer of cerebellum. There are huge masses of lipoid that have apparently destroyed the cells they first occupied and are now extracellular. (× 325.)

ting artifacts; no such artifacts were detectable in other structures examined. This was interpreted as an extreme degree of softness of this lipoid substance (Fig. 23). When these intracytoplasmic accumulations were extensive, the cells lost their structural integrity (Fig. 24). Peculiarly structured inclusions of various types were seen in the cells. Some were rod-shaped (Figs. 25 and 26), others rounded and vacuolated (Fig. 27). These variegated structures were considered to represent breakdown products of myelin which had been phagocytized. Sections of small blood vessels were difficult to interpret, but there were peculiar masses of lipoid material in the lumen, some of which were hard to distinguish from red cells by either light or electron microscopy (Fig. 28).

Summary of Pathologic Findings

The two most severely altered tissues were the central nervous system and the liver. The morphologic observations of the central nervous system suggest accumulation of abnormal substances which were associated with demyelination and cell breakdown. The materials were at least partially lipoid in nature. In the liver there was a fairly severe fibrosis but this was not accompanied by the presence of similar materials. Other changes included bronchopneumonia and a variety of less striking abnormalities not readily correlated with the hepatic and the nervous system pathology.

DISCUSSION

In mammals there is considerable evidence that pipecolic acid is an intermediate in lysine catabolism (Fig. 29a).[2, 3] It is therefore surprising, in view of the apparent block in pipecolate metabolism in our patient, that there was no delay in removal of added lysine from the blood. Recently Higashino, Isukada and Liebermann[12] have shown that lysine degradation in rat liver may proceed by a different route involving saccharopine formation. They propose the scheme shown in Fig. 29b in which pipecolic acid formation is a side-reaction from the main lysine pathway. The existence of a pathway other than that shown in Fig. 29a (perhaps through saccharopine) has also been supported by observations of Woody and his colleagues[8] and of Armstrong, Robinow and Andrews[13] in patients with hyperlysinemia who, presumably, have a block higher up in the metabolic path. These authors found only slightly increased amounts of Nϵ-acetyllysine in urine from their patients. This suggested that the pathway indicated in Fig. 29a was overloaded as a result of a block in some other main route for lysine degradation. In his initial report Woody[14] described excessive urine excretion of pipecolic and αaminoadipic acids in the presence of a markedly elevated blood lysine and a similar interpretation was made. Carson *et al.*[15] have described a patient who excreted large amounts of saccharopine and also had hyperlysinemia. They also concluded this indicated that saccharopine, not pipecolic acid, is on the main lysine catabolic path. The presence of pipecolic acid in all control livers at roughly the same concentration suggests it was formed there. These observations, taken together, indicate that the pathway shown in Fig. 29a is present in man but it is quantitatively a minor one for lysine. If the major path in man involves saccharopine, the observation of Rothstein and Miller[2] that lysine-ϵ-N^{15} gives rise to

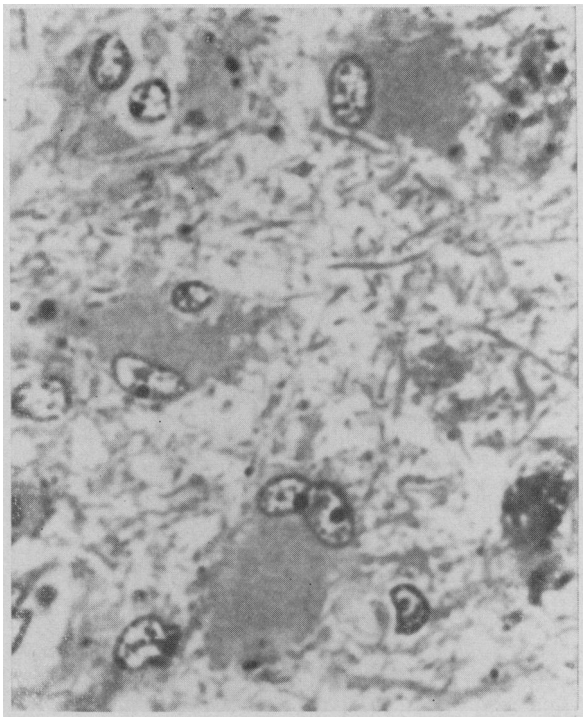


Fig. 21.—Atypical astrocytes proliferating in midbrain. These cells did not appear to be the site of the lipoid and crystalline inclusions, which probably occurred only in microglia.

