

THE STUDY OF INBORN ERRORS OF AMINO ACID
METABOLISM IN MENTALLY RETARDED SUBJECTS
IN ETHIOPIA

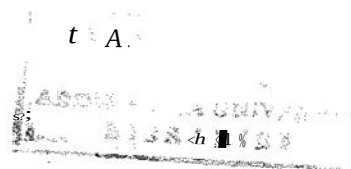
1
i
v> x i i : * J g

By

Chanyalew Zewdie

February, 1997

**JHETSTUDY OF INBORN ERRORS OF AMINO
ACID METABOLISM IN MENTALLY RETARDED SUBJECTS
LN ETHIOPIA**



**A Thesis Presented to
The School of Graduate Studies, Addis Ababa University
In Partial Fulfilment of the Requirements for the
Degree of Master of Science in Chemistry**

By

Chanyalew Ziewdie

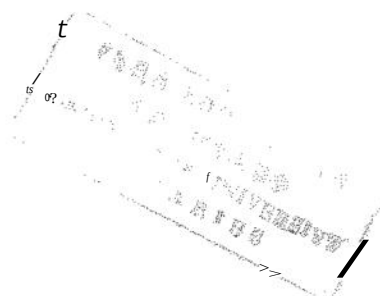
Februaity, 1997

To
my father Zewdie,
my mother Almaz,
my sisters Berhane, Adanech, Rahel, and Meskerem and
my brothers Gezahegn, shemelis and Amanuel,
without their understanding and sustained support,
my education couldn't have been completed.

CONTENT

ACKNOWLEDGEMENTS	I
CONTENT	II
List of Tables	IV
List of Figures	V
Abstract	VI
1. Introduction	1
1.1 General Consideration	1
1.1.1 Amino acids	I
1.1.2 Amino acid Nutrition	2
1.1.3 General Mechanism of amino acid Nutrition	3
1.1.4 Catabolism (Degradation) of amino acid	5
1.1.4.1 Deamination	5
1.1.4.2 Transamination	6
1.1.4.3 Decarboxylation	7
1.1.5 Reaction of amino acids	7
1.2 Inborn Errors of Amino Acid Metabolism	8
1.2.1 Concept and Definition	8
1.2.2 Genetics	9
1.2.3 Principles	9
1.2.3.1 The metabolic consequence of a genetically-deficient enzyme activity	9
1.2.3.2 Genetic Heterogeneity	12

1.2.4 Metabolic disorder of Amino acids	13
1.2.5 Overflow and renal aminoaciduria	15
1.2.6 Screening for inborn errors of amino acid metabolism	16
1.2.7 Analysis of amino acids	18
1.2.8 Inborn of errors of metabolism and mental retardation	19
2. Objective	22
3. Materials and Methods	23
3.1 Specimen Collection	25
3.2 Laboratory Procedures	26
4. Results	32
4.1 Study population	32
4.2 Chemical tests	34
4.3 Paper Chromatography	37
5. Discussions	40
5.1 Prevalence of Metabolic Abnormalities	43
6. Recommendations	46
7. References	47
8. Appendix	51



List of Tables

1.	Amino acids as significant constituents of body proteins	2
2.	Chemical tests used in the study	24
3.	Results of chemical tests	35
4.	Results of paper chromatography	37
5.	Results of Chemical tests and paper chromatography	38
6.	Metabolic disorders detected in the present study	42
7.	Comparison of results of the present study with other similar studies	45

[illegible]

List of Figures

1.	General structure of amino acid	1
2.	Scheme of general metabolic interrelations of amino acid	4
3.	The initial catalytic reaction in amino acid catabolism	5
4.	Hypothetical pathway which show the conversion of substrate A to the end product D through the successive actions of enzymes E_{AB} , E_{BC} , E_{CD}	9
5.	Metabolic pathway of galactose	10
6.	Metabolic pathway of methionine	11
7.	Metabolism of phenylalanine	14
8.	Age and sex distribution of patients	32
9.	Age and sex distribution of controls	34
10.	Connection between positive chemical tests and pathological chromatogram ...	41

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

ABSTRACT

Inborn errors of amino acid metabolism are directly related to the absence of an enzyme involved in the metabolism of one or more amino acids, so that those amino acids increase greatly in plasma concentration and urinary excretion rates. Most of these inborn errors of amino acid metabolism are associated with mental retardation.

Urine specimens from 345 mentally retarded subjects have been analyzed using qualitative chemical tests and one-dimensional paper chromatography in order to detect any inborn errors of amino acid metabolism which could be associated with mental retardation. The following amino acid abnormalities were discovered: Homocystinuria, 1 case (0.29%); Amino acid malabsorption, 1 case (0.29%); indicanuria, 1 case (0.29%); Generalized aminoaciduria, 6 cases (1.78%).

THE STUDY OF INBORN ERRORS OF AMINO ACID METABOLISM IN MENTALLY RETARDED SUBJECTS IN ETHIOPIA

1. INTRODUCTION

1.1 GENERAL CONSIDERATION

1.1.1 AMINO ACIDS

Amino acids are chemical compounds which possess an amino group ($-\text{NH}_2$); a carboxyl group ($-\text{COOH}$); and an R group or side chain, which is responsible for specific characteristics of the particular amino acid. The general structure of most amino acids can be represented by the formula shown in Fig. 1.

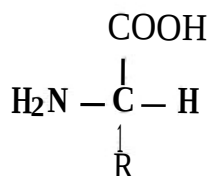


Fig. 1 General structure of amino acids.

About 21 amino acids (Table 1) are present in the body as significant constituents of proteins [1]. Some of these amino acids must be supplied by dietary intake, whereas others can be synthesized by various metabolic pathways.

Amino acids that possess an asymmetric carbon atom, i.e. those with different substituent

Table 1. Amino acids as significant constituents of body proteins.

Alanine	Glutamic acid	Isoleucine	Proline
Arginine	Glycine	Leucine	Serine
Aspartic acid	Histidine	Lysine	Threonine
Cysteine	Hydroxylysine	Methionine	Tryptophan
Cystine	Hydroxyproline	Phenylalanine	Tyrosine
Valine			

groups, have dextrorotatory (D) and laevorotatory (L) optical specificity. All optically active amino acids in human proteins are of the L-configuration.

1.1.2 AMINO ACID NUTRITION

Dietary protein is the principal source of amino acid nutrition. The role of protein in the diet is not to provide body proteins directly but to supply the amino acids from which the body can make its own proteins. Since the body can make glycine and serine for itself, the proteins in the diet need not contain these two amino acids. But there are some amino acids that the body can not make. These amino acids are referred to as essential amino acids; it is essential that they must be included in the diet. Eight amino acids are known to be essential for human adults and these are methionine, threonine, tryptophan, isoleucine, leucine, lysine, valine, and phenylalanine. An additional amino acid, histidine is also required by children and possibly by adults, while arginine is required by infants [2].

An important characteristic of a protein is therefore that it should supply all eight essential amino acids, plus enough of the non-essential ones to provide a source of nitrogen for the synthesis of others.

rates are of considerable medical importance, particularly in newborns and children.

The disease-oriented view of amino acid metabolism tends to focus attention on the initial catabolic events, while there are other aspects of equal importance to normal metabolic equilibrium which also merit attention.

1.1.4 CATABOLISM (DEGRADATION) OF AMINO ACIDS

The initial enzyme catalyzed attack upon the amino acid most often occurs around the α -

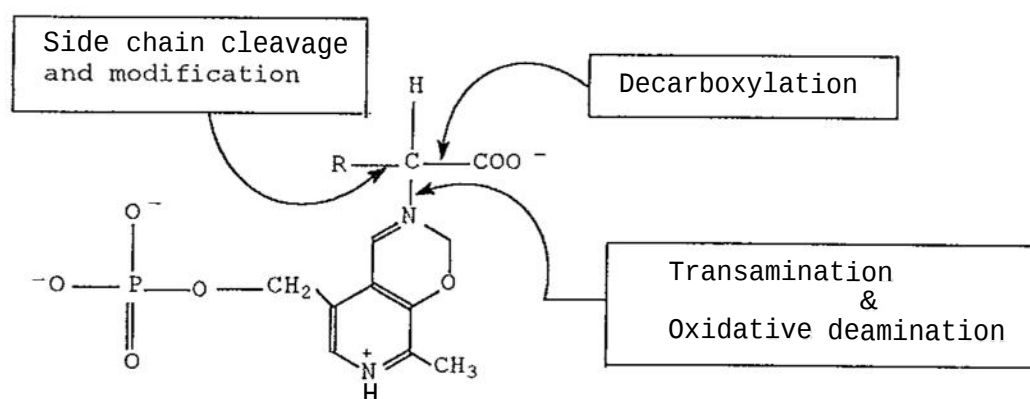
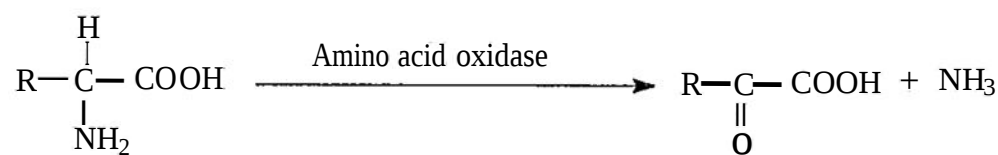


Fig. 3 The initial catalytic reaction in amino acid catabolism.

carbon atom. The result is either decarboxylation, removal of the α -amino group or cleavage and modification of the side chain. These enzyme-catalyzed reactions usually require pyridoxal-phosphate as coenzyme. The proposed relationship between the substrate and coenzyme, which facilitates electron mobilizations and transfer around the α -carbon atom, is shown in Fig 3.

