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# Partial adenosine deaminase deficiency without immunodeficiency: biochemical and genetic studies

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## 2.7. HEAT STABILITY MEASUREMENTS OF RED CELL ADA Hirschhorn *et al* 1979).

Neat haemolysates were diluted by mixing one part haemolysate with five parts of a 0.15M Tris/HCl buffer (pH 7.5), containing 0.05M NaCl (haemoglobin concentrations were between 35 and 50mg/mℓ). One hundred microlitre aliquots of diluted haemolysate were incubated at 57°C for periods of between 0 and 50 minutes. After heating, the aliquots were cooled in an ice water bath and their ADA activities determined by the radiochemical method. Heat stability was expressed as a half life, $t_{\frac{1}{2}}$, the time taken for the enzyme to lose half of it's original activity under the specified conditions.

## 2.8. ISOELECTRIC FOCUSING (IEF)

Isoelectric focusing of ADA was carried out in thick gels made of 1.25 percent w/v glycine, 4.85 percent w/v acrylamide, 0.15 percent w/v bis-acrylamide, 2 percent v/v ampholine, pH 4-6 (LKB 1809-116) and 0.83 percent v/v of 0.004 percent riboflavin. Polymerization was effected by exposing the gel solution of UV light. Contact between the gel and the electrodes of an LKB Multiphor apparatus was made by Whatman No 17 filter paper strips which had been soaked in 0.1M orthophosphoric acid (anode), and 0.5M sodium hydroxide (cathode). A cooling system maintained the gel at 4°C during the separation. Prefocusing was carried out for thirty

minutes during which time the voltage was gradually increased to 700V. Whatman No 3 filter paper squares were soaked with the samples to be focused and placed 2cm from the cathode. The voltage was then gradually increased until a maximum of 1200V was reached. Focusing then took place for a further three hours. The gel was stained for ADA activity in the way described under starch gel electrophoresis.

## 2.9. DEOXY-ATP MEASUREMENT

Red blood cell deoxy-ATP levels were determined by an assay employing DNA polymerase and a poly (dA.dT) template as described by Hirschhorn (personal communication 1985) who based her method on that of Solter *et al*[23].

In the assay, deoxy-ATP from red cells is used (together with added TTP), by DNA polymerase, to form double stranded poly (dA.dT) on a single stranded poly (dA.dT) template. The amount of labelled TTP incorporated by DNA polymerase serves as a measure of the amount of double stranded poly (dA-dT) formed and hence of the amount of deoxy-ATP present in the added red cell extract.

The reaction mixture for the assay of one sample of cell extract consisted of the following:

28.75µℓ buffer solution (0.1M Tris HCl, pH 8.3, 0.01M $MgCl_2$ and 0.002mM β-mercaptoethanol)

4µℓ $^3$H-TTP (40-60ci/mmol, 1µCi/µℓ)

16µℓ TTP, 0.015mM

12.5µℓ poly (dA.dT), 0.35mg/mℓ

2.5µℓ Bovine serum albumin (BSA), 1mg/mℓ

6.25µℓ water

20µℓ of protein free cell extract was added to 70 µℓ of the reaction mixture which was prepared just before the assay. Protein free cell extracts were prepared by perchloric acid precipitation. One hundred microlitres of packed red blood cells was added to 50µℓ of ice cold, 10% perchloric acid in an Eppendorf micro test tube and mixed well. After spinning for fifteen minutes at 0.000g inan Eppendorf centrifuge 50µℓ of clear protein-free extract was removed and neutralized with 15µℓ of 2.21M potassium hydroxide.

*Escherichia coli* DNA polymerase 1 (Materials) was diluted to an activity of 0.1 U/10µℓ with a 1mg/mℓ BSA solution and ten microlitres of this were added to the mixture described above in order to start the reaction. Reaction mixtures were incubated for one hour at 37°C with vortexing at thirty minutes. Forty-five micro litre aliquots of reaction mixture were then added to 2mℓ of ice cold, 10% trichloroacetic acid (TCA) and the resulting precipitate collected by suction aided filtration on to Millipore GS 0.22µm filters. Filters were washed with five 2.0mℓ aliquots of TCA and then placed in vials containing 10mℓ of scintillation fluid (Instagel II Hewlett Packard) in which they dissolved. The radioactivity present was then determined by scintillation

counting. Counts were converted to nmoles deoxy-ATP incorporated, by means of a standard curve which has been constructed by adding known amounts of deoxy-ATP (0.80pmoles) to the assay system including a blank with no deoxy-ATP. Deoxyadenosine triphosphate levels in red cells were finally expressed as nmoles deoxy-ATP/mℓ packed erythrocytes.

## 3. RESULTS

### 3.1. Family study (Fig 15)

The isozyme patterns of red cell ADA from all individuals in the family of the proband were examined as well as the white cell ADA isozyme pattern of the proband.

It was found that the proband's sister (II-2) also had partial ADA deficiency and that their father (I-1), in addition to being an obligate carrier of a partial ADA deficie[illegible]ele, possessed another rare ADA allele, probably [illegible] (Fig 16). The proband's other sister (II-1) also inherited the *ADA*$^{5}$ allele from her father as well as the *ADA*$^{1}$ allele from her mother (I-2). Starch gel electrophoresis failed to distinguish between the residual ADA of the proband and his partially ADA-deficient sister (II-2) and ADA individuals with the usual ADA-1 phenotype. The ADA isozyme pattern in white cell lysates of the proband, however, appeared to consist of only one band of activity, corresponding with the mobility of the major isozyme band of the ADA-1 pattern, instead of the usual triple-banded isozyme pattern (Fig 17).

Activity measurements with adenosine and deoxyadenosine as substrates confirmed that both the proband and his sister (II-2) had deficiencies of red cell ADA activity (Tables 2 & 3). The activities of the probands