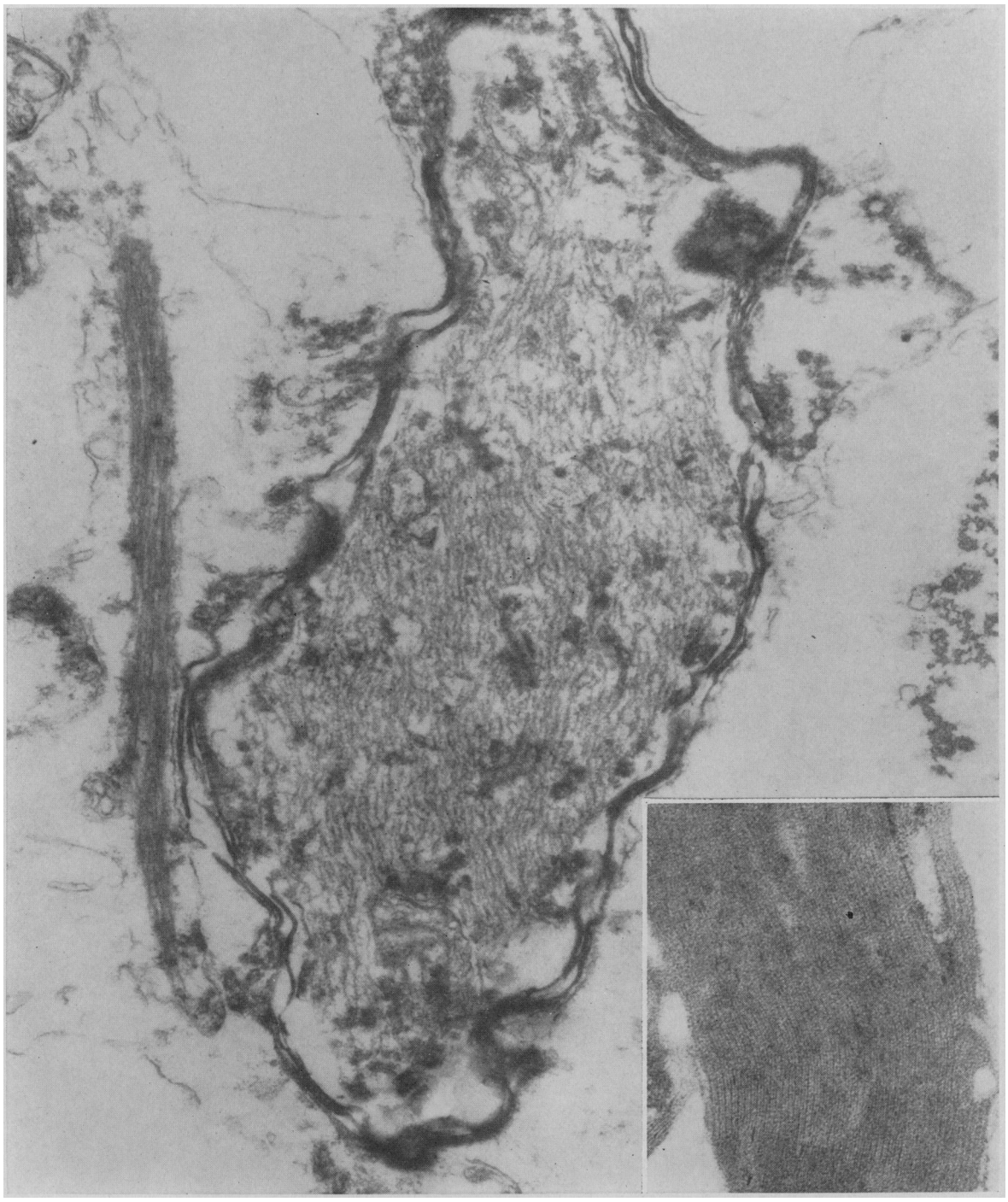


Fig. 22.—Electron micrograph of cerebellum showing degenerating and distorted myelin sheath surrounding an axon. Clumps of electron dense material are present, probably representing degenerated myelin. (× 31,400.) Fig. 22.—(Insert.) Section of midbrain showing that, where demyelination has not occurred, the structural details are well preserved and show normal myelin periodicity. (× 38,000.)

highly labelled pipecolic acid in rats, but lysine-α-N^{15} does not, must be explained on the basis of species differences.

Some of the intermediates of lysine metabolism yield clean peaks with the standard column runs for physiological fluids so that a relatively small increase in urine excretion is detectable. This is true for αaminoadipic acid and homoarginine. Pipecolic acid, homocitrulline and saccharopine* were found to separate poorly from each other following cystine and

*A generous gift from Prof. S. Darling, Arhus Tandlaegehojskole, Arhus, Denmark.