1.1.4.1 Deamination: Oxidative deamination is a catabolic reaction whereby the α -amino group of an amino acid is removed, forming an α -keto acid and ammonia. Deamination occurs

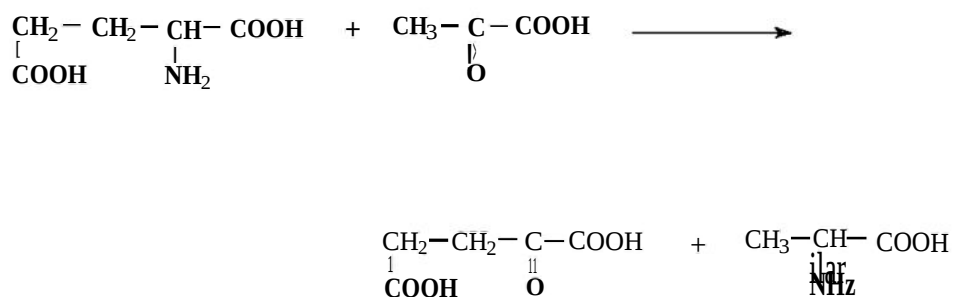
primarily in the liver and the kidneys under the catalysis of the enzyme amino acid oxidase.



The α -keto acid produced by this process can undergo several different types of reactions.

- i. It can be catabolized to carbon dioxide and water in the citric acid cycle with the release of energy.
- ii. It can be converted to carbohydrate (glycogen) or fat.
- iii. It may be reconverted to a different amino acid by a process called transamination.

1.1.4.2 Transamination: is a reaction whereby an amino group from amino acid is transferred to a keto acid. This reaction is catalyzed by the enzymes called transaminases. By this process the body can manufacture the amino acids that it needs and which it does not possess. An

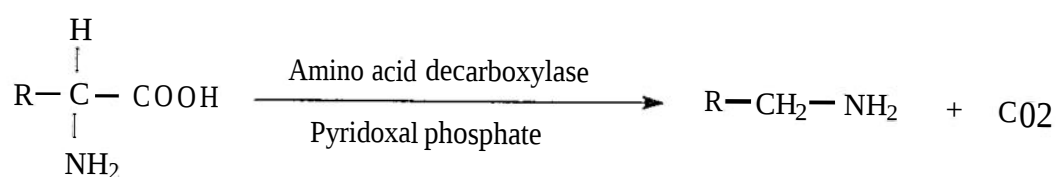


essential part of the active site of transaminases is pyridoxal phosphate, the coenzyme form of vitamin B₆.

An example of transamination is the reaction of glutamic acid (an α -amino acid) and pyruvic

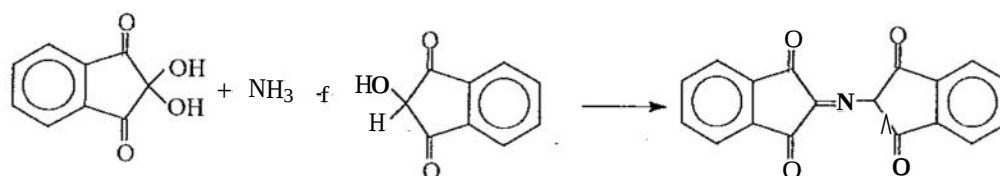
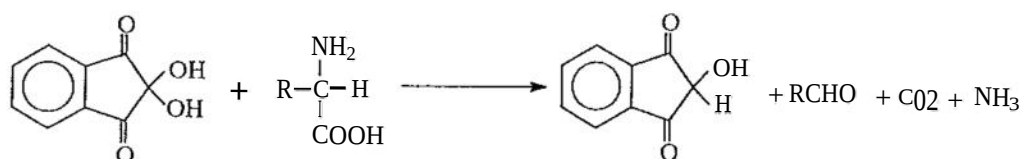
acid (an α -keto acid) to form α -ketoglutaric acid (another α -keto acid) and alanine (another α -amino acid).

1.1.4.3 Decarboxylation: The decarboxylation (removal of a $-\text{COOH}$ group) of an amino acid yields a primary amine. The carboxyl group that is removed is converted to carbon dioxide. The enzyme involved in a decarboxylation reaction requires pyridoxal phosphate as a coenzyme. The decarboxylation reaction may be summarized as follows.



1.1.5 REACTION OF AMINO ACIDS

The reaction of ninhydrin (triketohydrindene) with amino acids is of particular importance for the detection and quantitative estimation of amino acids. Ninhydrin is a powerful oxidizing



agent and elicits the oxidative deamination of the α -amino group, liberating ammonia, carbon dioxide, the corresponding aldehyde, and a reduced form of the ninhydrin. The ammonia then reacts with an additional mole of ninhydrin and the reduced ninhydrin to yield a purple substance.

1.2 INBORN ERRORS OF AMINO ACID METABOLISM

1.2.1 CONCEPT AND DEFINITION

The term "Inborn errors of metabolism" was first used by Garrod to describe his studies of alkaptonuria, albinism, cystinuria and pentosuria [3,4]. He postulated that each disease was due to a block in the normal metabolic pathway. This was later proved when Cori and Cori [5] in 1952 demonstrated absence of glucose-6-phosphate in the liver of patients with glycogen storage disease and Jervis [6] demonstrated in 1953 the absence of phenylalanine hydroxylase activity in the liver of patients with phenylketonuria.

Inborn errors of metabolism are defined as genetically caused metabolic diseases. Most of the diseases of this type are due to a defective gene. The genetic defect leads to synthesis of a defective enzyme or to an enzyme deficiency. A defect or absence of a specific enzyme causes a block in the metabolic reaction that the enzyme normally would catalyze. The metabolic blocks in turn lead to accumulation of a precursor, or metabolites. The product that accumulates, in abnormally high concentrations, can cause damage to body cells, with subsequent organ malfunctions [7].

Many metabolic defects are associated with dysfunction of the central nervous system, such as

mental retardation, seizure, degenerative disease or "failure to thrive".

1.2.2 GENETICS

Most of the inborn errors are transmitted in an autosomal recessive rather than dominant pattern of inheritance. The term "recessive" implies that the affected individual can escape symptoms if he has about half of the normal amount of the enzyme involved. It is apparent that the recessiveness or dominance of a trait might be affected by the environment; such as a substantial increase in the dietary intake of phenylalanine by an individual heterozygous for the phenylketonuria trait might lead to phenylketonuria.

1.2.3 PRINCIPLES

1.2.3.1 The metabolic consequences of a genetically-deficient enzyme activity:

The metabolic consequences of a genetically-deficient enzyme activity are depicted in Fig 4.

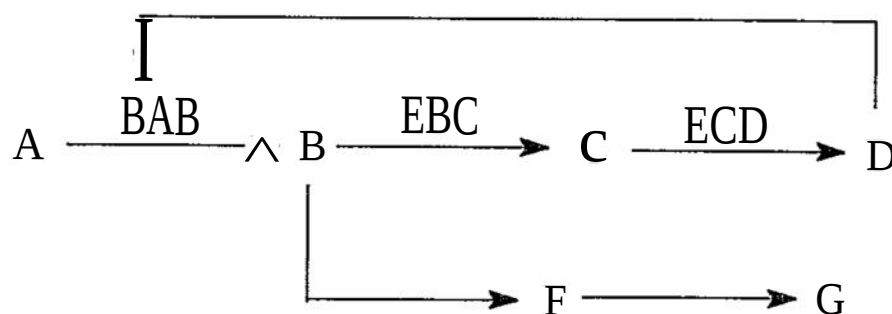


Fig. 4 Hypothetical metabolic pathway converting substrate A to the end product D through the successive actions of enzymes E₁B, EBC, and ECD. An alternative pathway

to F and G is indicated. The arrow from D to E_{AB} represents negative feedback control of the first enzyme in the pathway by the ultimate product of the sequence.

Precursor Accumulation: Let us consider a defect of E_{BC} (Fig. 4). Such a defect might lead to intracellular and extracellular accumulation of the immediate or remote precursor of the reaction. In some cases the substrate just before the block, in this case substrate B, not being converted to substrate C, will increase in concentration and may appear in abnormal quantities in blood and urine. Because most enzymatic reactions are reversible, substrate A may also pile up and be excreted. An example is galactosaemia. In the cases of galactosaemia [8,9], the defective enzyme is galactose-1-phosphate uridyl transferase, which normally converts galactose-1-phosphate to glucose-1-phosphate (Fig 5). In the mutant homozygote this step can not occur, and galactose-1-phosphate accumulates in the blood cells, liver and other tissues, damaging the liver, brain and kidney.

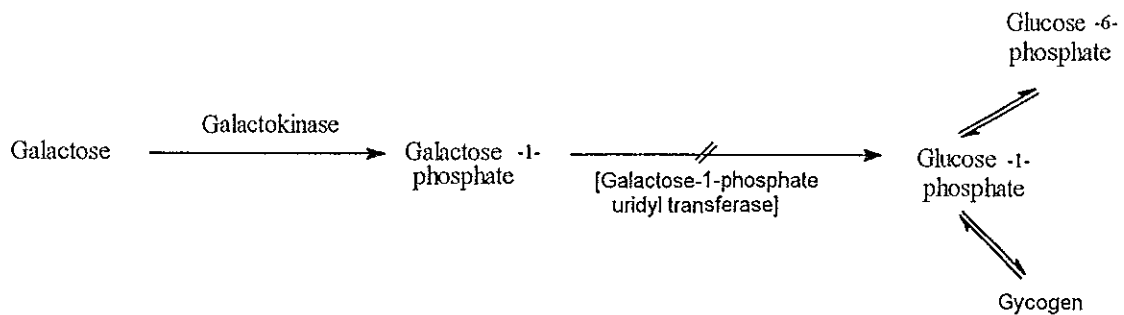


Fig. 5 Metabolic pathway of galactose. Site of block in galactosaemia is indicated.

Defect of E_{BC} (Fig. 4) may result in accumulation of A as well as B. Defect of E_{CD} could lead to the pile up of A, B, and C. An example is homocystinuria. In homocystinuria, due to cystathionine β -synthase deficiency [Fig 6], methionine, a remote precursor, accumulates as does homocystine, the immediate precursor of the blocked reaction [10,11].