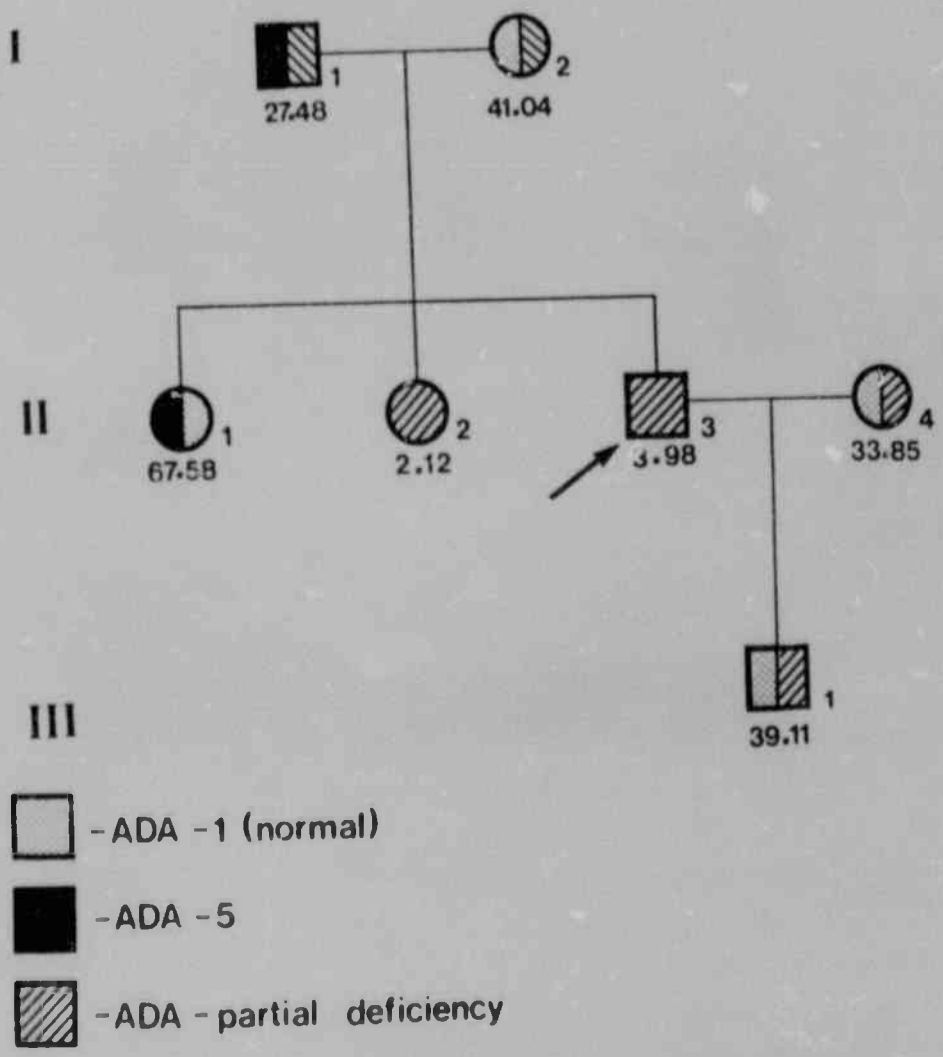



FIGURE 15 : Pedigree of the probands family. Haemolysate ADA activities (nmoles/ mgHb/hour) appear below the symbols. The normal activity level is 67.0 ± 13.5 nmoles/mgHb/hour.

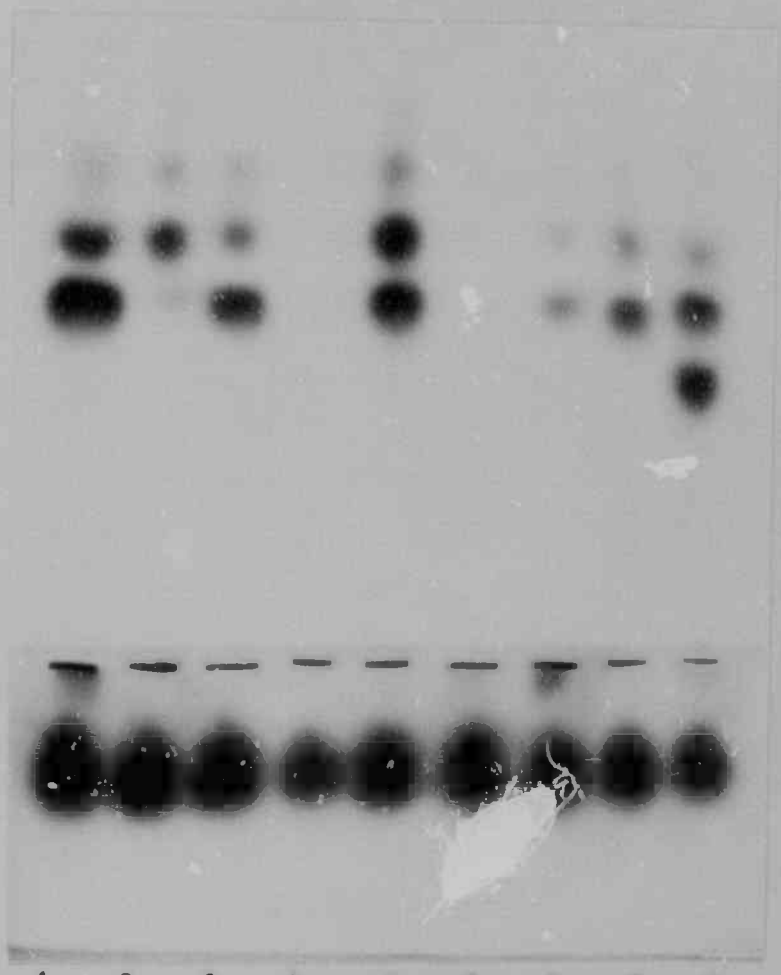



FIGURE 16 : Electrophoresis of red blood cell ADA on starch gel.

1 = ADA 1, control; 2 = I-1, AJ;
3 = I-2, JJ; 4 = II-2, VJ;
5 = II-1, EJ; 6 = II-3 KJ;
7 = III-1, Vuz; 8 = II-4, PR;
9 = ADA 2-1, control.

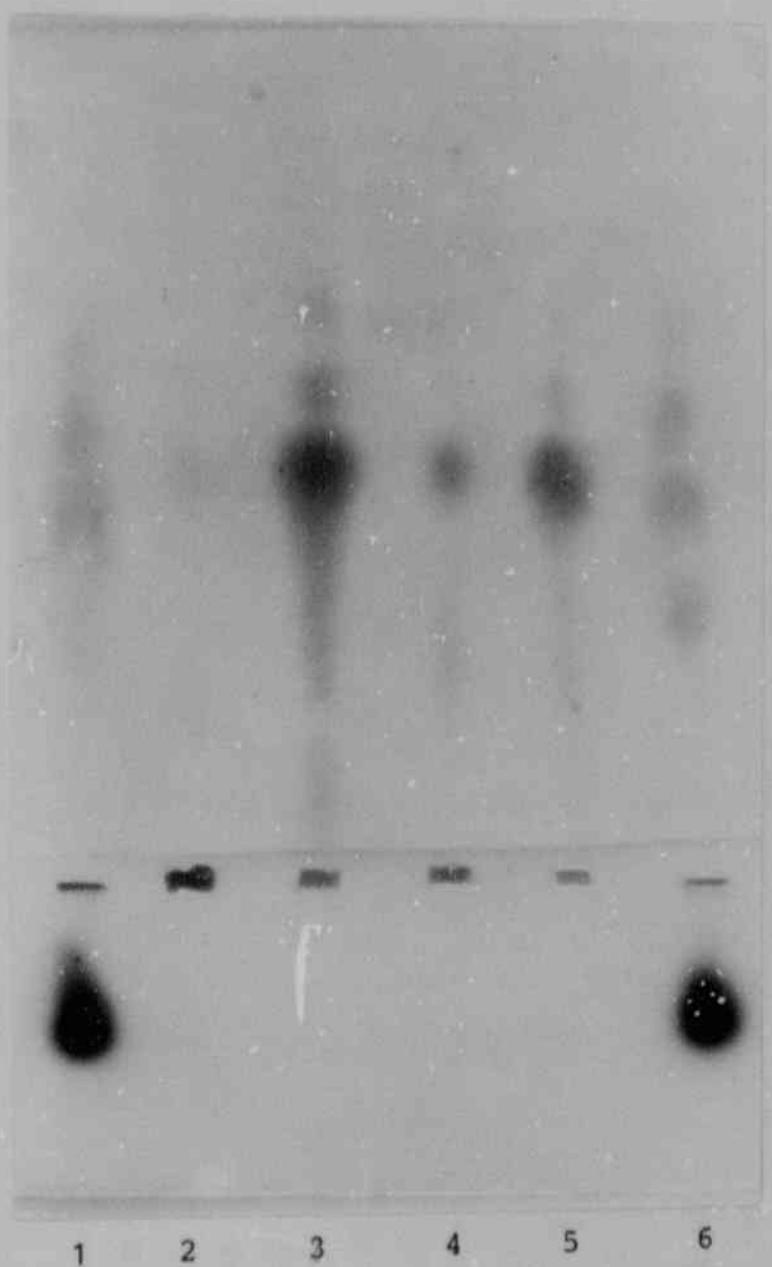



FIGURE 17 : Electrophoresis of white blood cell ADA on starch gel

1 = red cell ADA1, control; 2 = II-3 KJ;
3 = white cell ADA1, control;
4 = II-3 KJ; 5 = white cell ADA1, control;
6 = red cell ADA 2-1, control.

