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ORIGINAL ARTICLE

A Novel Approach To The Definitive Diagnosis Of Enzymopathies

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ABSTRACT

Enzyme estimations are invariably carried out using synthetic chromogenic or fluorogenic substrates, which are structurally very simple as compared to the natural substrates. A defective enzyme resulting from mutation in the structural gene loses its activity on the natural substrates, leading to a genetic disease. Such an enzyme, at times, may still retain substantial catalytic activity on the synthetic substrate. If the enzyme activity falls within the normal range, the situation poses a problem in the diagnosis of the patient. To circumvent this situation, we have invented a novel approach which has enabled us to arrive at an unambiguous, definitive diagnosis of patients. Our novel approach will be presented here.

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Introduction

Mutations in the structural genes of enzymes lead to alterations in their catalytically active structure. These alterations in mutant enzymes result in their reduced (or no) activity on the synthetic as well as natural or in-vivo substrates [2], [8],[9],[10],[20]. The deficiency of an enzyme results in a genetic disease. Each enzyme has a normal range of activity. For example, Arylsulphatase **A** (EC 3.1.6.1) is considered normal if its activity falls between 200 and 600 units (nmoles / mg protein / hour). A person with enzyme activity between 200 and 600 units will therefore, be considered normal. A carrier of the disease is also defined in terms of a range of enzyme activity. For Arylsulphatase **A**, a carrier will be one whose enzyme activity falls between, let us say, 50 and 200 units. Any individual showing activity below 50 units will be considered as a patient. All of these ranges of activities used for defining a patient or a carrier or a normal individual are conventional and are solely based on the experience and the data of different laboratories over a number of years. There are no sharp cut-offs between the three categories of individuals. At times, the patients may have enzyme activities extending into the carrier range or even beyond into the normal range. Indeed, cases have been documented where a patient showing fulminant clinical manifestations of Metachromatic Leukodystrophy (MLD) has normal Arylsulphatase **A** activity [15]. Cases have also been documented, where an individual with a zero

Arylsulphatase **A** activity is normal on all counts [5].

In all these methods of diagnosis of enzymopathies in the prior art, the enzyme estimations are done by using synthetic chromogenic or fluorogenic substrates. A single point enzyme activity measurement is made at the end of the incubation period. The diagnosis of the patient is based solely on this single point estimation.

We present here a novel approach, henceforth referred to as **Apte procedure** [1], to circumvent this problem of ambiguity in the diagnosis. Our invention led to a differential and definitive diagnosis of the patient, the carrier and the normal individual.

Materials and Methods

Leukocyte Enzyme Estimation

Blood samples (10ml) were collected in heparinised bulbs from the patient, the patient's father and mother as carriers, and a normal individual to serve as a control. Leukocytes were obtained from each of these samples by following the procedure described by Hans Galjaard (7). Leukocyte lysates were prepared by sonication. The sonicates were centrifuged to get clear supernatants which were used for quantitative enzyme estimations using the appropriate chromogenic or fluorogenic substrate for each enzyme.

Urinary Enzyme Estimation

About 10 ml of midstream urine samples were collected from the patient, the patient's father and mother as carriers and a normal person. 5 ml from each urine sample was dialyzed against distilled water for 18 hours in cold. The dialyzed urine samples were centrifuged at 5000 rpm for 10 minutes to remove any suspended matter, after which they were used for the quantitative estimation of the enzyme.

Serum Enzyme Estimation

Blood samples (3ml) were collected in plain bulbs containing no anticoagulant. The blood was allowed to clot. The tubes were centrifuged at 3000 rpm and the clear supernatant serum was collected and used for the estimation of enzyme.