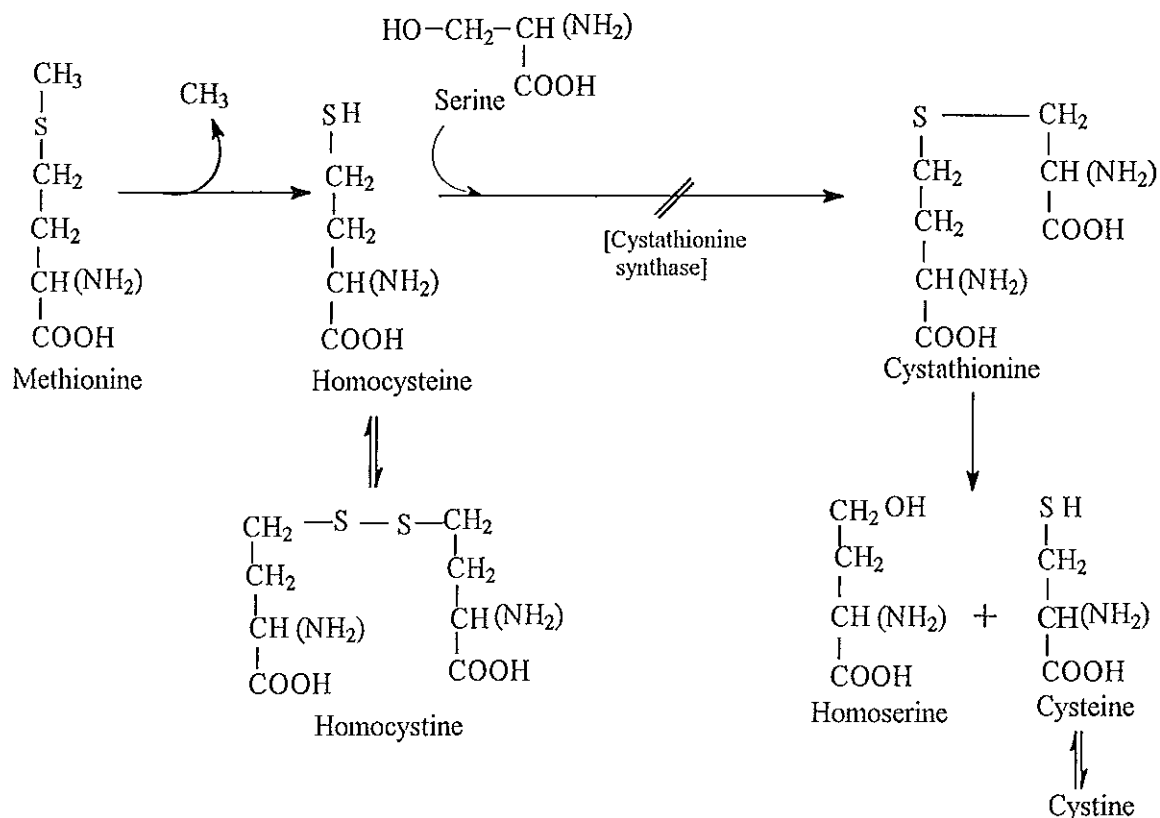


Fig. 6 Metabolic pathway of Methionine. Site of block in homocystinuria is indicated.

Alternate Pathway Utilization: If the conversion of B to C is impaired [Fig 4] by a defect of E_{BC} , not only will B accumulate, but the usual minor accessory pathway to F and G may become prominent. Phenylketonuria (Fig. 7) represents an example for such a case. In phenylketonuria, the absence of phenylalanine hydroxylase activity leads to gross over production and excretion of phenylpyruvic, phenylacetic, and phenyllactic acids [12,13]. These compounds are not usually detectable in blood or urine. Such alternate pathway augmentation may have important physiological significance if the products of the alternate pathway interfere with cell processes when present in more than minute concentrations.

Lack of Synthesis of End Product: Let us assume that D is the physiologically active product of the hypothetical reaction sequence [Fig. 4]. It is apparent that a block at any of the

steps from A to D may result in inadequate synthesis of D. The formation of thyroxine in the thyroid gland proceeds through such a series of reactions, involving first the transport of iodide into the gland and then its subsequent oxidation and organification. Several enzymatic defects have been described which lead to goitrous cretinism due to impaired synthesis of thyroxine [14].

Feedback Control Breakdown: In the hypothetical scheme [Fig. 4], the end product of the reaction sequence, D, is presumed to regulate the activity of E_{AB} , the first enzyme in this biosynthetic pathway. The phenomena of **end-product inhibition**, or **feedback inhibition** as it is more commonly called, has been noted in numerous microbial systems. Abnormal feedback control occurs in the adrenogenital syndrome. In this disorder there is a block at one of several steps in the biosynthesis of cortisol by the adrenal cortex. This deficiency stimulates excessive production of adrenocorticotrophic hormone (ACTH) by the pituitary, because the level of cortisol normally regulates the output of ACTH by a negative feedback mechanism. The increased ACTH levels, in turn, stimulate the adrenal cortex to increase synthesis of the cortisol precursors but, only as far as the block. Breakdown of the accumulated precursors by alternative pathways leads to the androgenic effects. In a female fetus, this may result in masculinization of the external genitalia [15].

1.2.3.2 Genetic Heterogeneity

A specific clinical or biochemical phenotype may be produced by more than a single genotype. The term **genetic heterogeneity** is used to describe this phenomenon. Such genetic heterogeneity exists for many of the inborn errors of metabolism. The study of the alpha and beta chains of human haemoglobin has revealed that the many mutant haemoglobins are

produced by different point mutations, unequal cross-overs, and deletions. Genetic heterogeneity may result from **allelic** or **nonallelic** mutations; that is, the common phenotype may be produced by more than a single mutation at one locus (allelic) or by mutations at different loci (nonallelic). Heterogeneity can be demonstrated by clinical (such as age of onset, severity and specific features), biochemical (such as constituents of blood, urine and cerebrospinal fluid (CSF), and enzyme activity), and genetic (such as chance mating, and mode of inheritance) investigations.

Genetic heterogeneity has clinical as well as scientific importance. The demonstration that elevated blood phenylalanine levels may be transient rather than permanent has obvious therapeutic implications. A low phenylalanine diet may be very beneficial to infants with classic phenylketonuria who lack phenylalanine hydroxylase activity. It may be equally harmful to infants who have a transient intolerance to phenylalanine at birth but who quickly develop normal phenylalanine hydroxylase activity within the first three months of life. In this instance and many others, appreciation of the biochemical and genetic heterogeneity in the inborn errors has important ramifications in family planning and genetic counselling.

1.2.4 METABOLIC DISORDER OF AMINO ACIDS

Amino acids, although essential for the synthesis of proteins and certain other compounds, a disorder in their metabolism may cause serious health problem. Many inherited disorders of amino acid metabolism are caused by a defective catabolic enzyme, leading to accumulation of an amino acid and often also its metabolites in plasma, urine and cerebrospinal fluid. Although a great number of inborn errors of amino acid metabolism are presently known, most of these diseases occur very rarely; phenylketonuria being considered the most frequent one.

Phenylketonuria is an inheritable disease with an incidence of about 1 in 10, 000 live births, characterized by a defect in the ability to metabolize the amino acid phenylalanine. The disease is caused by the absence or deficiency of the enzyme L-phenylalanine hydroxylase, which converts phenylalanine to the amino acid tyrosine. As a result, phenylalanine is excreted in the urine but a larger fraction is deaminated to phenylpyruvic acid and excreted in that form (Fig. 7).

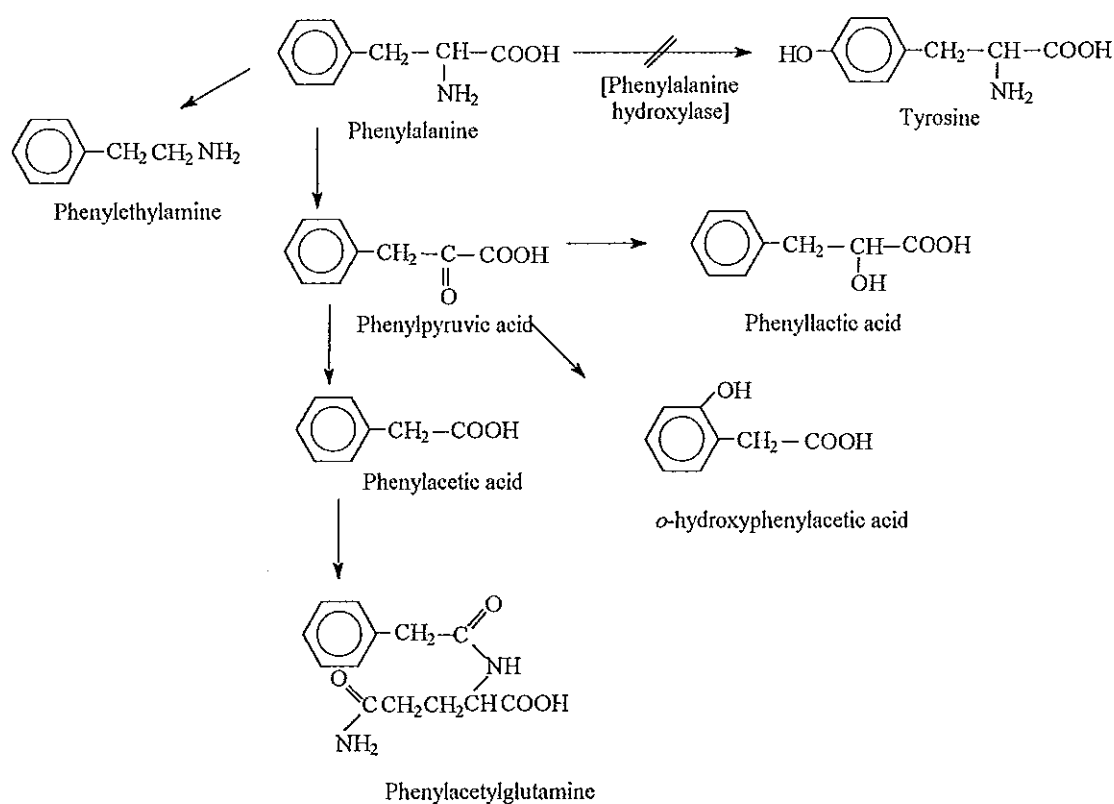


Fig. 7 Metabolism of phenylalanine. Site of block in phenylketonuria is shown.