| | | Spectrophotometric method | 100% of normal activity | Radiochemical method | 100% of normal activity |
|---|---|---|---|---|---|
| Normals | | 67.0 ± 13.5 (27) | - | 54.57 ± 8.2 (5) | - |
| II-3 | KJ | 3.98 ± 1.32 (5) | 5.9 | 4.97 | 9.1 |
| II-2 | VJ | 2.12 ± 0.54 (3) | 3.2 | 2.75 | 5.0 |
| I-1 | AJ | 27.48 | 41.0 | 26.99 | 49.5 |
| I-2 | JJ | 41.04 | 61.2 | 32.03 | 58.7 |
| II-1 | EJ | 67.58 | 100 | 88.14 | 161.6 |
| II-4 | PR | 33.85 | 50.5 | - | - |
| III-1 | Vuz | 39.11 | 58.4 | - | - |

TABLE 2 : ADA activity in RBC at adenosine substrate concentrations of 0.1mM for the spectrophotometric method and 0.09mM for the radiochemical method.
Activity expressed in nmole/mgHb/hour.

Numbers in brackets refer to the number of observations on the same individual or on different normal individuals.

| | ADA Activity (nmoles/mgHb hour) | 100% normal activity |
|---|---|---|
| Normals | 53.00 ± 8.60 (11) | - |
| II-3 KJ | 2.77 | 5.2 |
| II-2 VJ | 1.52 | 2.9 |

TABLE 3 : ADA activity in RBC at a deoxyadenosine substrate concentration of 0.1mM measured by the spectrophotometric method

The number in brackets refers to the number of observations in different normal individuals.

red cell ADA with adenosine and deoxyadenosine substrates were about 6-9% and 3% of normal, respectively. Activity levels for the proband's sister with adenosine and deoxyadenosine substrates were 3-5% and 5% of normal red cell ADA activity levels. Their sibling (II-1) who had the ADA 5-1 phenotype was found to have normal activity levels. The parents (I-1 and I-2) had, as expected, intermediate levels of ADA activity (about 41% and 61% respectively). The wife of the proband (II-4) coincidentally, also has intermediate levels of activity, suggesting that she too carries an allele for partial ADA deficiency. The proband's son (III-1), having intermediate levels of red cell ADA activity, would appear to have inherited a partial ADA deficiency allele from the proband, and an $ADA^{1}$ allele from his mother (II-4).

In contrast to his low red cell ADA activity, the level of activity in the white cells of the proband was about 26% of normal. The results of measuring white cell ADA activities of nine unrelated individuals were a mean of 863.3 ± 375nmoles/mg Hb/hour, whereas the activity of ADA in the white cells of the proband was 222nmoles/mg Hb/hour, a level which was not, in fact, significantly below the normal range ($0.2>p>0.05$).

Red cell ADA assays of both the partially deficient family members were performed with a duplicate incorporating EHNA, a powerful inhibitor of ADA, which revealed

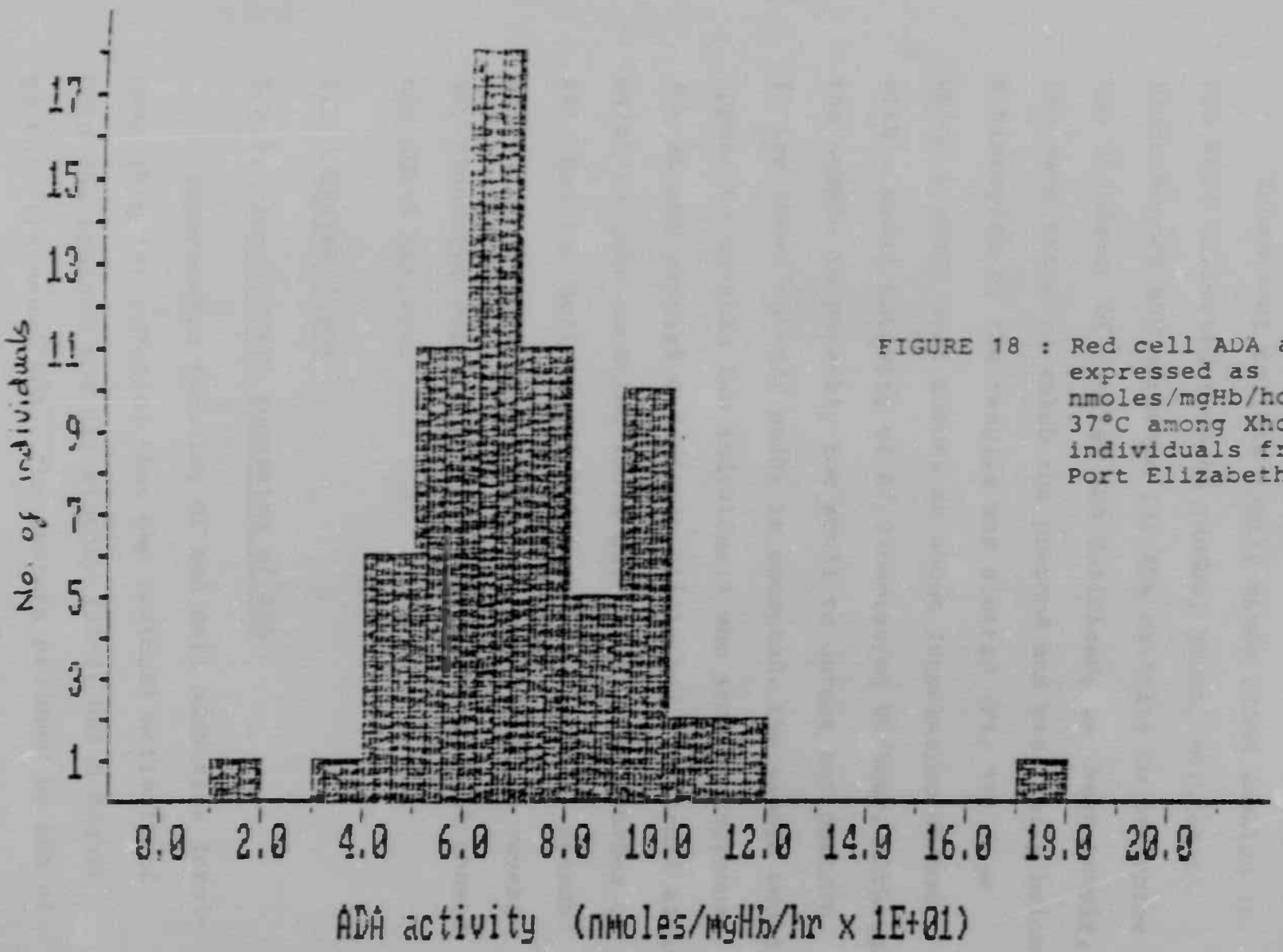



FIGURE 18 : Red cell ADA activity expressed as nmoles/mgHb/hour at 37°C among Xhosa individuals from Port Elizabeth.

