

TWO-DIMENSIONAL CHROMATOGRAPHY TO EVALUATE AMINO ACID EXCRETION

DERRICK LONSDALE, M.D.,

Department of Pediatrics

and

WILLARD R. FAULKNER, Ph.D.

Department of Clinical Pathology

COLLECTIVELY, amino acids may be determined quantitatively in terms of the α -amino nitrogen normally excreted in the urine. The amounts excreted show diurnal variation, and are affected by diet, by age of the person, and by disease. The purpose of this paper is to describe the chromatographic technic used for clinical screening of patients for abnormal amino acid excretion, to present results, and to discuss the usefulness of this technic as a clinical tool.

The term 'free α -amino nitrogen' has various meanings depending upon the method used to determine the concentration in a specimen and the interpretation placed upon it. As we use the term, it constitutes the collective group of compounds in an unhydrolyzed physiologic colorless specimen which upon reacting with ninhydrin become purple. It is assumed that the reacting amino radical of each of the compounds in this series yields the same color value. Although the color values differ considerably, such an analytic determination does furnish a clinically useful measurement. Included in the measurement are free amino acids, substituted free amino acids, and certain amino acid derivatives in which the amino group remains free. The free α -amino nitrogen is not to be confused with another entity generally known as the 'total α -amino nitrogen,' which has never been precisely defined, but empirically is the free α -amino nitrogen plus the extra yield of free α -amino nitrogen that appears after acid hydrolysis of the sample.

Material and Technic

From January, 1963, to March, 1964, the amino acid excretion of 168 subjects was studied by chromatography. Of this number, 29 were controls—children who were admitted to the Cleveland Clinic Hospital for surgical treatment of conditions that did not involve disturbance of metabolic processes. There were, therefore, 139 patients studied who were selected because of a clinical suspicion of a metabolic disorder.

A 24-hr. urine specimen was collected from each subject. Each sample voided was placed in a refrigerator until collection was complete. After arrival in the laboratory, all the specimens were thoroughly mixed and the volume was measured. Ammonia, which is ninhydrin-reactive and therefore a potential source of error, was eliminated by desiccation to dryness in the presence of alkali. The free α -amino

nitrogen was then estimated by the method of Rubinstein and Pryce.¹ For estimating the free α -amino nitrogen in plasma, the same method was applied to ultrafiltrates obtained by subjecting the sample to pressure of about 300 pounds per square inch from a nitrogen tank, and forcing it through a cellophane membrane.

Paper chromatography was employed to effect a two-dimensional separation and a roughly semiquantitative estimation of the individual ninhydrin-reactive substances. The procedure used was that devised by Armstrong and described by Meites and Faulkner.² This particular technic combines especially suitable solvent systems with a heavy grade of paper that obviates the desalting of urine specimens to eliminate grossly interfering substances.

For chromatograms of urine, a 1/10,000 aliquot of each 24-hr. specimen was spotted onto the paper; for those of plasma, 100 μ l. of the ultrafiltrate was employed in each case. The resulting chromatograms were compared with a chromatographic pattern of a standard mixture of ninhydrin-reactive substances made at the same time, and under the same conditions.

Results

Figure 1 shows the results of analysis of the data obtained from the urine of 29 control subjects. The range of α -amino nitrogen per kilogram of body weight per