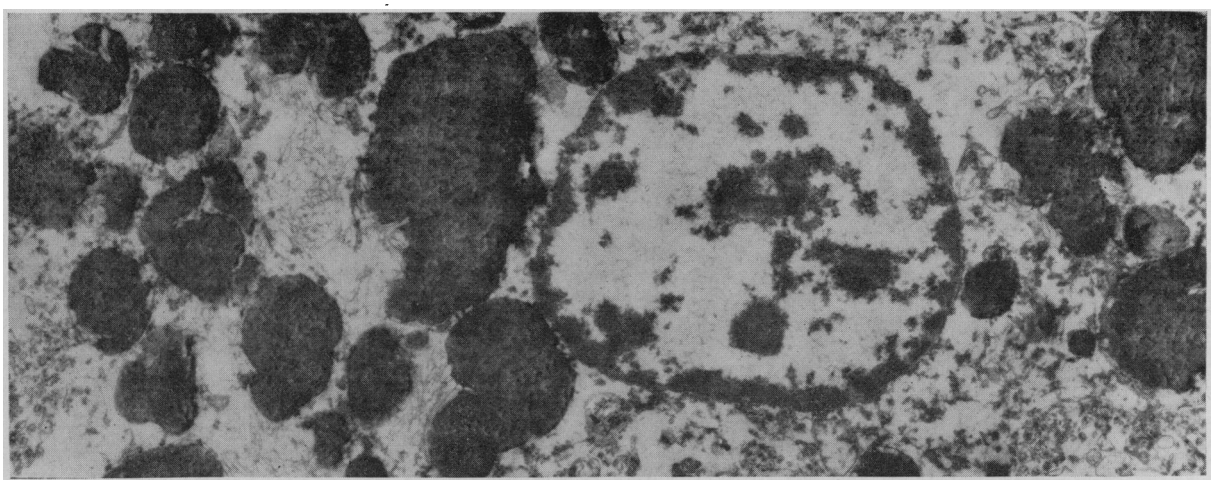


Fig. 23.—Electron micrograph of a microglial cell from same area of cerebellum as shown in Fig. 20. The cutting artifacts suggest very soft lipoid material, probably breakdown products of myelin. (× 13,100.)

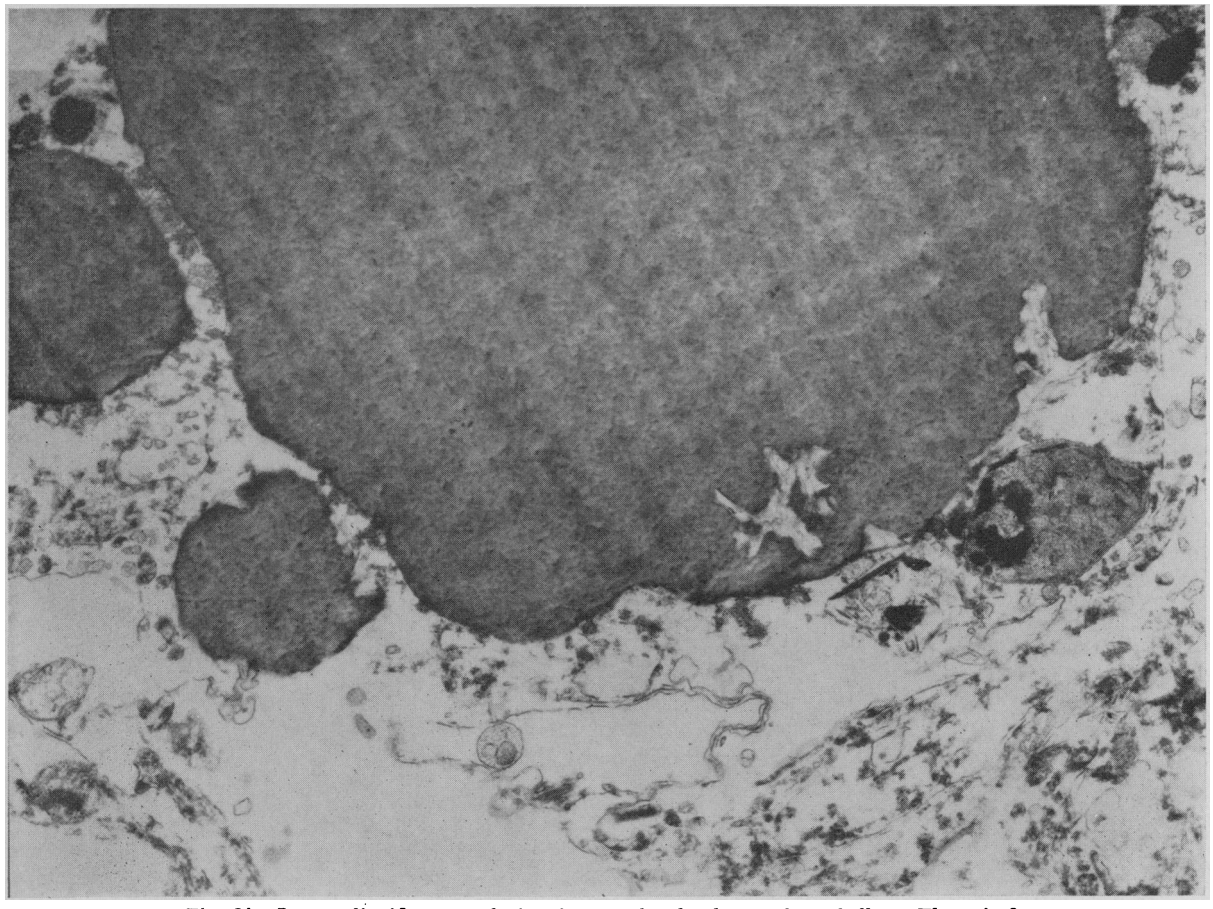


Fig. 24.—Larger lipoid accumulation from molecular layer of cerebellum. There is destruction of the microglial cell and the material is now extracellular. (× 18,800.)