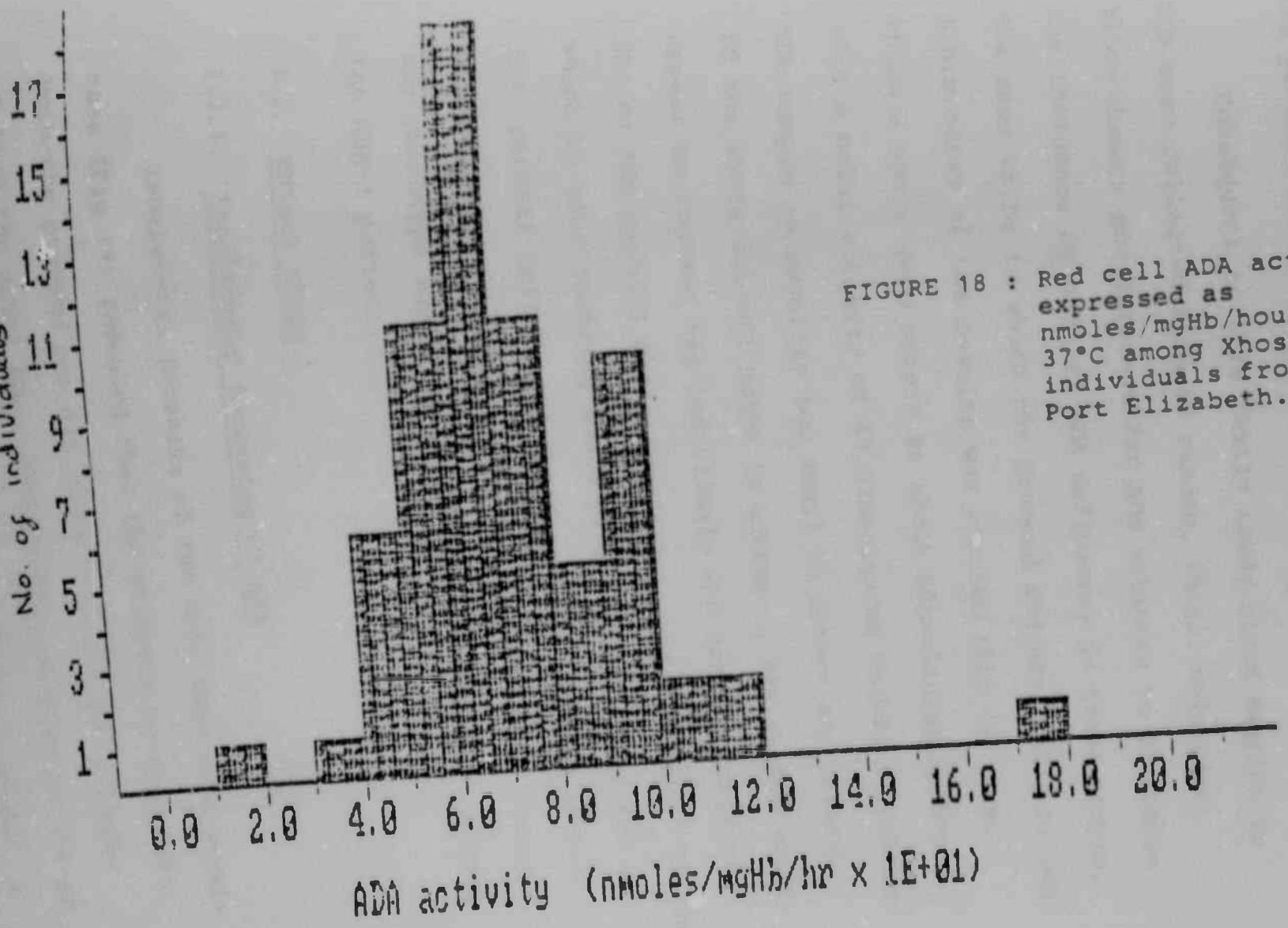



FIGURE 18 : Red cell ADA activity expressed as nmoles/mgHb/hour at 37°C among Xhosa individuals from Port Elizabeth.

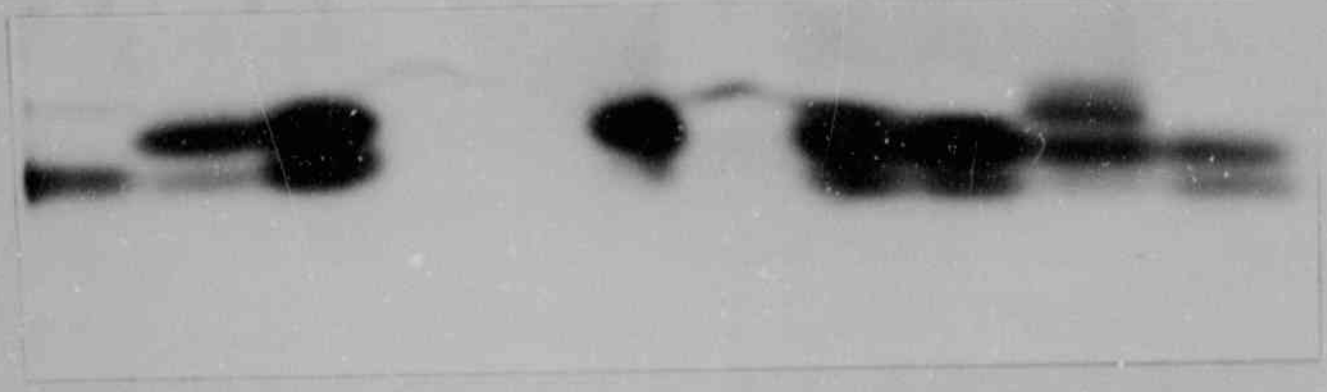
that all of the detectable red cell ADA activity of the proband was due to the major ADA, $ADA_1$.

Subsequent to this family study blood samples in ACD were collected from 68 random, Xhosa, volunteer blood donors and screened for ADA activity to determine the incidence of partial ADA deficiency in these people, the same tribe to which the proband and his family belong. A histogram of the results was plotted (Fig 18) from which a sharp drop occurs at about 40nmoles/mg Hb/hour with a modal activity of 67.01nmoles/mg Hb/hour, although the sample is possibly too small to detect any pattern. If the above cut-off point is accepted, the sample would appear to contain two individuals who are heterozygous for an ADA partial deficiency allele and the normal allele which by gene counting would give allele frequencies of $ADA$ 'partial deficiency' 0.015 and $ADA^1$ 0.985. Starch gel electrophoresis of these 68 samples did not reveal any phenotype variation in isozyme patterns, all showed the ADA-1 pattern.

## 3.2. ENZYME STUDY

### 3.2.1. Isoelectric focussing of ADA

Isoelectric focusing of red cell adenosine deaminase (Fig 19) revealed that the residual activity of both the proband and his sister, (II-2) had a higher pI than the usual ADA. The pattern produced by ADA of the proband's father (I-1) consisted of two bands; a



1 2 3 4 5 6 7 8 9 10

FIGURE 19 : Isoelectric focusing of ADA in red blood cells from the proband and his family.