α -AMINO NITROGEN

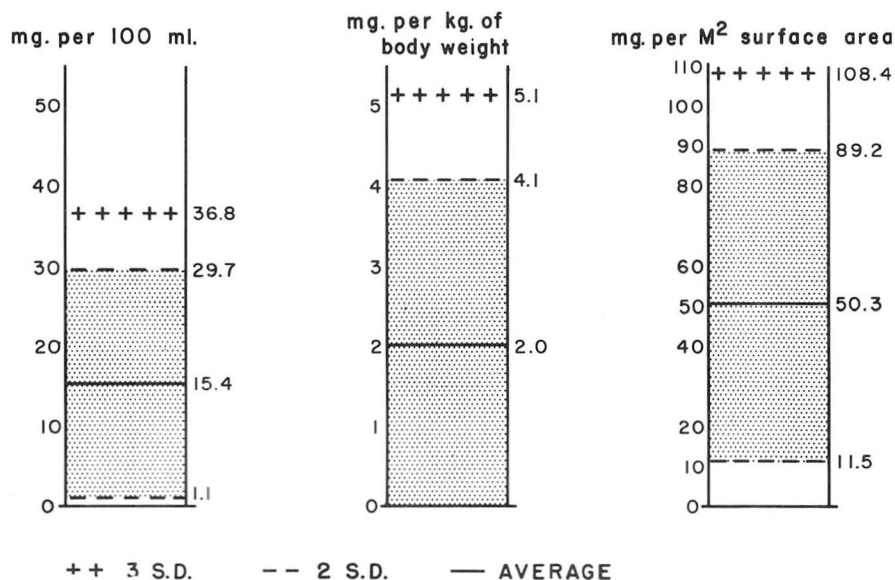


Fig. 1. Graph showing average values of α -amino nitrogen (in milligrams) in the urine of 29 control subjects.

24 hr. was from 0.8 to 3.4 mg., or an average of 2.0 mg., and this agrees with ranges reported by other authors,³⁻⁷ who regard the upper limit of normal as 4 mg. per kilogram of body weight, with an undefined lower limit. The two-dimensional chromatograms on the control subjects showed a range of from 9 to 22 ninhydrin-staining spots, with an average number of 15.8. *Figure 2* shows the frequency of appearance of metabolites. One or more unnamed substances occurred in 10 of the 29 chromatograms.

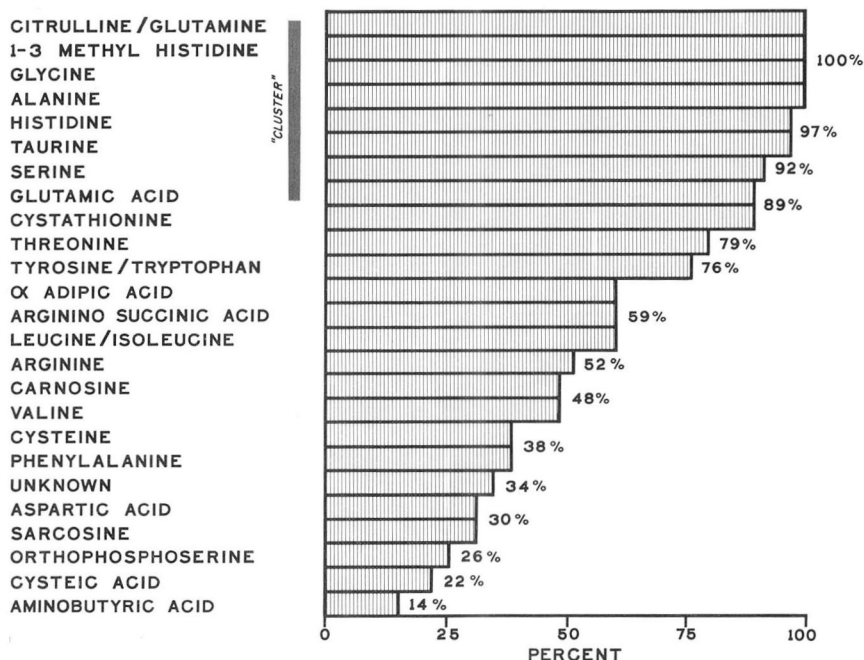


Fig. 2. Graph showing the frequency of appearance of ninhydrin-staining substances in the urine of 29 normal children.

Of the 139 patients who were studied, 104 were classified as normal. Of the 35 other patients, 23 had a generalized hyperaminoaciduria in which the total excretion of α -amino nitrogen was increased and the chromatograms showed a general increase both in the number and in the density of the ninhydrin-staining spots. In this group there were three cases of organic brain syndrome of unknown cause and two cases each of degenerative brain disease, of convulsive seizures of unknown origin, and of cirrhosis of the liver. There was one case each of pyridoxine dependency, necrotizing angitis, biliary atresia, hypogammaglobulinemia, degraded tetracycline toxicity, encephalitis, malabsorption syndrome, Wilson's disease, multiple congenital anomalies, vitamin-D resistant rickets, rhabdomyosarcoma, polymyositis, renal calculus, and hyperammonemia (now believed to be associated

with a deficiency of glutamic acid dehydrogenase, and under further study).