The phenylalanine accumulating in the tissue is apparently toxic, especially to developing brain tissue, and cause brain damage and progressive mental retardation. The injury to brain tissue begins within the second and third week of life and progresses with time, becoming maximal

at about 8 to 9 months. Brain damage can be minimized if the newborn child is placed on a low-phenylalanine diet soon after birth [16,17,18]. Even if diagnosis is made late, if phenylalanine is then removed from the diet, the rate of further mental deterioration can be decreased, provided that this is done before 4 to 6 months have passed.

1.2.5 OVERFLOW AND RENAL AMINOACIDURIA

Typically, a hereditary amino acid disorder is directly related to the absence of an enzyme involved in the metabolism of one or more amino acids, so that those amino acids increase greatly in plasma concentration and urinary excretion rates.

Increased concentrations of amino acids in plasma can be categorized as primary (hereditary) metabolic defects and secondary metabolic responses. Secondary responses involve most or all amino acids. Significant increase are usually a consequence of severe liver disease that inhibits the oxidative deamination of amino acids. Small increase in plasma concentration of many amino acids occur after ingestion of a protein rich-meal.

Increased urinary excretion of amino acids is of two major types-namely, overflow and renal (generalized). Overflow aminoaciduria are those that accompany increased plasma concentrations of amino acids when normally functioning kidney tubules are unable to reabsorb the increased concentrations of amino acids in the glomerular filtrate- i.e, the renal tubular maximum reabsorption capacity is exceeded.

Renal (generalized) aminoacidurias are those conditions associated with increased urinary excretion of one or more amino acids while plasma amino acid concentrations are normal.

These various conditions have in common a defect in the renal tubular transport mechanism that causes decreased reabsorption of amino acids from the glomerule filtrate. Renal aminoacidurias are classified as primary (hereditary) and secondary aminoaciduria. Primary or hereditary renal aminoacidurias are those involving a hereditary defect in renal tubular transport of one or several amino acids. For example, cystinuria results in an inability of the renal tubules to absorb not only cystine but also lysine, arginine, ornithine, and occasionally other amino acids as well. Secondary renal aminoacidurias are those resulting from acquired renal tubular disease, often a toxic etiology, such as heavy metal poisoning. Other etiologies include acute renal tubular necrosis; severe malnutrition; and various metabolic diseases otherwise unrelated to amino acid metabolism such as galactosaemia, hereditary fructose intolerance, and Wilson's disease.

1.2.6 SCREENING FOR INBORN ERRORS OF AMINO ACID METABOLISM

Newborn Screening: It is a common trend in many parts of the world to carry out early screening of infants for inborn errors of metabolism [19]. The trend has gained great momentum in recent years because it has been assumed that discovery of such abnormalities in the newborn period will allow the physician to initiate treatment so that the potential harm which would result from biochemical imbalance can be offset. The beneficial effect of the low-phenylalanine diet in phenylketonuria [20,21,22], of a milk free diet in galactosaemia [23,24], and of thyroid replacement therapy in several forms of cretinism have been repeatedly documented [25]. The neurological damage associated with maple syrup urine disease (MSUD), which leads either to death in infancy or to severe mental defect, can be prevented by the administration of a diet low in the branched-chain amino acids leucine, isoleucine and valine [26,27,28]. Homocystinuria, a disease which causes mental defect, dislocation of the

ocular lenses, skeletal malformations, and intravascular thromboses respond well to a diet low in methionine and supplemented with cystine, if these regimen is instituted early in infancy [29].

The primary role of screening would then appear to be preventive [30,31,32]. However other genetically determined biochemical diseases, characterized by the development of mental deficiency, for which no treatment is available, early diagnosis of this disorders is almost as important as for those conditions which are now treatable. If the diagnosis can be established by laboratory methods in early infancy long before mental retardation or characteristic physical signs appear, suitable genetic counselling of the baby's parents may prevent the birth of further defective children in that family [29,30].

Yet there are also other desirable objectives. Early detection of poorly understood diseases can provide an opportunity to study them and to interpret their pathophysiology; previously unknown problems may be discovered; and information about the ecology of human biochemical processes can be obtained [14].

Heterozygote Screening: Because the majority of inborn errors of amino acid metabolism are inherited as autosomal recessive traits, it is important to make the correct diagnosis and to identify heterozygotes in the family for counselling and for future family planing, regardless of whether or not treatment is effective for the homozygotes.

Investigation of high risk groups: Screening has also been conducted in institution for mentally retarded where there have been several reports of a high incidence of metabolic disorders. Most of the metabolic conditions revealed by these institutional studies have been

untreatable, but have pointed the way to better investigation of other patients in the future.

Although many studies showed that the prevalence of the hereditary aminoacidurias among livebirths and in general population is usually low, there are still significant regional and ethnic variations in the frequency of some conditions [33]. The prevalence of phenylketonuria was found to be high in Caucasian [34] while low in Negroes [35]. The neonatal screening program in Japan showed high incidence of histidinemia [34]. Homocystinuria was found to be the commonest disorder in North India [36]

1.2.7 ANALYSIS OF AMINO ACIDS

The analysis of amino acids play an important role in present day biochemistry and clinical chemistry research. Apart from the determination of the amino acid composition and sequence of proteins and peptides, the free amino acid content of physiological material such as blood, urine, cerebrospinal fluid, amniotic fluid and tissue is important in the diagnosis of inborn errors of amino acid metabolism, renal hyperaminoacidurias, malnutrition, collagen disease (hydroxyproline) and a few other diseases which do not seem to have an abnormal amino acid metabolism.

The technology of amino acid analysis is, in many ways, responsible for the present interest in disease of amino acid metabolism. The technology is ubiquitous and it determines the clinical investigation of patients. Diseases of amino acid metabolism have been the frequent target of mass screening programs because many of the available screening methods were designed to detect biochemical imbalance of amino acids in physiological fluids.

Many methods for analysis of amino acid in urine and blood have been described. These include paper [37,38] or thin layer chromatography [39,40], high voltage electrophoresis [41], microbiological assays, enzyme analysis and a number of chemical tests [1,29,39,42,43,44,45,46,47]. Automatic amino acid analyzer, gas chromatography (GC), mass spectrometry and high performance liquid chromatography (HPLC) are useful for quantitative identification of amino acids in both urine and blood [48].

Paper chromatography and chemical tests are suitable for screening a large number of patients. Chemical methods are the most important for investigating the metabolism of a particular amino acid. One- and two-dimensional chromatography are often effective for analysis of complex mixtures of amino acids such as urine. The advantage of chromatography techniques over the chemical methods is that one test can be used to detect several different compounds, and abnormal concentrations of one amino acid can be compared to those of others. The disadvantages are that the results are qualitative and demand considerable experience in their interpretation.

1.2.8 INBORN ERRORS OF METABOLISM AND MENTAL RETARDATION

Mental retardation, according to the American Association of Mental Deficiency (AAMD), refers to significantly sub-average intellectual functioning, existing concurrently with deficits in adaptive behaviour and manifest during the developmental period. Adaptive behaviour is measured by the responsibility, independence and social skills exhibited by a subject compared with those expected for his/her age. In gauging intellectual functioning, the intelligence quotient (IQ) is quite often taken as major criteria and is measured by a performance below two standard deviations from the mean on standardized tests [49].

Although IQ criterion is useful in the selection of patient material, in our situation it will not help since locally adaptive tests are not available. In such situation Egdell and Stanfield [50] suggested guidelines are applicable for the assessment of mental retardation. It was based on a combination of a) delay in milestone achievement- namely sitting, walking, speech (single words or short sentences); b) parental impression of less than expected social achievement; c) total clinical impression at interview and examination; d) school report (only occasionally available).

Many causes of mental retardation are unknown. In general, causes of mental retardation are being classified as being environmental, genetic or multifactorial (involving genetic-environmental interaction). About 50% of the cases of mental retardation can be attributed to genetic factors [49]. These include metabolic disorders. Many of these metabolic diseases are associated with mental retardation [51]. Among these phenylketonuria [52,53], homocystinuria [54,55], hyperglycinemia (non-ketotic form) [56,57] and galactosaemia [52] are responsible for the more severe degrees of mental retardation. However, there is no specific characteristic or feature of mental retardation that points to an inborn errors of metabolism. Specialized investigations are needed to identify many of the conditions precisely.

The association between mental retardation and inborn errors of metabolism continues to attract the interest of biochemical researchers. Thus a number of investigations had been carried out in many countries for the establishment of the frequency and distribution of inborn errors of metabolism associated with mental retardation [4,58,59,]. Others have established national genetic centres aimed at preventing the problems of congenital and hereditary disorders by identification of high risk families or groups of individuals by way of screening tests [60,61].

Studies on the prevalence of mental retardation in Ethiopia are rare. Tekle-Haimanot et.al. [62] in their study of neurological disorders in rural central Ethiopia, reported the prevalence rate of mental retardation to be 169/ 100, 000. However, the association of inborn errors of metabolism to the reported prevalence has not been studied.

2. OBJECTIVE

The objective of this work was to study the occurrence of biochemical disorders, mainly inborn errors of amino acid metabolism that might be associated with mental retardation.

3. MATERIALS AND METHODS

Mentally retarded subjects at special schools, Care Taking Centres and households were used as study population. When the study was initiated, no information was available on the etiology of the mental retardation of the subjects. Therefore, all the subjects at the institutes or households would form the clinical material for the study.

Urine specimens were used to study the metabolic disorders of amino acids. Urine specimens collected from these subjects were analyzed using a series of chemical tests (Table 2) in order to exclude obvious abnormalities such as phenylketones, sulphur containing amino acids, tyrosine and tyrosine metabolites, keto-acids, and excess reducing substances.

Ferric chloride test is positive when the urine contains phenylpyruvic acid. This test can detect phenylketonuria, histidinemia and tyrosinemia.

Cyanide nitroprusside test is positive when the urine contains excess cystine or homocystine. This test can detect cystinuria, homocystinuria and generalized aminoaciduria.