were further obscured by those amino acids (largely unidentified in urine) that are eluted with the buffer change. To improve this area of the chromatogram the temperature change was delayed to 130 minutes to enhance the valine-cystine separation and the buffer change was delayed to 160 minutes. Pipecolic acid then separated well from homocitrulline. Saccharopine and homocitrulline were eluted together before the buffer change. They could be differentiated by the relatively large 440/570 absorption of the former. Urine samples from the

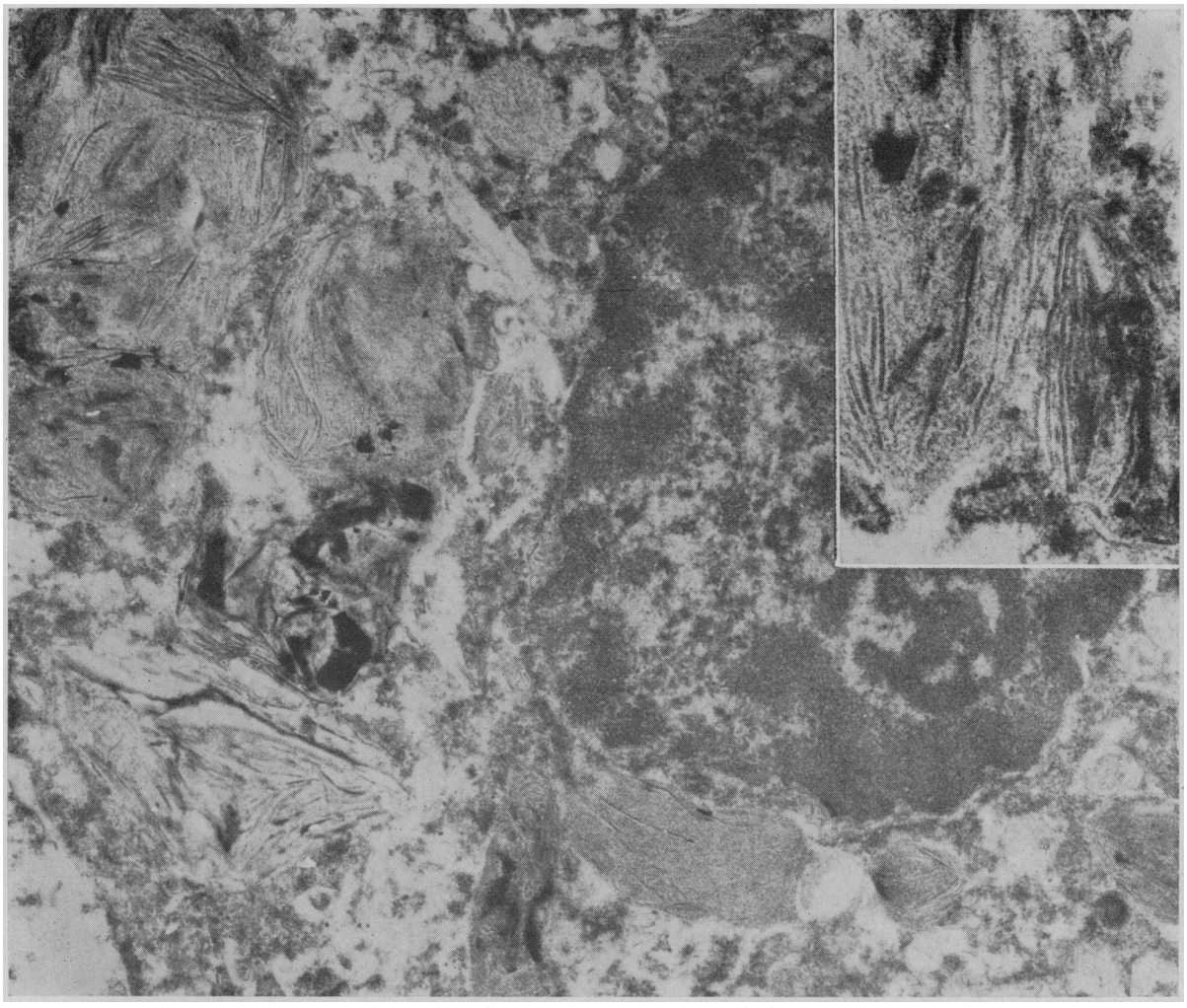


Fig. 25.—Cell from demyelinated area of pons showing rod-shaped inclusions suggestive of crystalline material. The material is intracellular and varies in size. (× 34,700.) Fig. 25.—(Insert.) Detail of rod-shaped material seen in Fig. 25. The structure of the rods is similar to myeline lamellae and the material may represent broken-up myelin. (× 55,700.)

patient often showed small peaks of aaminoadipic acid, homocitrulline and homoarginine but they were not larger than those of controls. No saccharopine peak was seen in any urine sample.

Ryan and Wells[16] and Woody, Ong and Pupene[8] obtained conversion of labelled lysine to homocitrulline and homoarginine in rats and man, respectively. Since homocitrulline and homoarginine give rise only to urea and more lysine, this route could be used for disposal of excess lysine only to the extent that homocitrulline and homoarginine are excreted. Whatever it is, the major lysine pathway yields a high level of CO_2 promptly in man,[17] so homocitrulline-homoarginine formation cannot be either an important normal fate of lysine or an alternate route for lysine disposal in our patient.