Of 11 patients whose chromatograms for urine were specifically abnormal, there were three with phenylketonuria which demonstrated the characteristic spot of phenylalanine, and two children with the easily recognizable pattern of maple syrup urine disease, which is associated with increased amounts of leucine, isoleucine, and valine. The urine specimen of one man, who had had cystine stones surgically removed, showed the presence of cystine, arginine, lysine, and ornithine, the four amino acids excreted in excessive amounts in cystinuria. The chromatograms of the other patients had changes that were abnormal but not sufficiently definitive for interpretation. One child, with organic brain disease, had a normal urinary α -amino nitrogen excretion, but a large spot of citrulline was seen on the chromatogram. Two children, one with convulsions, the other mentally retarded, each excreted an abnormal amount of glucosamine. Another child, also mentally retarded, excreted more than the normal amounts of leucine and isoleucine. A four-year-old girl with severe mental retardation and seizures was reported to have urine with the odor of maple syrup. The urine and plasma chromatograms were grossly abnormal and showed excessive amounts of all the branched-chain amino acids and glutamine. Enzyme studies showed that the transaminase activity for the branched-chain amino acids was normal, and that the decarboxylating enzyme common to the normal catabolic system for these acids was also present. An abnormal hydrazone formed in the urine with 2,4 dinitrophenylhydrazine, which migrated on thin-plate chromatograms with ketovaline.⁸ Urine and plasma from this child are under further study.

Finally, a child, with degenerative brain disease, had a normal urine chromatogram, but the plasma chromatogram showed a considerable increase in α -amino nitrogen excretion, but did not receive further study.

Discussion

The metabolism of amino acids has received an increasing amount of attention because of a realization of their importance. With the discovery of the mechanism of desoxyribonucleic acid (DNA) coding, and the method by which protein structure is built up in the body, more effort has been put into studying aberrations of this mechanism which are associated with disease. This is particularly important in childhood, the period in which most of the genetically determined errors of metabolism are first identified. A large group of these errors is now recognized, and one of the most interesting features is the fact that mental retardation and seizure disorders appear to be the common denominators in so many of them. This correlation has resulted in a serious effort to 'screen' urine specimens from many mentally retarded children, such as the recent illuminating study by Carson and associates.⁹ Such diseases are rare, because their appearance or expression is governed by the mechanism of recessive inheritance, but, in addition they are difficult to recognize,

and the fact that they are associated with severe mental retardation is discouraging and will often result in such a patient's receiving little or no definitive evaluation.

'Generalized aminoaciduria' is the term used to indicate an increased excretion of all the normal physiologic amino acids. It is detected by finding an excessive amount of α -amino nitrogen in urine. Such a finding, however, is nonspecific, reflecting as it does any one of a number of metabolic disturbances. It may arise from failure of catabolism of amino acids and derivatives, as in disease of the liver, the so-called overflow mechanism, or as a failure in the normal resorption of amino acids in the proximal tubules of the kidney, hence the term 'renal aminoaciduria.'

Two separate steps in the urinalysis require comment: the quantitative expression of α -amino-nitrogen, and the evaluation of the two-dimensional chromatogram. Since the α -amino nitrogen excretion is affected by the age of the person, expressing it as a 24-hr. total urinary excretion makes it difficult to compare that of one child with that of another, and complicates the normal range of values. The α -amino nitrogen is dependent on urinary volume, and expressing it in terms of this volume fails to take into account the dilution factor. For this reason three indexes are used:

(1) Milligrams of α -amino nitrogen per kilogram of body weight per 24 hr.^{4,6,10} We have found this index to be the most useful; it fails only when there is an extremely abnormal discrepancy between height and weight, and in this situation relating α -amino nitrogen to body surface is probably more accurate. A 24-hr. urine specimen is required.