1 = I-1,AJ; 2=I-2, JJ; 3 = II-1, EJ
4 = II-2, VJ; 5 = ADA-1, control; 6 = II-3, KJ
7 = III-2, VJ; 8 = II-4. PR; 9 = ADA 2-1, control
10 = ADA 1, control.

cathodal band, corresponding to enzyme coded for by the same *ADA* deficiency allele as that present in the proband and his sister, and a more anodal band due to his purported $ADA^5$ allele. The proband's mother (I-2) however, does not show the cathodal band due to the type of ADA deficiency allele which is present in her husband but rather an ADA-1 pattern. Yet she has intermediate levels of adenosine deaminase activity suggesting that she is a carrier of a partial deficiency allele and she is, of course, an obligate heterozygote for it.

The proband's wife who has approximately half of the normal red cell ADA activity shows the cathodal component suggesting that she is heterozygous for the same partial deficiency allele as her husband and for the normal $ADA^1$ allele. The proband's son (III-1) appears to have inherited the cathodal component due to an allele of ADA for reduced activity which must have come from his father as well as the normal ADA-1 component from his mother.

The ADA-5 component of the father (I-1) and his daughter II-1 were detected as bands which migrated more anodally than ADA-1.

### 3.2.2. $K_m$ determination

The residual red cell ADA of the proband was found to have a $K_m$ for adenosine of 47.9 ± 15.8μM, a value which is not significantly different from that estimated

| | Adenosine (μM) | Deoxyadenosine (μM) |
|---|---|---|
| Normals | 51.7 ± 11.4 (5) | 50.7 ± 22.2 (3) |
| II-3 KJ | 47.9 ± 15.8 (8) | 39.9 ± 11.4 (3) |
| I-1 AJ | 59.54 (3) | |

TABLE 4 : Estimation of Michaelis constant ($K_m$) for adenosine and deoxyadenosine as determined from standard Lineweaver-Burk plots after measuring ADA activity by the radiochemical method.

Numbers in brackets refer to the number of observations on the same individual or on different normal individuals.

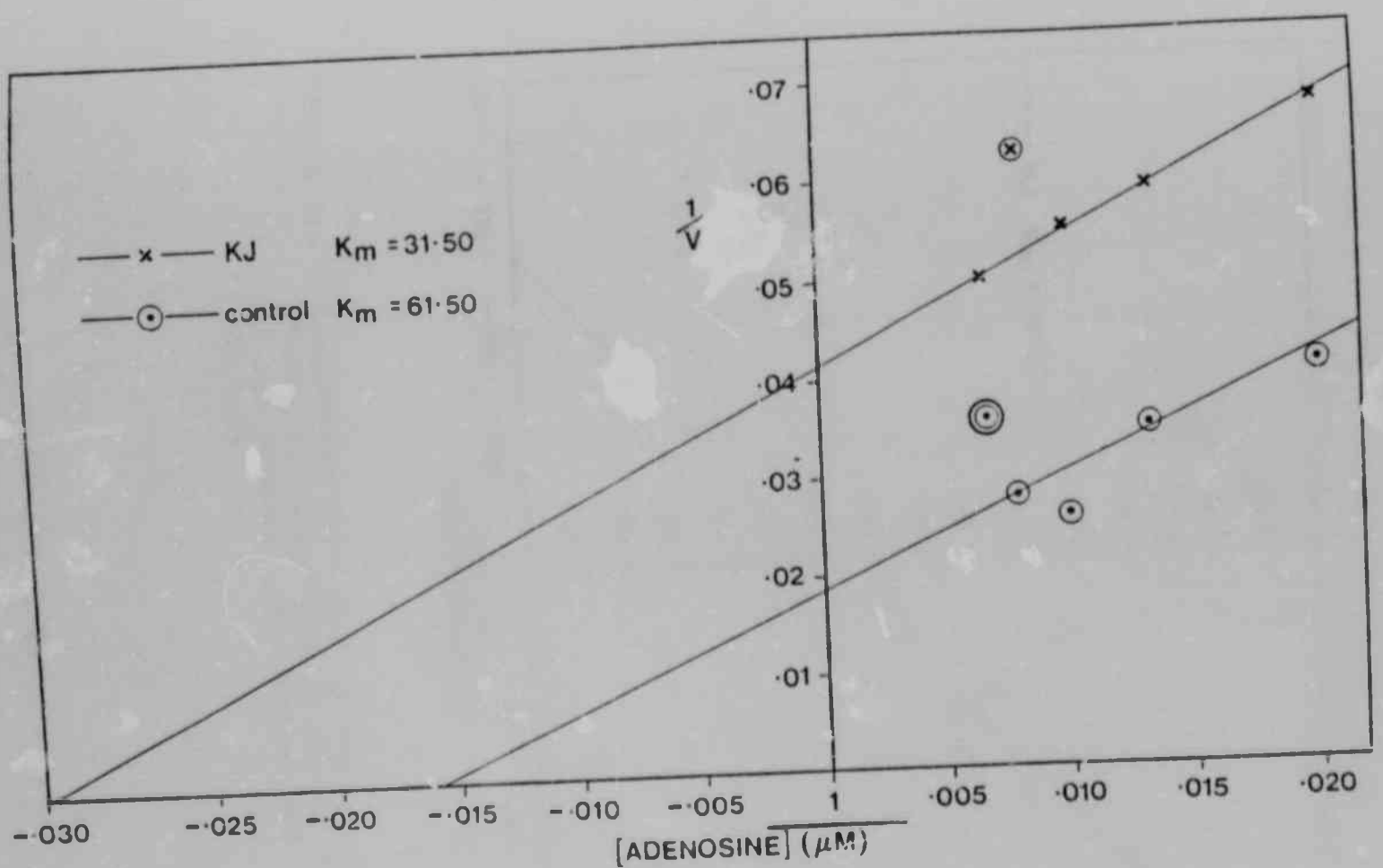



FIGURE 20 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of a normal individual (61.5μM) and the proband with partial ADA deficiency (31.5μM).