We describe below, the application of the **Apte procedure** to the study of one leukocyte and urinary Arylsulphatase **A** (EC 3.1.6.8) [4],[11],[12] and one enzyme in serum, Biotinidase (EC 3.5.1.12), as illustrative examples. All the enzymes were assayed according to the published procedures [7]. Arylsulphatase **A** was assayed by using p-nitrocatechol sulphate as the synthetic, chromogenic substrate [15], [7], [11]. Biotinidase was assayed using biotin-4-amidobenzoic acid as synthetic chromogenic substrate [18], [19]. All enzyme estimations were carried out at 37^0 C and one-point readings were taken at the end of the incubation period. The leukocyte lysates, the urine and the serum samples were stored at -10^0C after enzyme estimations. The enzyme estimations on the same samples were repeated every 24 hours for the next three days. The percentage of enzyme activity retained was calculated for each day, assuming the enzyme activity in each sample on the first day to be 100 %.

Results

Studies on one leukocyte enzyme, one urinary enzyme and one serum enzyme are presented here as illustrative examples.

Example 1

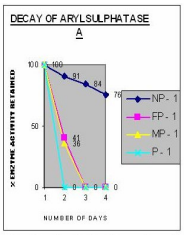
Deficiency of Arylsulphatase **A** causes Metachromatic Leukodystrophy (MLD, Mckusick 250100) in which the patient suffers from gait disturbance, mental regression, peripheral neuropathy, progressive dementia and muscle weakness. The enzyme was quantitatively estimated in the leukocytes of a 2-year-old patient (P-1), his father

(FP-1) and mother (MP-1) as carriers and 2-year-old normal child (NP-1), as described in the materials and methods. The enzyme activity was followed up for three consecutive days. The percentage of the enzyme activity retained on each day was calculated, assuming the enzyme activity on the first day of estimation to be 100%. The results of the enzyme estimations in all these samples for Arylsulphatase **A** are shown in [Table/Fig 1] and graphically in [Table/Fig 2].

(Table/Fig 1) Decay of Arylsulphatase A

	% ENZYME ACTIVITY RETAINED DAYS			
	1	2	3	4
NP-1	100 (412)	91(375)	84(346)	76(313)
FP-1	100 (87)	41 (36)	0	0
MP-1	100 (66)	36 (24)	0	0
P-1	100 (38)	0	0	0

Normal range of Arylsulphatase A activity = 200 – 600 units (nmoles/mg protein/hr). All values in the parentheses are actual readings.
NP-1 = Normal person 1 FP-1 = Father of patient 1
MP-1 = Mother of patient 1 NP-1 = Normal person 1



(Table/Fig 2) Decay of leukocyte Arylsulphatase A activity over a period of four days. The enzyme activity obtained on the first day was considered 100 %. The enzyme activities obtained on the 2nd, 3rd and the 4th day are plotted as percentage of the original enzyme activity retained on that day.

Example II

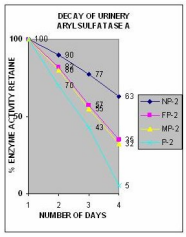
Deficiency of Arylsulphatase **A** causes MLD, as described before. This enzyme can also be measured in urine [4]. Urine samples of a 2-year-old patient **P-2** and the patient's father **FP-2** and mother **MP-2** as carriers and a normal 2-year-old child **NP-2** were collected and processed as described in the materials and methods. The enzyme activity was followed up for three consecutive days and the percentage of the enzyme activity retained on each day was calculated, assuming the activity on the first day to be 100%. The results of the enzyme estimations in all of these urine samples for Arylsulphatase **A** are shown in [Table/Fig 3] and graphically in [Table/Fig 4].

(Table/Fig 3) Decay of urinary Arylsulphatase A

SUBJECTS	% ENZYME ACTIVITY RETAINED NUMBER OF DAYS			
	1	2	3	4
NP – 2	100(7.4)	90(6.66)	77(5.7)	63 (4.66)
FP – 2	100(4.5)	82(3.69)	57(2.57)	35(1.58)
MP – 2	100(4.0)	80(3.2)	55 (2.2)	32(1.28)
P – 2	100(5.8)	70(4.06)	43(2.49)	5(0.29)

Normal range of urinary Arylsulphatase A activity ≥ 5 units (μgm/ml/hr). All values in the parentheses are actual readings.

NP – 2 = Normal person 2 FP – 2 = Father of the patient 2
MP – 2= Mother of the patient 2 P- 2 = Patient 2



(Table/Fig 4) Decay of urinary Arylsulphatase A activity over a period of four days. The enzyme activity obtained on the first day was considered 100 %. The enzyme activities obtained on the 2nd, 3rd and the 4th day are plotted as percentage of the original enzyme activity retained on that day.