(2) Milligrams of α -amino nitrogen as a percentage of the total nitrogen excretion in milligrams per 24 hr.^{4-6,10} We have not used this index but it has the advantage that no 24-hr. specimen is required. Berglund¹¹ reported that interpretation of a morning spot-specimen appeared to correlate well with an evaluation from a 24-hr. specimen.

(3) Milligrams of α -amino nitrogen in relation to the creatinine excretion in milligrams per 24 hr.^{10,12} This index depends on the supposedly constant excretion of creatinine. We have found this index to be erratic, and it will certainly be inaccurate when muscle atrophy occurs as in mental defectives. Furthermore, it has been shown that creatinine is not a constant factor and is both actively secreted and absorbed by the renal tubules.¹¹

Many attempts have been made to semiquantitate a two-dimensional chromatogram in terms of the density and the number of ninhydrin-staining metabolites. We have found it difficult to draw any conclusions from this approach, and rely on the general appearance of the chromatogram on a strictly qualitative basis.

In the technic used by us, most of the amino acids that are excreted in normal urine appear on the chromatogram as a central ring or cluster. It is found that the so-called 'cluster' group increases in density and size of the individual spots (Fig. 2) as the α -amino nitrogen excretion increases. Proportional to this increase 'satellite' spots appear in the constellation as ninhydrin-reactive substances that are not seen

on the chromatogram in the normal state, being present in insufficient concentration. A 1/10,000 aliquot of the 24-hr. specimen must always be used for this phenomenon to be observed. If the total amount of α -amino nitrogen is increased by increasing the aliquot of urine placed on the filter paper, the satellite spots appear in the normal chromatogram. In order to have urine chromatograms comparable, the same aliquot of urine must be used in each study. Basing the applied aliquot on the total α -amino nitrogen present leads to a uniform statement of all chromatograms, but using a constant volume fails to take into account the dilution factor, and results in overstatement of the chromatogram of concentrated urine. We have found that the best method is to use a constant proportion of the total urinary volume. The chromatograms are then reasonably comparable. This method is equivalent to using an amount of urine that is excreted per unit time.

The two-dimensional chromatogram has its greatest use in the diagnosis of disorders involving the excessive excretion of a specific metabolite, as in phenylketonuria. In such a disorder the α -amino nitrogen value may be normal or even quite low. The excessive excretion of one substance is frequently compensated for by a decrease in the excretion of normal metabolites. Reading the chromatogram is likened by Dent¹³ to ". . . a picture that can be remembered and subsequently recognized like somebody's face."

This study reports 35 abnormal findings from 139 cases studied, and suggests a high positive yield when using this technic. The data have to be reviewed critically, and may be considered in two parts with reference first to 'generalized' and second to 'specific' aminoaciduria. If rigid criteria are applied, then a generalized aminoaciduria may be defined as an excretion in excess of normal quantity. The difficulty is to determine exactly when such excretion really does become abnormal, and Berry¹⁴ considers that the excretion pattern should not be considered abnormal unless the amounts are from five to ten times the so-called normal values. In routine screening she finds that between 95 and 98 percent of the specimens tested fall within normal limits. Our high yield of abnormality reflects the use of more rigid criteria and the clinical selection of patients in whom aminoaciduria could be considered a strong possibility. Unfortunately, the finding of such a generalized hyperexcretion is quite nonspecific, somewhat like the yield of information from a blood sedimentation rate, and merely reflecting a change in protein metabolism. A reversion to a normal pattern, however, can be a useful index of remission of disease. Finding a specific abnormality may be diagnostic, and three cases of phenylketonuria and two cases of maple syrup urine disease certainly represent a rewarding yield in the field of rare disease. In addition, there were six patients with gross, but undecipherable, abnormalities, pointing to the fact that little is known still of brain disease governed by errors of metabolism. Further evaluation of these problems involves erudite and time-consuming biochemical study that is difficult to perform outside a full-time research program.