Nitrosonaphthol test is positive when tyrosine is present in the urine. This test can be used to detect tyrosinemia and amino acid malabsorption. It is a non-specific screening test and should be confirmed by paper chromatography or quantitative serum assay of tyrosine.

2,4-Dinitrophenylhydrazine test is used to indicate the presence of α -keto amino acids in urine. A positive result is seen with maple syrup urine disease (MSUD) and possibly in

Table 2. Chemical tests used in the study

Procedure	Detectable Substance	Suspected disease if substance is present
1. Ferric Chloride test	Phenylpyruvic acid, Imidazolpyruvic acid	Phenylketonuria, histidinemia, tyrosinemia
2. Cyanide Nitroprusside test	thiol-and disulphide substances, cystine, cysteine, homocystine, homocysteine	Cystinuria, homocystinuria, generalized aminoaciduria
3. Nitrosonaphthol test	Tyrosine, <i>p</i> -hydroxyphenylpyruvic acid, <i>p</i> -hydroxyphenyllactic acid /or <i>p</i> -hydroxyphenyl acetic acid	Tyrosinemia, Amino acid malabsorption
4. 2,4-dinitrophenylhydrazine test	α -Ketoacids, homogentisic acid	Phenylketonuria, histidinemia, maple syrup urine disease, tyrosinemia
5. Benedict's test	Reducing substances	Galactosaemia, hereditary fructose intolerance, pentosuria
6. Sulfosalicylic acid test	Protein	Proteinuria, useful to indicate galactosaemia, Lowe's syndrome
7. Ninhydrin test	Excess amino acids	Generalized aminoaciduria
8. Cuprizone test	Histidine	Histidinuria

phenylketonuria, histidinemia and methionine malabsorption. The test is also positive with ketonuria due to other inherited diseases and other causes. A Preliminary screening test for ketones should be performed.

Benedict's test is used to indicate the presence of abnormal amount of glucose or other reducing sugars. If the test is positive, paper chromatography for sugars should be done, and this procedure can detect galactosaemia, hereditary fructose intolerance, and pentosuria.

Sulfosalicylic acid test is a test for urine protein. It is likely to be positive in untreated galactosaemia and in Lowe's syndrome (cerebro-oculo-renal syndrome). The test is also useful as an indication of many forms of renal disease.

Cuprizone test is positive when histidine is present in the urine in excessive amount, i.e. exceeding 60 mg/100 ml.

Ninhydrin test is positive in many different diseases involving aminoaciduria, but is especially useful for detection of generalized aminoacidurias. Positive urine specimens should be examined further by paper chromatography for amino acids. A very concentrated urine may give a false positive ninhydrin screening test. Conversely, a very dilute urine may contain a significant excess of one amino acid and yet fail to yield a purple color in 5 minutes.

In addition, urinary excretion of amino acids and reducing sugars were studied using one-dimensional paper chromatography (PC).

3.1 SPECIMEN COLLECTION

Urine specimens in 12 hour portions or early morning were collected in plastic bottles (1 lt) containing 1-2 ml toluene or thymol as preservative. The specimens were shipped as soon as possible to the laboratory. At the laboratory about 50 ml sample was taken and divided into two portions; one used for analysis and the other kept frozen at -25°C for control analysis.

3.2 Laboratory Procedures

i) Ferric Chloride Test

Reagent Preparation: The reagent was made by dissolving 1.0 g of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and 1 g of ferrous ammonium sulphate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) in 100 ml of 0.02 N HCl. The reagent appears to be stable at room temperature indefinitely.

Procedure: The test was performed by placing 1 ml of ferric chloride in a test tube, adding 10 drops of urine, and mixed by shaking. The resulting color was observed within two to three minutes. A green color indicates positive result.

ii) Cyanide-Nitroprusside Test

Reagent Preparation: a) The sodium cyanide reagent was prepared by dissolving 5.0 g of sodium cyanide in 100 ml of water. b) The sodium nitroprusside reagent was prepared by dissolving 5.0 g of sodium nitroprusside ($\text{Na}_2\text{Fe}(\text{CN})_3 \cdot \text{NO} \cdot 2\text{H}_2\text{O}$) in 100 ml of water. The reagents were then stored in a refrigerator to prevent gradual decomposition of these compounds in aqueous solution. For best results, fresh sodium cyanide solution was prepared once every month.

5 mg cystine in 10 ml 0.1 N HCl diluted to 100 ml with normal urine was used as a positive control.

Procedure: The test was performed by placing 1 ml of urine in a test tube and adding 0.4 ml

of sodium cyanide solution. The content of the tube was mixed by agitation, and allowed to stand for 5 minutes at room temperature. One drop of the sodium nitroprusside solution was then added and the tube was shaken. An immediate pink to beet-red color indicated a positive test.

iii) Nitrosonaphthol Test

Reagent Preparation: a) 2.63 N nitric acid was prepared by adding one part of concentrated nitric acid to five parts of water. b) The sodium nitrite solution was prepared by dissolving 2.5 g of sodium nitrite in 100 ml of water. c) The nitrosonaphthol reagent was prepared by dissolving 100 mg of 1-Nitroso-2-naphthol in 100 ml of 95% ethanol. Reagents b and c were stored in the refrigerator, while the nitric acid reagent was kept at room temperature.

Procedure: The test was performed by placing 1 ml of the 2.63 N nitric acid in a test tube. One drop of the sodium nitrite reagent was added, followed by 0.1 ml of the nitrosonaphthol reagent. The content of the tube was agitated and without undue delay, three drops of urine were added and the content of the tube was again mixed by agitation. The development of an orange-red color within two to five minutes indicate a positive test, while persistence of the original yellow color indicate a negative test.

iv) 2,4-Dinitrophenylhydrazine Test

Reagent Preparation: Dinitrophenylhydrazine solution was prepared by dissolving 100 mg of 2,4-Dinitrophenylhydrazine in 100 ml of 2 N HCl. The reagent was stored in a brown bottle in the refrigerator.

25 mg of ketoglutaric acid in 100 ml normal urine was used as a control.

Procedure: 10 drops of reagent was added to 1 ml of clear (the supernatant centrifuged) urine in a test tube. The content of the tube was mixed by agitation, and was allowed to stand for 10 minutes at room temperature. The appearance of a light yellow cloudy precipitate in the tube indicates a positive test and suggests the need for a more detailed examination of the urine for α -keto acids.

v) Benedict's Test

Reagent Preparation : a) Copper sulphate solution was prepared by dissolving 17.3 g of copper sulphate ($\text{CuSO}_4 \cdot 3\text{H}_2\text{O}$) in 100 ml of water; b) Citrate-carbonate solution was prepared by dissolving 173 g of sodium citrate and 100 g of anhydrous sodium carbonate in 700 ml of boiling water. The solution was filtered and the volume of the filtrate was adjusted to 850 ml with water; c) The citrate-carbonate solution (b) was poured into a large beaker, and with constant stirring the copper sulphate solution (a) was slowly added to it. The volume was then adjusted to 1000 ml with water. The solution was stable indefinitely at room temperature.

Procedure: The test was performed by pipetting 1 ml of the Benedict's solution into a test tube, adding three drops of urine and mixing and heating the tube in a boiling water bath for not less than five minutes. A yellow orange or red precipitate indicates a positive test.

vi) Sulfosalicylic acid

Reagent Preparation: The reagent was prepared by dissolving 20 g of sulfosalicylic acid in

100 ml of water. The reagent was stable indefinitely at room temperature.

Procedure: The test was performed by adding three drops of the 20% sulfosalicylic acid to 1 ml of clear urine in a test tube. A cloudy precipitate indicates an abnormal amount of protein in the urine.

vii) Ninhydrin Test

Reagent Preparation : The ninhydrin reagent was prepared by dissolving 1 g of ninhydrin in 500 ml of 95% ethanol. The solution was kept in the refrigerator when not in use.

Procedure: The test was performed by placing 1 ml of the ninhydrin reagent in a test tube and adding three drops of urine. The content of the tube was mixed by agitation. The tube was then allowed to stand at room temperature and was examined after two and five minutes. The presence of a distinct blue or purple color, particularly in two minutes, indicates that the urine may contain an excessive amount of one or more amino acids.

viii) Cuprizone Test

Reagent Preparation: a) copper solution was prepared by mixing 0.5 parts of 0.02 M copper sulphate, 1.56 parts of 1 M Sodium citrate and 250 parts of 0.1 M tris (hydroxymethyl)aminomethane (pH 7.4); b) Cuprizone reagent was prepared by mixing 1 g of cuprizone (Oxalic bis(cyclohexylidenehydrazide)), 0.5 ml of ethyl alcohol, and 0.5 ml of water.

Procedure: Four ml of the copper solution, 0.2 ml of urine and 0.25 ml of cuprizone solution

were added sequentially with mixing in a test tube. The test tube was allowed to stand at room temperature for 5 minutes. If the resulting solution was colorless or only very slightly blue tingled, the result was considered positive. The concentration of histidine in such urine is very likely to be greater than 60 mg/100 ml, the patient may have a significant amount of histidinuria and should be examined for elevated plasma histidine.

ix) Amino Acid paper Chromatography (PC)

Preparation of standard amino acid solution: Solution A: 5 mmol/l each of leucine, phenylalanine, tryptophan, valine, proline, threonine, glycine, aspartic acid, and lysine in 0.1 molar HCl was prepared. Solution B: 5 mmol/l each of isoleucine, methionine, tyrosine, alanine, glutamic acid, serine, arginine, histidine and cystine in 0.1 molar HCl was prepared.

Preparation of Ninhydrin Reagent: The ninhydrin reagent for spray of amino acid chromatogram was prepared by dissolving 0.2 g ninhydrin in 100 ml acetone.

Procedure: 10 ml aliquots of undesalted filtered urine and standard amino acid solutions were spotted, 2.5 cm apart and 3 cm from the edge, on a 28.5 x 32 cm sheet of Whatman No 3 paper. Two to four papers were placed at once in a tank for ascending chromatography. These were developed overnight in butanol: acetic acid: water (12:3:5 by volume) solvent mixture. The papers were removed from the tank, and air dried. The detection of amino acids on chromatogram was carried out by spraying the paper with ninhydrin reagent. The chromatogram was allowed to dry at room temperature, and then heated for 5-10 minutes in oven at 90°C.

x) Reducing Sugar paper Chromatography

Preparation of standard sugar solution: Standard solution of galactose was prepared by dissolving 200 mg of galactose in 100 ml 10 % (v/v) aqueous isopropanol.