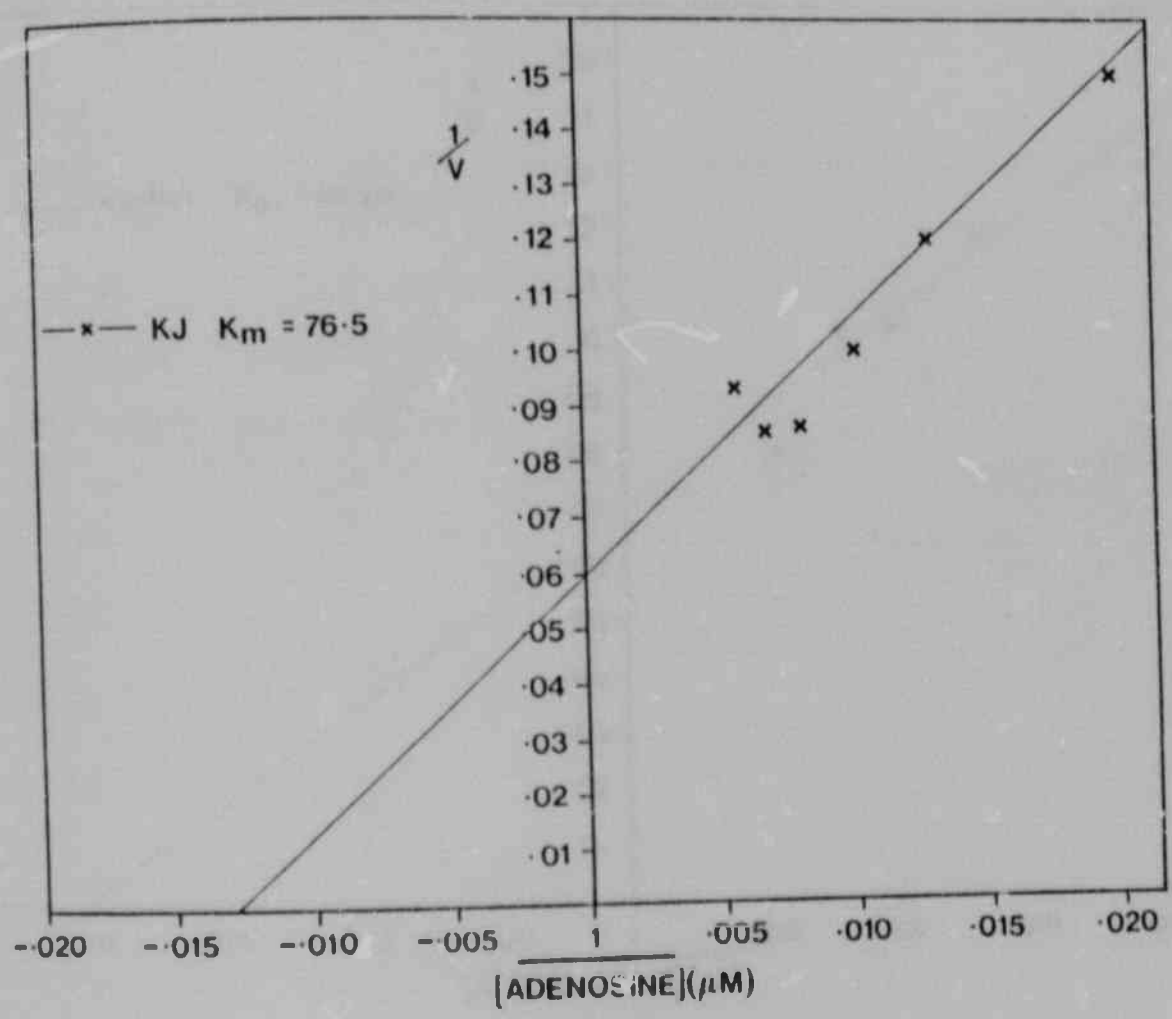



FIGURE 21a : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysate of the proband with partial ADA deficiency (76.5μM).

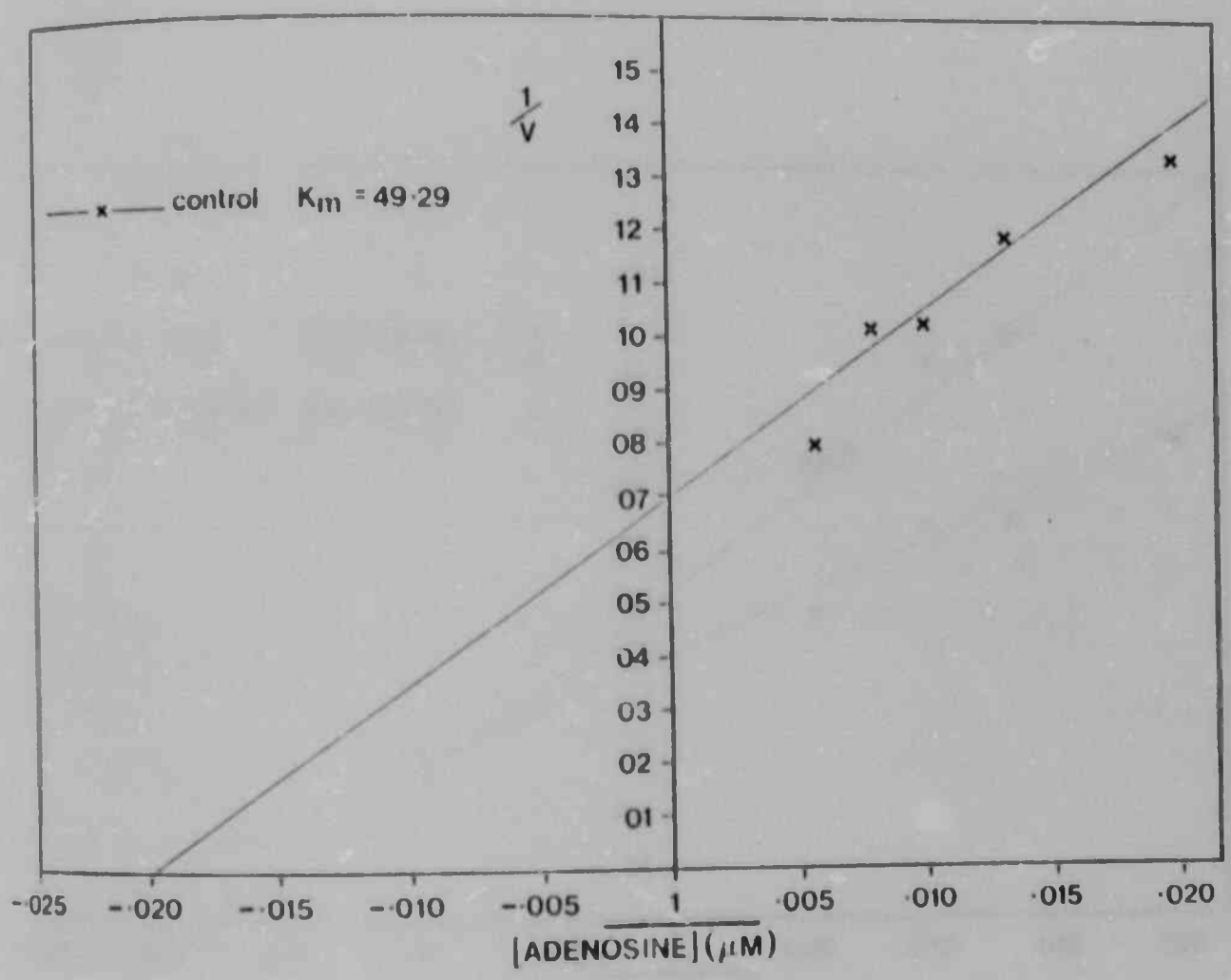



FIGURE 21b : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysate of a normal individual (49.29μM).