Example III

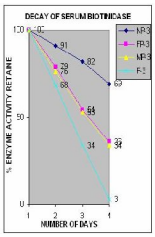
Defective Biotinidase activity causes Multiple Carboxylase Deficiency (McKusick 253260). This results in ataxia, muscular hypotonia, seizures, alopecia, periorificial cutaneous eruptions, and episodic metabolic acidosis. The enzyme Biotinidase was estimated in serum. Blood sera of a 6-month-old patient **P – 3**, the patient's father **FP –3** and mother **MP – 3** as carriers and a normal 6-month-old child **NP – 3** were collected as described in the materials and methods. The enzyme activity was followed up for three consecutive days and the percentage of the activity retained on each day was calculated, assuming the activity on the first day of assay to be 100 %. The results of the Biotinidase estimation in all the samples are shown in [Table/Fig 5]and graphically in [Table/Fig 6].

(Table/Fig 5) Decay of serum Biotinidase

SUBJECT	% ENZYME ACTIVITY RETAINED			
	NUMBER OF DAYS			
	1	2	3	4
NP – 3	100(6.4)	91(5.8)	82(5.25)	69(4.4)
FP – 3	100(3.2)	78(2.5)	53(1.7)	36(1.15)
MP – 3	100(3.5)	76(2.66)	53(1.86)	34(1.19)
P - 3	100(3.9)	68(2.65)	34(1.33)	3(0.12)

Normal range of serum Biotinidase = 4.3 – 7.5 units (nmoles/ml/min). All values in the parentheses are actual readings.

NP – 3 = Normal person FP – 3= Father of the patient 3
MP – 3 = Mother of the patient 3 P – 3 = Patient 3



(Table/Fig 6) Decay of serum Biotinidase activity over a period of four days. The enzyme activity obtained on the first day was considered 100 %. The enzyme activities obtained on the 2nd, 3rd and the 4th day are plotted as percentage of the original enzyme activity retained on that day.

Example IV

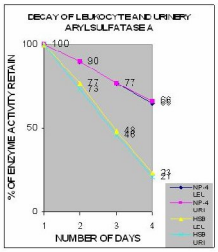
We now describe a case of a 56-year-old male patient, **HSB**, whose Arylsulphatase A activity was 400 units, which was well within the normal limits. However, the patient exhibited a classical picture of MLD with fulminant clinical manifestations such as ataxia, muscular hypotonia, dementia, etc. All of these clinical manifestations were progressive and had started at the age of 52 years. **HSB** was investigated before he came to us for various possible enzymopathies. However, he was not given any definitive diagnosis. We decided to apply the **Apte procedure** to measure the Arylsulphatase **A** activity in his leukocytes as well as in his urine sample. Leukocytes and urine sample from a normal person, **NP-4**, of the same age were also run together with **HSB**'s samples as controls. The results of this experiment are shown in [Table/Fig 7] and graphically in[Table/Fig 8]

(Table/Fig 7) Decay of leukocyte and urinary Arylsulphatase A

SUBJECT	% ENZYME ACTIVITY RETAINED			
	NUMBER OF DAYS			
	1	2	3	4
NP – 4 LEU	100(258)	90(232)	77(199)	65(168)
NP – 4 URI	100(8.6)	90(7.7)	77(6.6)	66(5.68)
HSB- LEU	100(400)	77(308)	48(192)	23(92)
HSB- URI	100(3.9)	73(2.85)	46(1.79)	21(0.82)

Normal range of leukocyte and urinary Arylsulphatase A activity is as in (Table/Fig 1) and (Table/Fig 3) respectively.

NP – 4 = Normal person 4 HSB = Patient
LEU = Leukocyte sample URI = Urine sample



(Table/Fig 8) Decay of leukocyte and urinary Arylsulphatase A activity over a period of four days. The enzyme activity obtained on the first day was considered 100 %. The enzyme activities obtained on the 2nd, 3rd and the 4th day are plotted as percentage of the original enzyme activity retained on that day.