Preparation of Aniline-phosphate reagent: This reagent was used for spray of reducing sugar chromatogram. The reagent was prepared as follows. To 20 ml aniline, 200 ml water, 180 ml glacial acetic acid and 10 ml of concentrated phosphoric acid were added with mixing after each addition. Just before use 2 volumes of this mixture was diluted with 3 volumes of acetone by mixing. The reagents were stored at 4°C.

Procedure: The papers with the urine specimens and standard sugar solution of galactose prepared in the same manner were developed for the reducing substances with ethyl acetate:pyridine:water (12:5:4 by volume) and stained with aniline phosphate reagent. The chromatogram was allowed to dry at room temperature, and then heated for 5 minutes in oven at 105°C to develop the color.

4. RESULTS

4.1 STUDY POPULATION

A total of 345 (233 males and 112 females) mentally retarded subjects were studied. The study population was identified by the survey conducted in the first phase of the study. Information regarding the condition of the patient and drug intake was recorded. Epilepsy, speech defect, growth retardation, and skeletal deformity were found to be some of the conditions associated with the patients. Some showed signs of Down's syndrome and microcephaly.

The age and sex distribution of the subjects are shown in Fig 8. Most of our subjects were between 6-20 years of age.

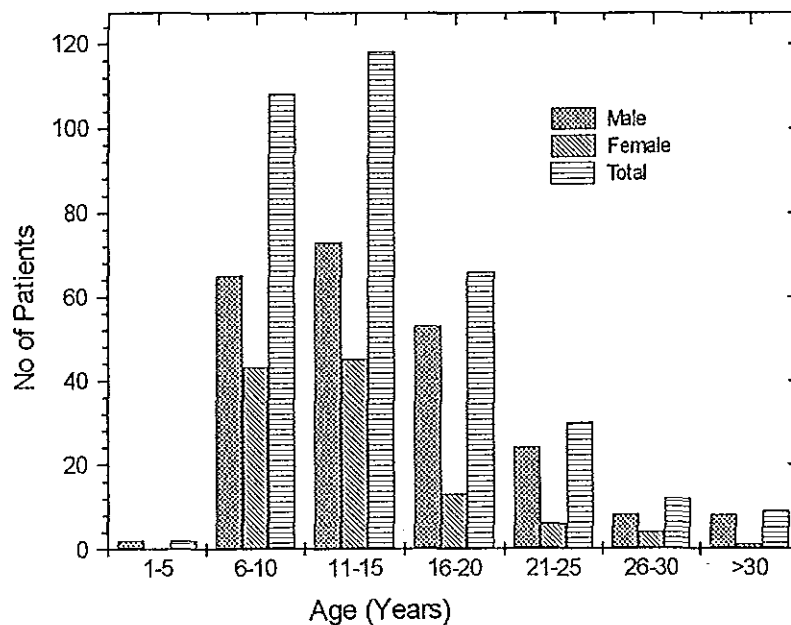


Fig. 8 Age and sex distribution of subjects.

Out of the 345 mentally retarded subjects 263 were from Addis Ababa. These were identified by survey conducted in the first phase of the study. Seven institutions were involved in the survey.

- i. Ethiopian Evangelical church: Leteresut Project for Mentally Retarded Children (Mekenisa and Kazanchiz)
- ii. Integrated Holistic Approach Urban Development Project: Special Unit for Mentally Retarded (Higher (H) 3, Kebele (K) 41)
- iii. Concern: Special Unit for Mentally Retarded (H.4 K.37)
- iv. Kokebe Tsebah Elementary School: Special Unit for Mentally Retarded
- v. Yekatit 23 School: Special Unit for Mentally Retarded
- vi. Missionary of Charity (Sisters) (H.13 K.O3)
- vii. Missionary of Charity (Brothers) (H.10 K.22)

The first three are non-governmental schools, the next two are governmental school and the last two are residential centres for mentally retarded subjects.

82 mentally retarded subjects (59 males and 23 females) were from Butajira; a district situated 130 km south of Addis Ababa. These subjects were from the 103 cases identified by Tekle-Haimanot et.al. [62] during their study of neurological disorder at Butajira between 1986-88.

88 normal subjects (60 males & 28 females) were included to serve as control in the study. The age and sex distribution of the controls are shown in Fig 9.

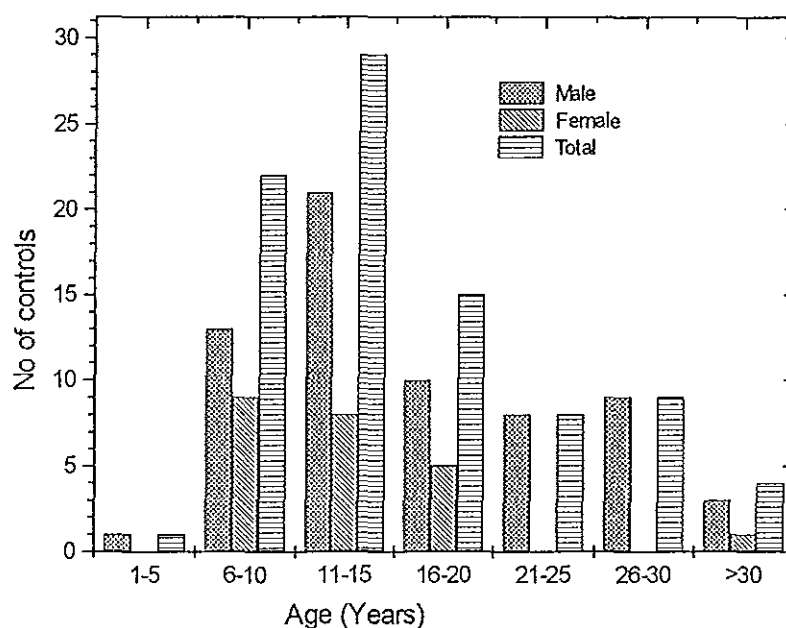


Fig. 9 Age and sex distribution of controls.

4.2 CHEMICAL TESTS

The results of chemical tests for mentally retarded subjects as well as normal controls are summarized in Table 3.

Out of 345 urine specimens of mentally retarded subjects studied by different chemical tests 3 (0.87%) were positive for cyanide-nitroprusside test, 12 (3.49%) were positive for nitrosonaphthol test, 5 (1.45%) were positive for sulfosalicylic acid test, 14 (4.05%) were positive for Benedict's test, and 30 (8.70%) were positive for ninhydrin test.

Out of the 3 positive cyanide nitroprusside urine specimens, one urine specimen was positive for ninhydrin test and one urine specimen was positive for ninhydrin and Benedict's tests.

Table 3 Results of chemical tests.

Procedure	Pos.	Patient (%) (345)*	Pos.	Control (%) (88)*
1. Ferric Chloride	-	-	-	-
2. Cyanide nitro- prusside	3	(0.87)	-	-
3. Nitrosonaphthol	12	(3.49)	2	(2.27)
4. Dinitrophenyl- hydrazine	-	-	-	-
5. Sulfosalicylic acid	5	(1.45)	-	-
6. Benedict's	14	(4.05)	3	(3.34)
7. Ninhydrin	30	(8.70)	4	(4.55)
8. Cuprizone	-	-	-	-

* The number of subjects examined is shown in the parentheses

Out of the 12 positive nitrosonaphthol urine specimens, 4 urine specimens were positive for ninhydrin test, one for ninhydrin and Benedict's tests and one urine specimen was positive for Benedict's test.

Nine urine specimens were not only positive for Benedict's test, but also positive for ninhydrin test.

In cyanide nitroprusside test a positive reaction was identified by the formation of magenta red color. A positive cyanide-nitroprusside test indicated an increased excretion of sulphur containing amino acids such as cystine or homocystine. The differentiation between cystine and homocystine was made by paper chromatography.

Positive nitrosonaphthol test appeared orange red in color. The reaction was non-reproducible

and was further studied by chromatography. Positive nitrosonaphthol test indicate that the urine contains excessive amount of one or more of the following compounds: tyrosine, p-hydroxyphenylpyruvic acid, p-hydroxyphenyllactic acid, or p-hydroxyphenylacetic acid.

In Benedict's test a positive reaction was yellow to brick red in color, depending upon the amount of reducing substances in the urine. Benedict's test was positive when the urine contains glucose, galactose, fructose, lactose, mannose or other reducing substances (in phenylketonuria, alkaptonuria and tyrosinemia).

In ninhydrin test the appearance of a distinct blue or purple color indicated that the urine may contain an excessive amount of one or more amino acids. The test was positive in many different diseases involving hyperaminoaciduria, but was especially useful for detection of generalized hyperaminoaciduria. Positive urine specimens were further examined by paper chromatography for amino acids.

There were no positive reactions with ferric chloride, 2,4-Dinitrophenylhydrazine and cuprizone tests.

Indican was detected by distinctly blue-colored urine.

Out of 88 urine specimens of normal control subjects 2 (0.87%) were positive for nitrosonaphthol test, 3 (3.34%) were positive for Benedict's test and 4 (4.55%) were positive for ninhydrin test.

4.3 PAPER CHROMATOGRAPHY (PC)

All 345 urine specimens from mentally retarded subjects were also analyzed with one-dimensional paper chromatography and abnormal amino acid pattern were observed in 9 urine specimens (Table 4). The chromatogram was interpreted by comparing the unknown with known amino acid standards. Abnormal patterns were seen for homocystine, tyrosine, and different excessive amino acids.