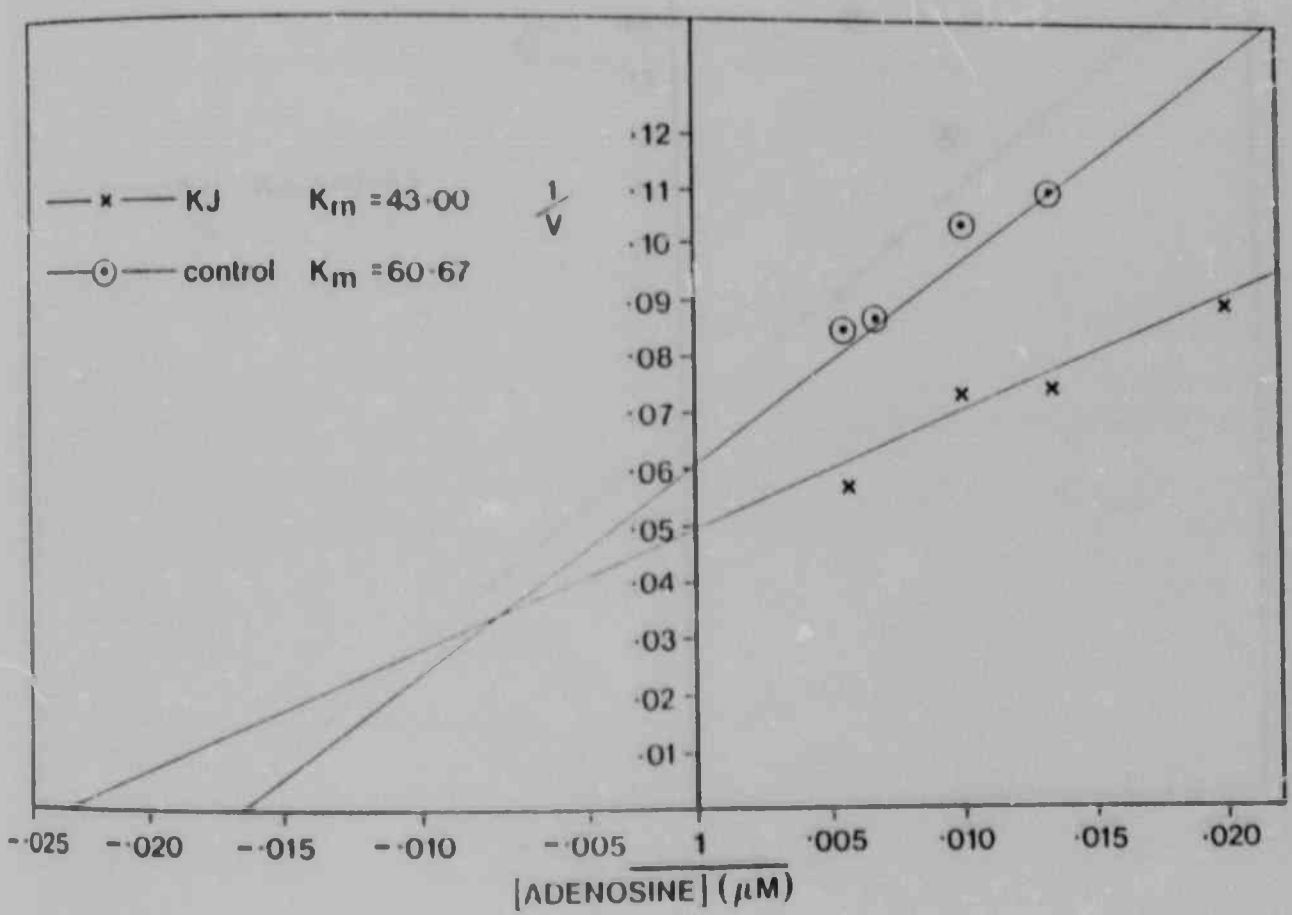



FIGURE 22 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA haemolysates of a normal individual (60.67μM) and the proband with partial ADA deficiency (43.00μM).

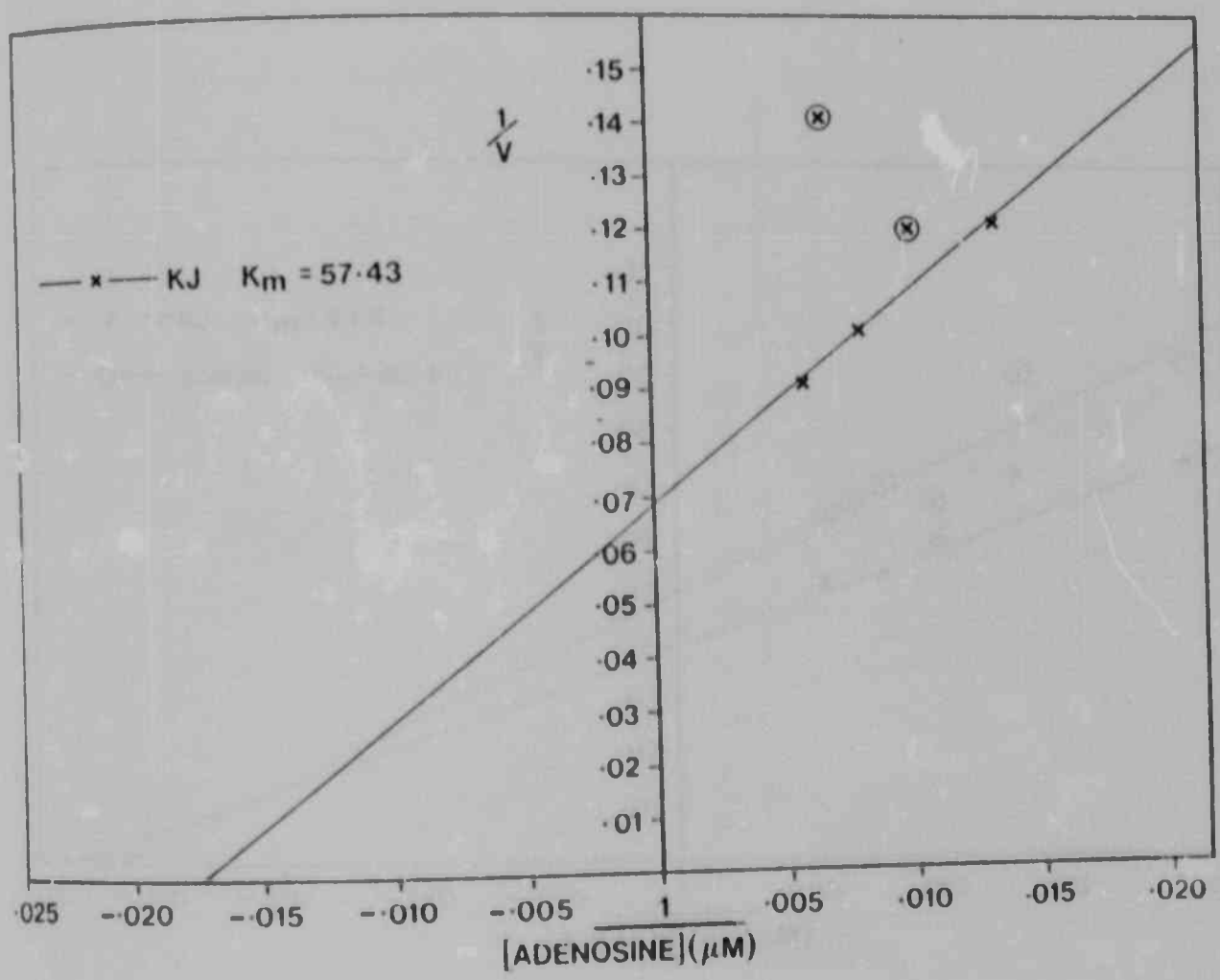



FIGURE 23 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysate of the proband with partial ADA deficiency (57.43μM).