Pipecolic acid has been found in the serum of some patients with kwashiorkor at unspecified but presumably high concentrations in view of the method used.[18] While our patient had hepatomegaly, his serum proteins, other serum amino acids and usual liver function tests were normal. There was no suggestion of malnutrition in our patient or in those in the control series whose serum pipecolic acid values fell in the upper range. High concentrations in the kwashiorkor patients may have had a dietary explanation as pipecolate intake was apparently not controlled.

The other well-documented report of pipecolic acid in man is that of Jagenburg,[19] who found it in the urine of some premature infants.

Recently Kasé, Kataoka and Miyata[20] demonstrated production of piperidine from pipecolic acid using guinea pig brain homogenates. They refer to the CNS depressant and convulsant effects of administered piperidine, and Perry, Hansen and MacDougall[21] have demonstrated its presence in human brain. As Kasé and his

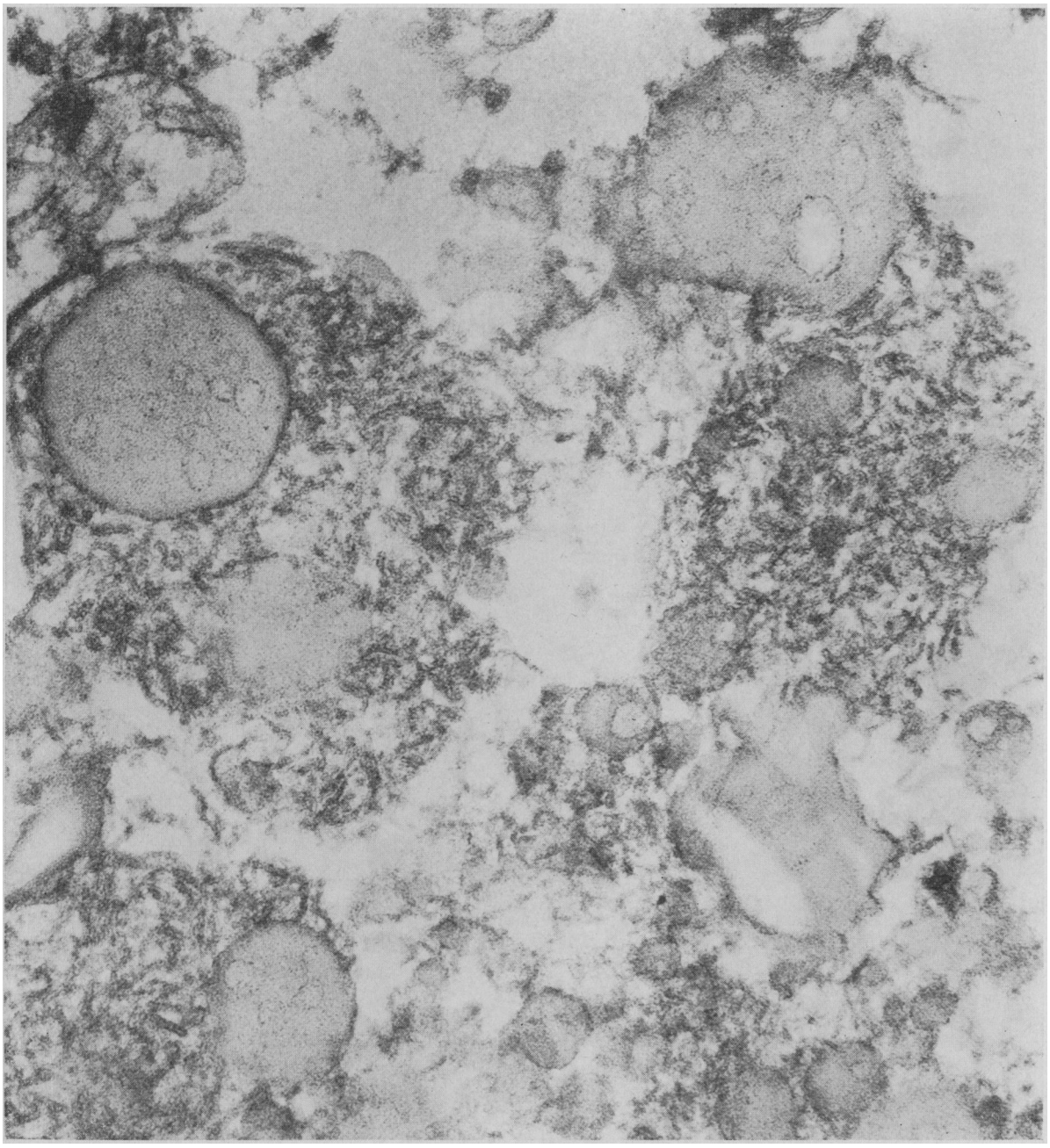


Fig. 26.—Round and blunt cytoplasmic inclusions of material different from that seen in Figs. 25 and 25, insert. The identity of the cell is not established; the short fibrils may represent degenerating neurofibrils. (× 85,000.)

colleagues suggest, piperidine formation from pipecolic acid may occur under physiological conditions; however, no symptoms arise when pipecolic acid is ingested in many foods. Since intracellular pipecolic acid concentrations in our patient were probably much higher than those that normally occur following ingestion of foods rich in pipecolate, piperidine formation still remains a possible pathogenetic mechanism. It was not measured in this case.