Discussion

[Table/Fig 1] shows the decay of Arylsulphatase **A**, over a period of four days. The day on which the enzyme activity is measured first, was the first day. Arylsulphatase **A** activity came down to 76 % in the normal person (**NP-1**) on the fourth day, whereas the activity of this enzyme in the patient (**P-1**) went down to zero on the second day itself. The enzyme activities in the patient's father (**FP-1**) and mother (**MP-1**) who are carriers of the disease, became zero on the third day.

The example of the decay of Arylsulphatase **A** in urine shown in (Table/Fig 3) and (Table/Fig 4) is interesting, as it follows the same pattern of decay as that in the leukocytes. Normal subject, **NP-2**, retained 63% of his original enzyme activity as against the patient, **P-2**, who retains only 5%. The patient's father and mother, **FP-2** and **MP-2** respectively, retained 35% and 32% of their initial enzyme activity on the 4th day.

The example of the decay of Biotinidase is of particular interest. The enzyme

activities in both carriers of the disease **FP-3** and **MP-3,** , the father and the mother, were lower than the patient, **P-3**, to start with. However, the patient retained only 3% of his initial enzyme activity on the fourth day of the assay, whereas the patient's father and mother retained 36% and 34% of their initial enzyme activity, respectively. The normal person, **NP-3**, retained 69% of his initial Biotinidase activity.

Another interesting observation made, was in the decay of leukocytes and urinary Arylsulphatase **A** in the patient, **HSB**, and the normal person. The patient, **HSB,** had a much higher leukocyte Arylsulphatase **A** activity of 400 units, as compared to the corresponding control, **NP-4**, who had 256 units. The patient's urinary Arylsulphatase **A** was in the carrier range (3.8 units). The rate of decay of the leukocyte enzyme of the patient followed hand in hand with that of the urinary enzyme. The rates of decay of the leukocytes and the urinary Arylsulphatase **A** in the normal person were much lower than that of **HSB**.

The patient retained only 23% of the initial enzyme activity on the fourth day of the assay, whereas the normal person retained 66%, thus allowing us to differentiate and diagnose the patient with certainty. This problem had precluded confirmed diagnosis for **HSB** before we applied the **Apte procedure** to this case.

We carried out another interesting experiment to ascertain the validity of the **Apte procedure**. We had a patient, **MS**, who started showing the clinical manifestations of MLD at 46 years of age. The decay kinetics of his leukocyte and the urinary Arylsulphatase **A,** confirmed the diagnosis. He has a daughter. His wife was found to be unaffected for MLD, with her urinary Arylsulphatase **A** activity of 7.4 units with the decay kinetics, corresponding to that of a normal person. Thus, **MS** was a patient and his wife was normal. Therefore, their daughter was suspected to be a carrier. The urine samples of **MS**, his wife **BS** and their daughter **DS** were collected and processed as described in the materials and methods. The decay of Arylsulphatase **A** in these urine samples was followed up and is shown in [Table/Fig 9] and graphically in [Table/Fig 10]. The kinetics of the decay of the enzyme of the patient's daughter, **DB**, falls in between that of her father and the mother, thus confirming the diagnosis of **DB** as a carrier.

The **Apte procedure** was successfully applied to the prenatal diagnosis of a case of suspected MLD. The previous sibling had died with MLD at 3 years of age. The enzyme in the chorionic villi showed a normal rate of enzyme decay. Both parents showed the decay of the enzyme activity, which was consistent with their carrier status. A healthy child was born to the couple who is now a 12 year old, school going boy.

The question here is, why the enzymes in the patient, the carriers and the normal person showed different rates of decay of activity?

The amino acid sequence of an enzyme is the unique determinant of the three dimensional structure and, therefore, of its active state. A change of an amino acid due to a missence mutation will alter the structure and hence affect the activity. Such a structure will be fragile and will fall apart quite easily. It is also known that denatured proteins are excellent substrates for proteolytic enzymes. An enzyme with an altered, fragile structure will also form a better substrate for proteolysis than the enzyme in its natural, native state.