Table 4. Results of paper chromatography

Abnormal amino acid pattern	Positive	% (345)*
Homocystine	1	0.29
Tyrosine	1	0.29
Different amino acid	6	1.78
Unclassified	1	0.29
Total	9	2.61

* The number of subjects examined is shown in the parentheses

Pathological spot for homocystine was observed in one urine sample. The spot was identified after treatment of the urine with H_2O_2 and ammonium molybdate. Treatment of the urine converts homocystine to homocysteic acid which appeared on chromatogram [47]. Pathological spot for tyrosine was observed in one urine sample. Abnormal amino acid pattern involving neutral amino acids (two cases) and leucine, isoleucine, valine, phenylalanine, methionine and tyrosine (four cases) were observed. All urine specimens from normal subjects showed normal amino acid pattern.

17 Benedict positive urine specimens (14 specimens from patient + 3 specimens from normal

subjects) were further analyzed by one-dimensional paper chromatography for the presence of excessive galactose. None of these showed abnormal pattern. The chromatogram was interpreted by comparing the unknown with the known galactose standard.

The summary of the diagnostic biochemical tests in the positive cases (Table 5) were as follows.

One urine specimen was positive for cyanide-nitroprusside test and gave pathological spot on chromatogram for homocystine.

Two urine specimens which appeared positive for cyanide-nitroprusside test showed a generalized aminoaciduria by chromatogram.

Table 5. Results of chemical tests and paper chromatography.

Abnormal amino acid pattern	Results of chemical tests	Results of paper chromatography	No. of Pos. pt.	%
Homocystine	cyanide-nitroprusside +	spot for homocystine	1	0.29
Indican	all tests neg. blue colored urine	-	1	0.29
Tyrosine	Nitrosonaphthol+	spot for tyrosine	1	0.29
Excess amino acids	Ninhydrin +	spots for amino acids	6	1.78
Unclassified	Negative	spot	1	0.29
Total			10	2.91

Indican was neither detected by any of the chemical screening tests nor by paper chromatography. The urine was found to be blue on standing after several hours.

One urine specimen was positive for nitrosonaphthol test and gave pathological spot on PC for tyrosine.

Six urine specimens showed abnormal pattern for different amino acids on paper chromatography. All urine specimens were positive for ninhydrin test. Furthermore one urine specimen was positive for cyanide-nitroprusside test and another one for cyanide-nitroprusside and nitrosonaphthol tests. Two urine specimens were positive for Benedict's test and 2 urine specimens were positive for sulfosalicylic acid test.

5. DISCUSSIONS

Most of the chemical tests used in the study detect excess metabolites derived from elevated amino acids due to inborn errors of amino acid metabolism. These screening tests are sensitive, but not specific. The sensitivity of ferric chloride test is 5 to 10 mg per 100 ml. Beta-imidazolepyruvic acid is the substance other than phenylpyruvic acid that gives the typical green color with ferric chloride. Qualitative Benedict's test is sensitive not only to glucose but also to any reducing substances.

The cyanide-nitroprusside test is positive in the presence of excess homocystine. Positive tests will also be obtained in the presence of excess cystine. Acetone and other drugs may give false positive results.

One dimensional paper chromatography was carried out on the identical urine specimens used for chemical tests. Because the amino acids are not completely separated, the one dimensional paper chromatography is used mainly to confirm results obtained by the chemical tests and to identify abnormal amino acid patterns.

Every chromatogram include two amino acid standard mixture and one urine control. The amino acid standard mixture was used to check for the resolution of chromatography, the color reagent, and to the positions of some key amino acids. The urine control helps to distinguish abnormal from normal urinary amino acid patterns.

In this study chemical tests in combination with one dimensional paper chromatography were

used in the detection of metabolic abnormalities in urine. The relationship between positive chemical test and pathological chromatograms is presented in Fig 10.

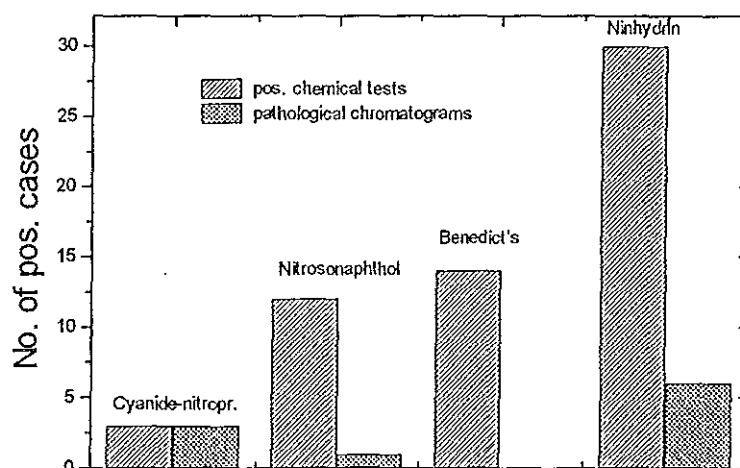


Fig. 10 Relationship between positive chemical tests and positive pathological chromatograms.

It is evident that in most urine specimens which gave one or more positive chemical tests, a hyperaminoaciduria or abnormal pattern of amino acids have been also demonstrated. All urine specimens which gave negative screening tests were also negative in chromatography.

The amino acid disorders detected using the combination of chemical tests and one dimensional paper chromatography is shown in Table 6.

Positive cyanide nitroprusside test and pathological spot for homocystine on PC probably suggested that the patient may had **homocystinuria**. Homocystinuria may be caused by a deficiency of cystathionine β -synthase, but it is now apparent that homocystine accumulation can be produced by either acquired or inherited blocks in the methyltetrahydrofolate-homocysteine methyltransferase reaction as well [13]. Chemically, cystathionine β -synthase

deficiency is characterized by elevated plasma concentrations of methionine and homocystine and by excretion of homocystine in the urine. Therefore further confirmation by blood analysis is required.

Table 6. Metabolic disorders detected in the present study.

Metabolic disorder	No. of positive Subjects	%
Homocystinuria	1	0.29
Indicanuria	1	0.29
Amino acid malabsorption	1	0.29
Generalized Aminoaciduria	6	1.78
Unclassified	1	0.29
Total	10	2.91

It is important to distinguish between homocystinuria and cystinuria. These conditions both give a positive urinary nitroprusside test and result in the excretion of the mixed disulfides of homocysteine and cysteine. Selective excessive excretion of cystine, lysine, arginine, and ornithine is observed in cystinuria but not in homocystinuria and can be detected easily by paper chromatographic or electrophoretic techniques. Homocystinuria is featured clinically by dislocated lenses, mental retardation, and cutaneous flushing.

One urine sample turned blue on standing and is believed to be a sign of Indicanuria. The blue coloration was caused by the dye indigotin, an oxidation product of indican. A specific defect in tryptophan absorption is postulated as the basic underlying abnormality [63].

Positive nitrosonaphthol test and pathological spot for tyrosine on chromatogram probably

suggested that the patient had **amino acid malabsorption** [57] and indicates the need for more definite tests of blood and urine. A patient with an amino acid malabsorption syndrome was reported by Reinecke et.al. [59]. Amino acid malabsorption is characterized by persistent high excretion of tyrosine catabolites. This was originally interpreted as indicating persistent hypertyrosinemia.

Although sulfosalicylic acid test was used to detect proteinuria (the presence of detectable amounts of protein in the urine) it is likely to be positive in untreated galactosaemia and in Lowe's syndrome (cerebro-oculo-renal syndrome). A Positive sulfosalicylic acid test was also useful as an indication of many forms of renal diseases [29]. Therefore, further study in the positive cases is required.

Positive ninhydrin test and pathological spots of amino acids on chromatogram probably suggest the presence of **Generalized Aminoaciduria**. A condition in which generalized aminoaciduria occurs in association with mental retardation and clinical and chemical phenomena has been reported by Lowe et.al [64]. Generalized aminoaciduria is usually accompanied by proteinuria, and occasionally by glycosuria.

5.1 PREVALENCE OF METABOLIC ABNORMALITIES

In the present study metabolic abnormalities were detected in 2.91% of our subjects (Table 6). Homocystinuria, Indicanuria and amino acid malabsorption syndrome each observed in 0.29% of subjects. Metabolic screening of 2560 children with psycho-motor retardation in North India [36] showed abnormal pattern of urinary amino acid excretion in 6.13% of patients. Another study among mentally retarded children by Davi et. al. [65] from India have reported

2.4% of children with disorders of amino acid metabolism. The present study showed comparable results with that reported by Davi and et.al..

Generalized (renal) aminoaciduria was detected in 1.78% of our subjects. The report from North India [36] showed that 3.52% of patients have Generalized aminoaciduria. Reinecke et.al. [59] had reported that 0.77% of the patients have mild generalized aminoaciduria after study of 1568 institutionalized mentally retarded subjects. The results of our findings showed less occurrence of generalized amino aciduria compared to that reported for North Indian but much greater than that reported by Reinecke et.al..

Further comparison of our results with similar surveys reported from South Africa by Reinecke et.al. [59] and Henderson et.al. [35] is shown in Table 7. Our findings differ from those obtained in the two surveys with regard to the prevalence of phenylketonuria. Henderson et. al. reported 3 patients (0.28%) and Reinecke et. al. reported 2 patients (0.13%). In our study no phenylketonuria cases were observed. Of course our sample size was small. Normally the incidence of phenylketonuria in the general population was 1 in 10,000 [35]. The average worldwide incidence of phenylketonuria among institutionalized mentally defective was 0.64% [66]. Carson and Neill [54] reported the prevalence of 2.4% of phenylketonuria among mentally backward individuals in Northern Ireland while in North India found to be 0.08% [36]. Screening of newborns in the USA has revealed a very low occurrence of phenylketonuria among Negroes [35].

The survival rate of phenylketonuric patients was reported by Lang [67]. According to Lang, the ages of 500 reported patients indicate that half died by 20 years of age, and three-quarters by 30 years of age.

Table 7. Comparison of results of the present study with other similar studies .

Metabolic disorder	Henderson et.al. No. of pt. (%) (1087)*	Reinecke et.al No. of pt.(%) (1568)*	Present study No. of pt.(%) (345)*
Amino acid mal-absorption	-	1 (0.06)	1 (0.29)
Glucosuria	-	3 (0.19)	-
Cystinuria	2 (0.18)	-	-
Hartnup disease	1 (0.09)	-	-
Histidinuria	-	2 (0.13)	-
Homocystinuria	-	-	1 (0.29)
Indicanuria	-	-	1 (0.29)
Generalized aminoaciduria	-	12(0.77)	6 (1.78)
Phenylketonuria	3 (0.28)	2 (0.13)	-
Unclassified	-	-	1 (0.29)
Total	6 (0.55)	20(1.28)	10(2.91)

* The number of subjects screened in each study is shown in parentheses.