The identity of the peak emerging at 252′ in brain and CSF column chromatograms seems quite well established as homocarnosine. This dipeptide has been found in brain[22] and its regional distribution has been described.[10] In CSF this is the biggest peak in this area of the chromatogram and it appears to be identical with that previously tentatively attributed (with a different column system) by Dickinson and Hamilton[23] to carnosine. Perry *et al.*[24] have recently independently identified this peak as homocarnosine. They reported high CSF con-

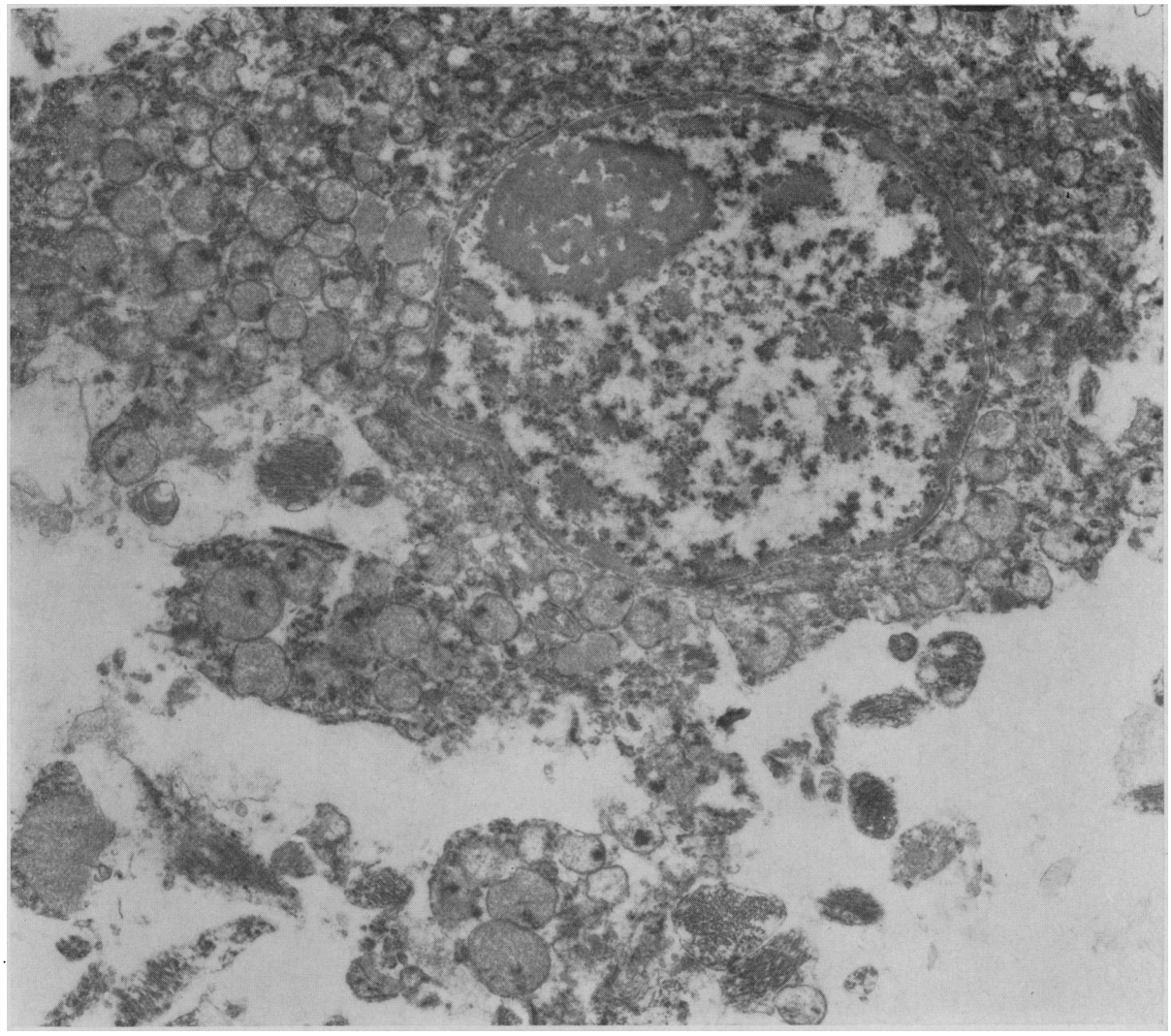


Fig. 27.—Round vacuoles containing fibrillar material in macrophages from pons. These may represent either inclusions or degenerating mitochondria. The cell is partially broken down although the nucleus is intact. (× 18,000.)

centrations in a retarded patient with a defect in carnosine metabolism.