Studies on the inactivation of the highly purified bacterial α-galactosidase enzyme at the single molecular level have shown that thermal denaturation of the enzyme resulted in an all-or-none inactivation [13]. In contrast to this observation, it is shown that enzyme inactivation caused

by prolonged storage, results in a population of molecules with intermediate levels of activity ranging from five to one hundred percent of that present in the native α galactosidase molecule [14]. One of the hypotheses proposed for this observation was that the molecules of the aged enzyme are in a state of lower total free energy than those of the freshly synthesized enzyme. This implies that different active conformational states can be obtained with identical amino acid sequences. The rapid loss of enzyme activity in our experiments could be explained partially by this hypothesis, provided, it is proved that the number of intact molecules of the enzyme remain constant even on the fourth day.

Experiments on αgalactosidase were done with highly purified, homogeneous preparations. Our system is crude. It consists of the total leukocyte extract or the urine or the serum containing myriads of other components, including a large number of proteolytic enzymes. It is therefore, highly likely, that the loss of enzyme activity is due to the physical loss of the enzyme molecules and not due to their conformational alterations. The patient's enzyme would have had the most fragile structure, which would have made it most amenable to the proteolytic attack. The normal person would have had a tighter, native enzyme structure, making it less accessible to proteolysis.

All of the enzymes under the present study are multimeric and had a quaternary structure. Therefore, various possibilities of association of different subunits – native and the mutant ones – in carriers makes the picture even more complex [6]. If the hybrid structure assumed normal conformation, it would behave like the native enzyme. The decay of these molecules would be like those of the native enzyme. Reduced rates of activity decay in carriers could well be explained on this basis. Structural studies on the enzymes in the normal person, the carriers and the patient will clinch this issue.

On the contrary, there may be a large number of other epigenetic factors, which would go to make an active enzyme structure. In αGalactosidase for instance, there are at least two other factors, which are involved in shaping the final catalytic structure of the enzyme. A glycoprotein called Cathepsin A and a substance called Saposin B. Glycoproteins stabilize αGalactosidase. The same glycoprotein can activate Neuraminidase in the Lysosomes [3], [16],[17]. Saposin B, on the other hand, can act as a physiological detergent, facilitating the interaction of the substrate and the enzyme. One can consider defects in the structure of these two additional entities, which would result in the defective αGalactosidase. There are many such examples in the human enzyme systems and they should be kept in mind while formulating a model for the decay of enzyme activities when stored at -10^0C. Then there are chaperoning proteins, which guide the enzymes to their appropriate destination. The hydrolases will be guided to the lysosomes. The oxidases and peroxidases will be guided to the peroxisomes. Any mistake in the tag of the enzyme will result in the enzyme(s) not reaching the final destination, or its leakage out of the cell. This can affect the 'stability' of the enzyme in terms of its activity, as measured by the conventional procedure. We should keep all these options open when we think of the 'stability' of the enzyme.

We have extended our studies of the application of the **Apte procedure** to several other enzyme systems such as, αGalactosidase, α-Iduronidase, Arylsulphatase **B**, αGlucuronidase, etc, and in all cases, the procedure has enabled us to differentially diagnose the patients, the carriers and the normal persons without any ambiguity. The most satisfying part of the procedure is the

generality of the application of the procedure to any pathological sample and to any enzyme system. Let us say that Arylsulphatase **A** is well within normal limits in the leukocytes of a patient. It may be in the carrier range in his plasma and may be in a range, which raises suspicion about the individual as to whether he is a patient. When the decay kinetics of the enzyme is carried out in all his samples, the rate of decay in all the samples is exactly identical, thus providing an unambiguous result and hence a definitive diagnosis.

The generality of the application of the **Apte procedure** to the study of enzymes in any pathological sample makes it highly appealing. The clear-cut results given by the procedure put the test subject under one of the three categories- a patient or a carrier or a normal person. One of the drawbacks of the procedure, as we saw, was the sample size. Since the assays were done on four consecutive days, four times more sample was required than when it was done only once. The results of the diagnostic test will now take three additional days and hence the patient will get his report three days later. The **Apte procedure** would also increase the cost of the test as the assays have to be carried out four times. This will result in extra expense on the part of the patient.