Finally it should be emphasized that the evaluation depends, like any other

laboratory technic, on a certain amount of clinical information. The height, weight, and age of the patient are necessary, together with an accurately collected 24-hr. specimen of urine. The clinical diagnosis is necessary in order that special technics may be used to detect diseases that are not readily demonstrable by routine study. This is best illustrated by histidinuria¹⁵ for which a search for the abnormal metabolites must be made by specialized methods when the clinical suspicion arises. In our own experience, the urinalysis of an infant with maple syrup urine disease demonstrated a normal amino acid pattern and a normal α -amino nitrogen excretion. Only a chromatographic analysis of plasma revealed the diagnostic pattern and confirmation of the clinical findings. The branched-chain amino acids, although increased in concentration in the blood, were being adequately resorbed from the proximal renal tubules, and therefore there was no excessive branched-chain aminoaciduria.

Summary

Two-dimensional chromatograms of ninhydrin-reactive substances in the urine were performed on 168 persons from January, 1963, to March, 1964, at the Cleveland Clinic. There were 29 control subjects, from whom were derived normal values for α -amino nitrogen and the normal chromatographic pattern, and 139 patients, selected because of a clinical suspicion of a metabolic disorder. Twenty-three of the patients studied showed a generalized hyperaminoaciduria, and there were 11 patients whose chromatograms for urine were specifically abnormal. One patient had a generalized elevation of the plasma amino acids with a normal chromatogram. Criteria of definition for a generalized aminoaciduria in terms of various indexes are discussed. The inclusion of certain clinical information when this special study is requested is critically important.

References

1. Rubinstein, H. M., and Pryce, J. D.: Colorimetric estimation of alpha-amino nitrogen in tissue fluids. *J. Clin. Path.* 12: 80-84, 1959.
2. Meites, S., and Faulkner, W. R.: *Manual of Practical Micro and General Procedures in Clinical Chemistry*. Springfield, Illinois: Charles C Thomas, 1962; 350 p.; p. 249, 301.
3. Zellweger, H.: Amino-aciduria and mental retardation. *Clin. Chem.* 7: 315-327, 1961.
4. Childs, B.: Urinary excretion of free alpha-amino acid nitrogen by normal infants and children. *Proc. Soc. Exper. Biol. & Med.* 81: 225-226, 1952.
5. Huisman, T. H.: L'élimination des acides aminés chez des enfants normaux d'âges différents. [Elimination of amino acids in normal children of various ages.] *Arch. franç. pédiat.* 14: 166-180, 1957.
6. Berger, H.: Aminoaciduria and hyperaminoaciduria (physiology and pathology of amino-acid excretion in urine and its clinical significance). *Bibl. paediat.* no. 71, suppl. *Ann. paedit.*: 1-174, 1959.

7. Van Geldersen, H. H., and Dooren, L. J.: Excretion of free α -amino acids in children. *Arch. Dis. Child.* **39**: 261-264, 1964.
8. Dancis, J.: Personal communication, April, 1964.
9. Carson, N. A. J.; Cusworth, D. C.; Dent, C. E.; Field, C. M. B.; Neill, D. W., and Westall, R. G.: Homocystinuria: new inborn error of metabolism associated with mental deficiency. *Arch. Dis. Child.* **38**: 425-436, 1963.
10. Ghadimi, H., and Schwachman, H.: Evaluation of amino-aciduria in infancy and childhood. *A.M.A. J. Dis. Child.* **99**: 457-475, 1960.
11. Berglund, F.: Urinary excretion patterns for substances with simultaneous secretion and reabsorption by active transport. *Acta physiol. scandinav.* **52**: 276-290, 1961.
12. Fowler, D. I.; Norton, P. M.; Cheung, M. W., and Pratt, E. L.; Observations on urinary amino acid excretion in man; influence of age and diet. *Arch. Biochem.* **68**: 452-466, 1957.
13. Dent, C. E.: Clinical applications of amino acid chromatography. *Scandinav. J. Clin. & Lab. Invest.* **10** (supp. 31): 122-127, 1958.
14. Berry, H. K.: Personal communication, July, 1964.
15. Ghadimi, H.; Partington, M. W., and Hunter, A.: Familial disturbance of histidine metabolism. *New England J. Med.* **265**: 221-224, 1961.