There is no obvious metabolic link to explain the coincidence of high pipecolic acid and low homocarnosine. Their marked structural dissimilarities argue against any simple explanation such as absence of an enzyme catalyzing a precursor to product conversion. It may be that there has been a selective destruction of cell types or subcellular organelles that normally accumulate homocarnosine. Other possibilities include inhibition of enzyme activity by an accumulating metabolite, or the (presumed) mutation giving rise to a defect in pipecolate metabolism may have affected production of other enzymes. Perhaps the pipecolate abnormality is an incidental finding and the disease-producing defect involves homocarnosine, carnosine or some other unidentified compounds. In this respect it is of interest that preliminary results indicate that in addition to brain homocarnosine, muscle carnosine (288 ± 77 μmoles per kg.) was less than 10% of normal in our patient.

While those other amino acids that are present in high concentration and are easily identified were normal in our patient's brain, there were three small peaks emerging at about 265′, 274′ and 284′ in basic runs that were not detectable with similar amounts of control brain. Identification of these peaks may help clarify the connection between pipecolic acid and the histidine peptides.

Our patient was referred from a local general hospital where he was found to have a slight generalized aminoaciduria by paper chromatography. Paper chromatograms done on three urine specimens here were interpreted as show-

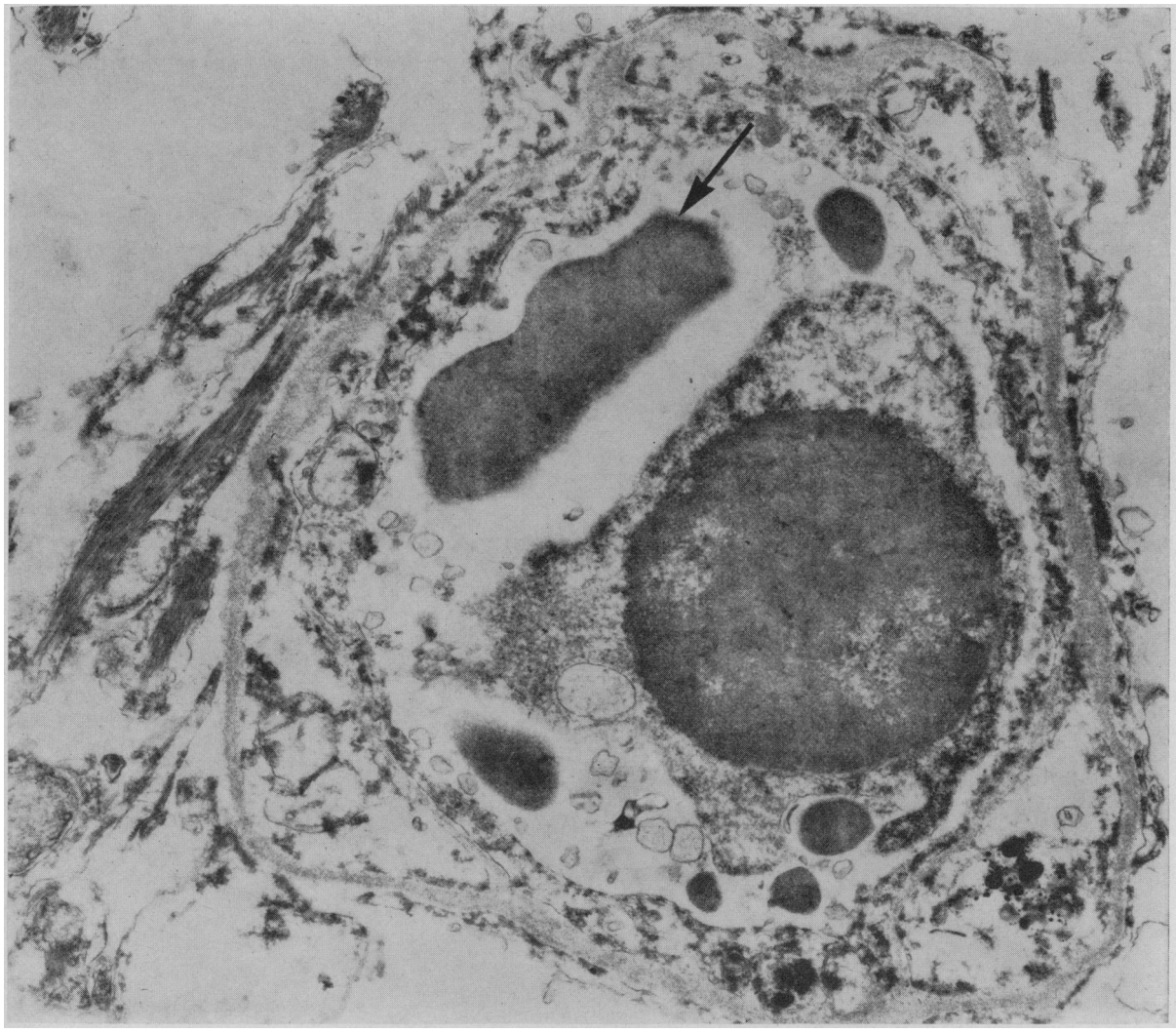
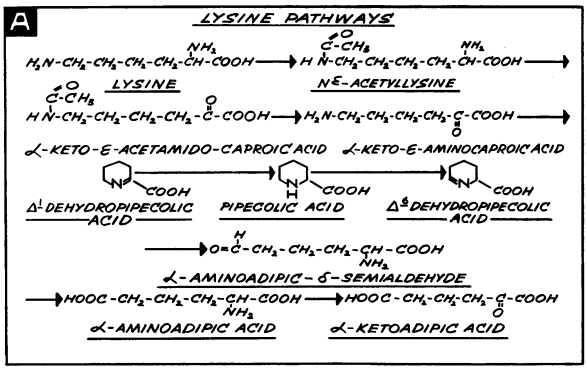
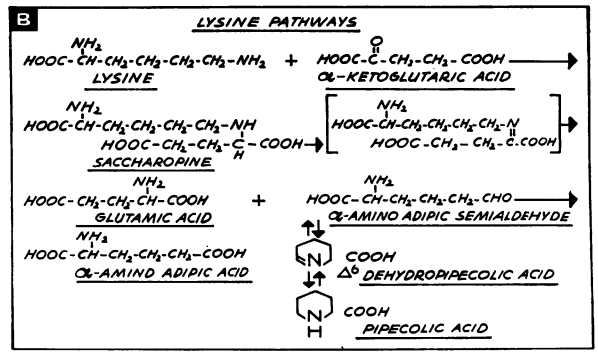