The other difference is in the case of homocystinuria. No figures were reported by the two studies. Our results showed one homocystinuric patient (0.29%). Homocystinuria is the most common disorder in North India [36].

6. RECOMMENDATIONS

Based on the present study, the following recommendations are made.

1. Further studies with large number of study population would be required to determine the occurrence of inborn errors of amino acid metabolism among mentally retarded subjects.
2. Referral laboratories should be organized for screening of inborn errors of metabolism. These laboratories apart from giving the laboratory service, they will serve as a source of information for researchers.
3. Screening for inborn errors of metabolism in newborns should be carried out.
4. The tests used in this study are only screening tests, and abnormal results are not diagnostic of any disease. All abnormal results must be substantiated by more precise laboratory evaluation and must be interpreted in the light of the clinical state of the patient.

7. REFERENCES

1. Henry, J.B. *Clinical Diagnosis and Management by Laboratory Methods*, W.B. Saunders Company: Philadelphia, 18th ed., 1991.
2. Whiney, E.; Hamilton, M. *Understanding Nutrition*, West Publishing Company, St. Paul, 1977.
3. Hsia, D.Y.; Inoune, T. *Inborn Errors of Metabolism*, Part 1: Clinical Aspect, Year Book Medical Publishers, Inc.: Chicago; 1969.
4. Renuart, A.M. *New Eng. J. Med.* 1966, 274, 387.
5. Cori, G.T.; Cori, C.F. *J. Biol. Chem.* 1952, 199, 661.
6. Jervis, G.A., in Bondy, P.K.; Rosinberg, L.E. *Metabolic Control and Disease*, W.B. Saunders Company: Philadelphia, 8th ed.; 1980.
7. Trauner, D.A. *Childhood Neurological Problems: A text Book for Health Care Professionals*, Year Book Medical Publishers, Inc.: Chicago; 1979.
8. Segal, S.; Cuatrecasas, P. *Am. J. Med.* 1968, 44, 340.
9. Tedesco, T.A.; Mellman, W.J. *J. Clin. Invest.* 1969, 48, 2390.
10. Laster, L.; Spaeth, G.L.; Mudd, S.H.; Finkelstein, J.D. *Ann. Intern. Med.* 1965, 63, 1117.
11. Mudd, S.H.; Finkelstein, J.D.; Irreverie, F.; Laster, L. *Science* 1964, 143, 1443.
12. Penrose, L.; Quastel, J.H. *Biochem. J.* 1937, 31, 221.
13. Folling, A. In Bondy, P.K.; Rosinberg, L.E. *Metabolic Control and Disease*, W.B. Saunders Company: Philadelphia, 8th ed.; 1980.
14. Bondy, P.K.; Rosinberg, L.E. *Metabolic Control and Disease*, W.B. Saunders Company: Philadelphia, 8th ed.; 1980.
15. Nora, J.J.; Fraser, F.C. *Medical Genetics: Principles and Practice*, Lea and Febiger,

Philadelphia, 4th ed. 1994.

16. Armstrong, M.D.; Tyler, F.H. *J. Clin. Invest.* **1955**, *34*, 565.
17. Knox, W.E. *Pediatrics* **1960**, *26*, 1.
18. Sriver, C.R.; Katz, L.; Clow, C. *Can. Med. Ass. J.* **1968**, *98*, 125.
19. Sriver, C.R. *Pediatr. Clin. North. Am.* **1965**, *12*, 807.
20. Blainny, J.D.; Gulliford, R. *Arch. Dis. Child.* **1956**, *31*, 452.
21. Woolf, L.I.; Griffiths, R.; Moncrieff, A.; Coats, S.; Dillistone, F. *Arch. Dis. Child.* **1958**, *33*, 31.
22. Brimblecombe, F.S.W.; Stoneman, M.E.R.; Maliphant, R. *Lancet* **1959**, *1*, 609.
23. Holzel, A.; Komrower, G.M.; Schwarz, V. *Am. J. Med.* **1957**, *22*, 703.
24. Komrower, G.M. Lee, D.H. *Arch. Dis. Child.* **1970**, *45*, 367.
25. Refetoff, S. *Med. Clin. North. Am.* **1975**, *59*, 1147.
26. Westall, R.G. *Arch. Dis. Child.* **1963**, *38*, 485.
27. Snyderman, S.E. et.al. *Pediatrics*. **1964**, *34*, 454.
28. Committee for Improvement of Hereditary Disease Management, *Can. Med. Ass. J.* **1976**, *115*, 1005.
29. Perry, T.L.; Hansen, S.; MacDougall, L. *Can. Med. Ass. J.* **1960**, *95*, 89.
30. Wu, J.T.; *Ann. Clin. Lab. Sci.* **1991 Mar-Apr**, *21(2)*, 123.
31. Holmgren, G. *Umea University Medical Dissertations*, No 16; 1974.
32. Seashore, M.R. *Semin. Perinatal* **1990 Dec**, *14(6)*, 431.
33. World Health Organization Technical report series, **1968**, 401.
34. Tada, K.; Tateda, H. Arashima, S. et.al. *Eur. J. Pediatr.* **1984**, *142(3)*, 204.
35. Henderson, H.E.; Goodman, R.; Schram, J.; Diamond, E.; Daneel, A. *S. Afr. J. Med.* **1981**, *60*, 731.

36. Kaur, M.; Das, G.P.; Verma, I.C. *J. Inherit. Metab. Dis.* **1994**, *17*(2), 230.
37. Dent, C.E. *Biochem. J.* **43**: 169, 1948.
38. Efron, M.L.; Young, D.; Moser, H.W.; MacCready, R.A. *New Eng. J. Med.* **1964**, *270*, 1378.
39. Tietz, N.W. *Fundamentals of Clinical Chemistry*, W.B. Saunders Company: Philadelphia; 1982.
40. Cooper, G.R. (ed) *Standard Methods of Clinical chemistry*. Academic Press, New York, Vol. 7, 1972.
41. Holmgren, G.; Jeppson, J.O.; Samuelson, G. *Scand. J. Clin. Lab. Invest.* **1970**, *26*, 313.
42. Bradley, G.M. *Human Pathol.* **1971**, *2*, 309.
43. Dahlqvist, A. *Scand. J. Clin. Lab. Invest.* **1968**, *22*, 87.
44. Brand, E.; Harris, M.M.; Biloon, S. *J. Biol. Chem.* **1930**, *86*, 315.
45. Gerber, M.G.; Gerber, D.A. *Pediatrics* **1969**, *43*, 40.
46. Buist, N.R.M. *Brit. Med. J.* **1968** June **22**, 2(607), 745.
47. Buist, N.R.M.; Jhaveri, M. *J. Pediatr.* **1973**, *82*, 511.
48. Watanabe, Y.; Imai, K. *J. Chromatography* **1984** Aug 10, 309(2), 279.
49. Op't Hof, J. *S. Afr. Med. J.* **1983**, *63*, 8.
50. Egdeell and Stanfield in Tekle-Haimanot, R. et.al. *Umea University Medical Dissertations*, No. 293, 1990.
51. Reine, D.N. *Brit. Med. J.* **1972**, *2*, 329.
52. Pitt, D. *Med. J. Aust.* **1971**, *1*, 378.
53. Pertington, M. W. *Can. Med. Ass. J.* **1961**, *84*, 985.
54. Carson, N.A.J.; Neill, D.W. *Arch. Dis. Childh.* **1962**, *37*, 505.
55. Waisman, H.A. *Am. J. Dis. Child.* **1966**, *113*, 101.
56. Gerristen, T.; Kaveggia, E.; Waisman, H.A. *Pediatrics*. **1965**, *36*, 882.

57. Baumgartner, R.; Ando, T.; Nyhan, W.L. *J. Pediatr.* 1969, 75, 1022.
58. Bell, L.S.; Blair, W.C.; Lindsay, S.; Watson, S.J. *J. Pediatr.* 1950, 36, 427.
59. Reinecke, C.J.; Mienle, L.J.; Hitzeroth, H.W.; Op'thof, J. *S. Afr. Med. J.* 1983, 63, 14.
60. Hoffman, G.F. *Eur. J. Pediatr.* 1994, 153(suppl 1), S2.
61. Stanbury, J.B.; Wyngaarden, J.B.; Fredrickson, D.S. (eds) *The metabolic bases of Inherited Diseases*. McGraw-Hill Book Company, New York, 2nd ed., 1966.
62. Tekle-Haimanot, R. et.al. *Umea University Medical Dissertations*, No. 293, 1990.
63. Drummond, K.N.; Michael, A.F.; Ulstron, R.A. *Med. J. Am.* 1964, 37, 928.
64. Lowe, C.U.; Terry, M.; Maclachan, E.A. *Am. J. Dis. Childh.* 1952, 83, 164.
65. Davi, A.R.R.; Rao, N.R.; Bittles, A.H. *J. Med. Genet.* 1987, 24, 362.
66. Guthrie, R.; Susi, A. *Pediatrics* 1963, 32, 338.
67. Lang, H. in Stanbury, J.B.; Wyngaarden, J.B.; Fredrickson, D.S. (eds) *The metabolic bases of Inherited Diseases*. McGraw-Hill Book Company, New York, 2nd ed., 1966.

8. APPENDIX

Homocystinuria: A 9 years old girl and the younger of 2 siblings. Walked at the age of 4 years. The eyes were dislocated. Mental retardation, speech defect, and growth retardation were observed in this patient.

Indicanuria: A girl of 7 years old. Mental retardation, speech defect and growth retardation were the clinical symptoms observed in this patient.

Amino acid malabsorption: 18 years old male with mental retardation.

Generalized aminoaciduria: One girl and five boys of 10-20 years of age. All of them have mental retardation.