We believe that the unambiguity and the certainty in the results more than offset all the above drawbacks. The consulting doctor giving out the report will feel confident about the diagnosis, which is of paramount importance for the patient. With the definitive diagnosis, the management of the patient will be done in the most appropriate manner.

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References

[1]. Apte BN A novel approach to the diagnosis of enzyme deficiency, abstracted in the Bombay Hospital Journal 2000; 42: 531.

[2]. Baglioni C (1963) Correlation between genetics and chemistry of human haemoglobins.*Molecular Genetics, part I* (eds), Academic Press, New York, 405- 38.

[3]. Bruns GAP, Leary AC, Regina VM, Gerald PS Lysosomal α -D-Galactosidase in man-hamster somatic hybrids. Cytogenet Cell Genet 1978; 22: 177-81.

[4]. Baum H, Dodgson KS, Spencer B The assay of arylsulphatase A and B in humanurine. Clin Chim Acta 1959; 4: 453-60.

[5]. Dubois G, Turpin JC, Baumann N Absence of ASA activity in healthy father of a patient with Metachromatic leukodystrophy. N Engl J Med 1975; 293:302-10.

[6]. Epstein CJ, Goldberger RF, Young DM, Anfinson CB A study of the factors influencing the rate and extent of enzymatic reaction during reoxidation of reduced ribonuclease. Arch Biochem Biophys 1962; suppl I: 223-30.

[7]. Galjaard H (1980) Genetic metabolic diseases: Early diagnosis and prenatal analysis (eds) Elsevier/North Holland Biomedical press, New York. Oxford.

[8]. Garen A, Levinthal C A fine structure genetic and chemical study of the enzyme alkaline phosphatase in E. coli. Biochim Biophys Acta 1960; 38: 470-63.

[9]. Jacob F, Monod J Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol 1961;3: 318-56.

[10]. Kolodny EH, Fluharty AL (1995) Metachromatic leukodystrophy and multiple sulphatase deficiency: sulphatide lipidosis. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds) in The metabolic and molecular bases of inherited disease, 7th edn, New York: McGraw-Hill, 2693-2739.

[11]. Neufeld EF Lysosomal storage diseases. Ann Rev Biochem 1991; 60: 257-80.

[12]. Porter MT, Fluharty AL, Kihara H Metachromatic leukodystrophy: arylsulphatase A deficiency in skin fibroblast cultures. Proc Natl Acad Sci,USA 1969; 62: 887-93.

[13]. Rotman B Measurement of activity of single molecules of α

galactosidase. Proc Natl Acad Sci, USA 1961; 47: 1981-85.
[14]. Rotman B (1970) Partial loss of activity of individual molecules of aged α galactosidase. In Beckwith JR, Zipser D (eds, The lactose operon, Cold Spring Harbor Laboratory, 279-89.
[15]. Shapiro LJ, Alek KA, Kaback MM, et al Metachromatic leukodystrophy without arylsulphatase A deficiency. Ped Res 1979;13: 1179-84.
[16]. Shows TB, Scrafford-WolfL, Brown JA, Meister M Assignment of Galactosidase gene (α-gal A) to chromosome 3 in man. Cytogenet Cell Genet 1978;22:219-22.
[17]. Sips HJ, de Wit-Verbeek HA< de Wit J, Westerveld A, Galjaard H The Chromosomal localization of human αGalactosidase revisited: a locus for Galactosidase on human chromosome 3 and for its protective protein on human chromosome 22. Hum Genet 1985; 69: 340-45.
[18]. Wolf B (1995) Disorders of biotin metabolism. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 7th edn,New York: McGraw-Hill, 3151-77.
[19]. Wolf B, Greer RE, Allen RJ, Goodman SI, Kien CL Biotinidase deficiency: the enzymatic defect in late onset multiple carboxylase deficiency. Clin Chim Acta 1983;131: 273-81.
[20]. Yanofsky C The tryptophan synthetase system Bact Rev 1960; 24: 221-45.