Fig. 28.—Small blood vessel from cerebellum containing electron opaque masses in the lumen. Some of these may represent lipid, some are clearly particulate amorphous material, whereas the largest mass (arrow) is difficult to distinguish from a RBC. (× 24,200.)

ing a generalized increase in amino acid excretion. When the automatic column became available and more quantitative estimates were done, the amino acid concentrations were found to be only at the upper range of normal of our control group. The mean values were, however, in several instances two to three times the mean of the controls. A mild defect in renal tubular reabsorption seems to have been present. The apparent discrepancy between the paper and column techniques may have been due in part to the presence of sizeable peaks in some urine column chromatograms that have not yet been identified. These were not consistently present and probably arose from changes in diet. There was no proteinuria and no loss of concentrating ability. The efficient renal reabsorption of pipecolate itself is striking in the presence of blood values elevated about one hundred-fold. Pipecolic acid could only be detected in urine with the solvent modifications noted and was not seen with the standard paper or column chromatographic runs.

The pathological lesions in the liver and central nervous system furnish a good explanation of the physical findings and clinical course, but are not readily correlated with the biochemical abnormalities found. It is most unlikely that any of the material described in the cells represents pipecolic acid and thus the relation between abnormal accumulations and the biochemical lesion is not as direct as it is, for example, in the case of glycogen storage disease or Gaucher's disease. The situation may be more analogous to that seen in tyrosinemia, where the abnormal biochemical product is not stored although its direct effects may be seen. The fibrotic liver lesions appear to be the result of





Figs. 29a and b.—Suggested alternative pathways of lysine and pipecolic acid metabolism.

toxic damage similar to that seen in tyrosinemia. The dilated tubules seen in the present case are reminiscent of similar changes in other metabolic disorders with aminoaciduria.

After the death of the patient a second normal sibling was born. Fasting serum pipecolic acid concentrations are normal in all four living family members.

Summary A child with a degenerative neurological disease and hepatomegaly was found to have grossly elevated blood concentrations of pipecolic acid with mild generalized aminoaciduria. Loading studies indicated there was impaired pipecolic acid metabolism but, contrary to expectations, no impairment of lysine or casein metabolism. This was interpreted as support for the emerging concept that in man the pipecolic acid pathway is a minor route for lysine catabolism. Dietary manipulations did not affect the course of the illness. The main pathological change in the nervous system was demyelination at all levels, especially in the pons, medulla and cerebellum. Accumulation of lipoid material in glia and neuronal degeneration appeared to be secondary to the demyelinating process.

Some unusual problems arising in the assay of pipecolic acid are described and normal values for serum are presented.

Résumé De fortes concentrations sériques d'acide pipécolique et une aminoacidurie plus ou moins généralisée ont été constatées chez un anfant souffrant de troubles neurologiques de type dégénératif et d'hépatomégalie. Les épreuves de charge ont mis en lumière que le métabolisme de l'acide pipécolique était troublé, mais contrairement à toute attente, que le métabolisme de la lysine et de la caséine était normal. Les auteurs considèrent ce fait comme une preuve du bien-fondé de l'hypothèse que le cycle de l'acide pipécolique na guère d'influence sur le catabolisme de la lysine. La principale atteinte du système nerveux était une démyélinisation existant à tous les niveaux mais surtout accentuée à la protubérance annulaire, au bulbe et au cervelet. Les dépôts lipidiques existant dans le tissu glial et les neurones dégénérés étaient une manifestation secondaire du processus de démyélinisation.

On discute plusieurs problèmes exceptionnels rencontrés au cours des essais biologiques de l'acide pipécolique et donne les valeurs sériques normales.

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