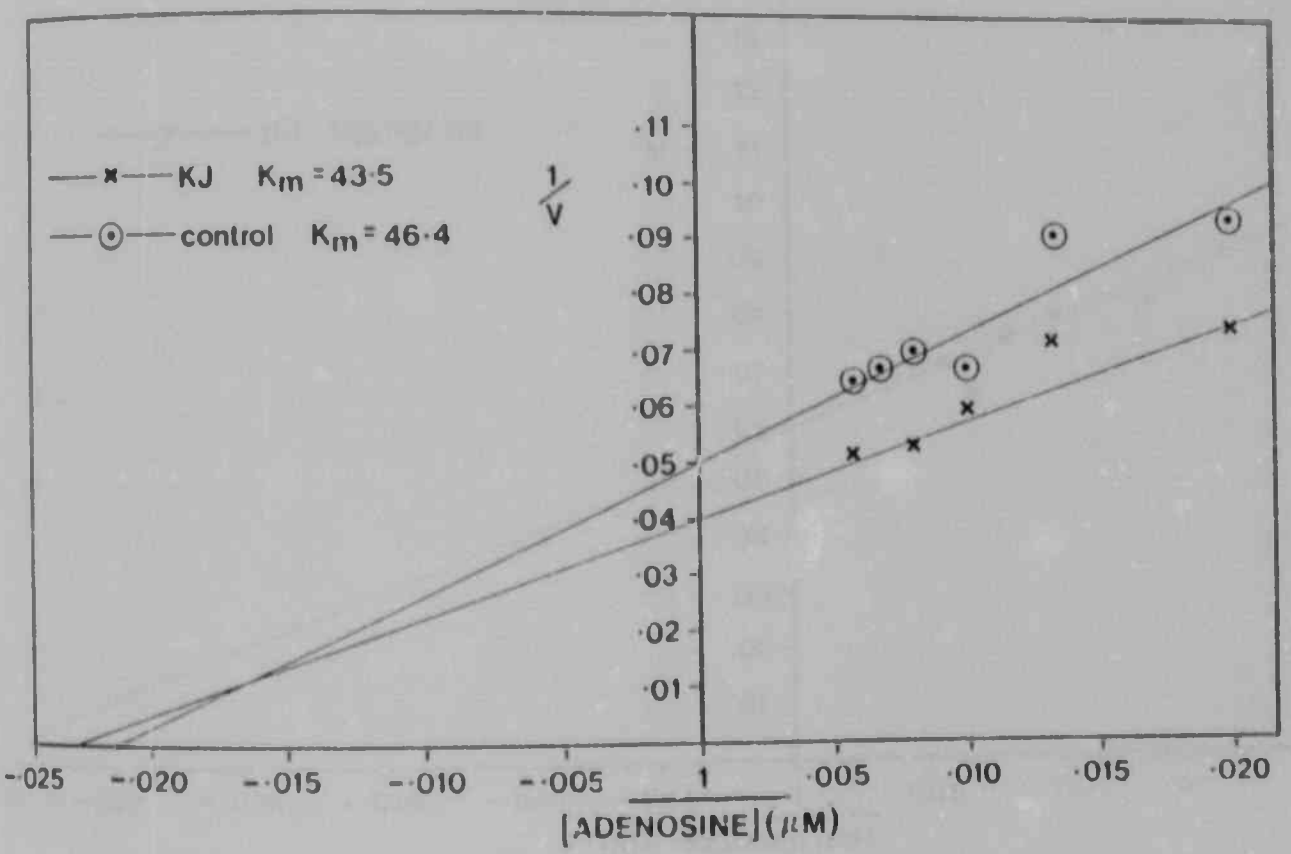



FIGURE 24 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of a normal individual (46.4μM) and the proband with partial ADA deficiency (43.5μM).

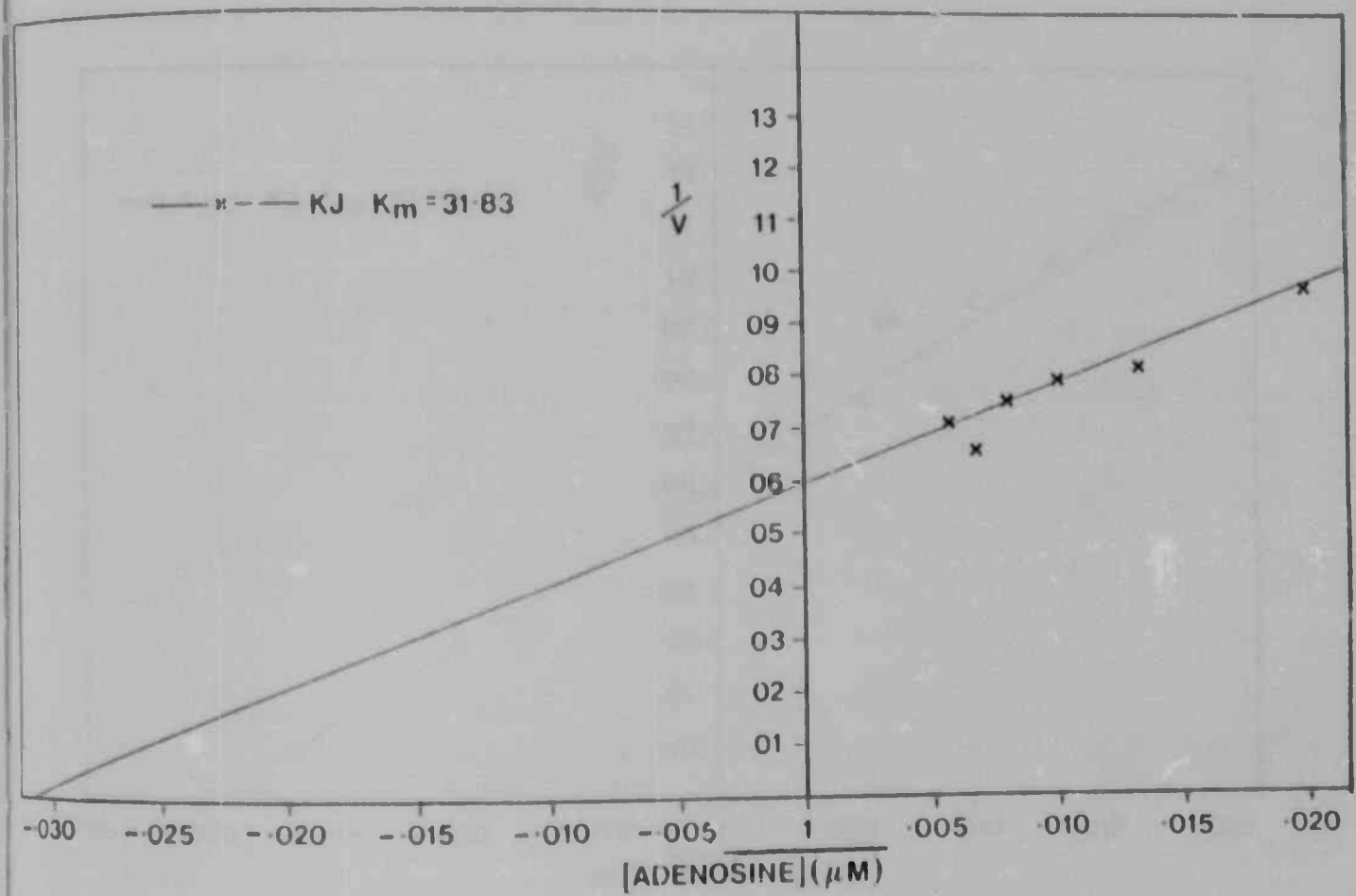



FIGURE 25 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of the proband with partial ADA deficiency (31.83μM).

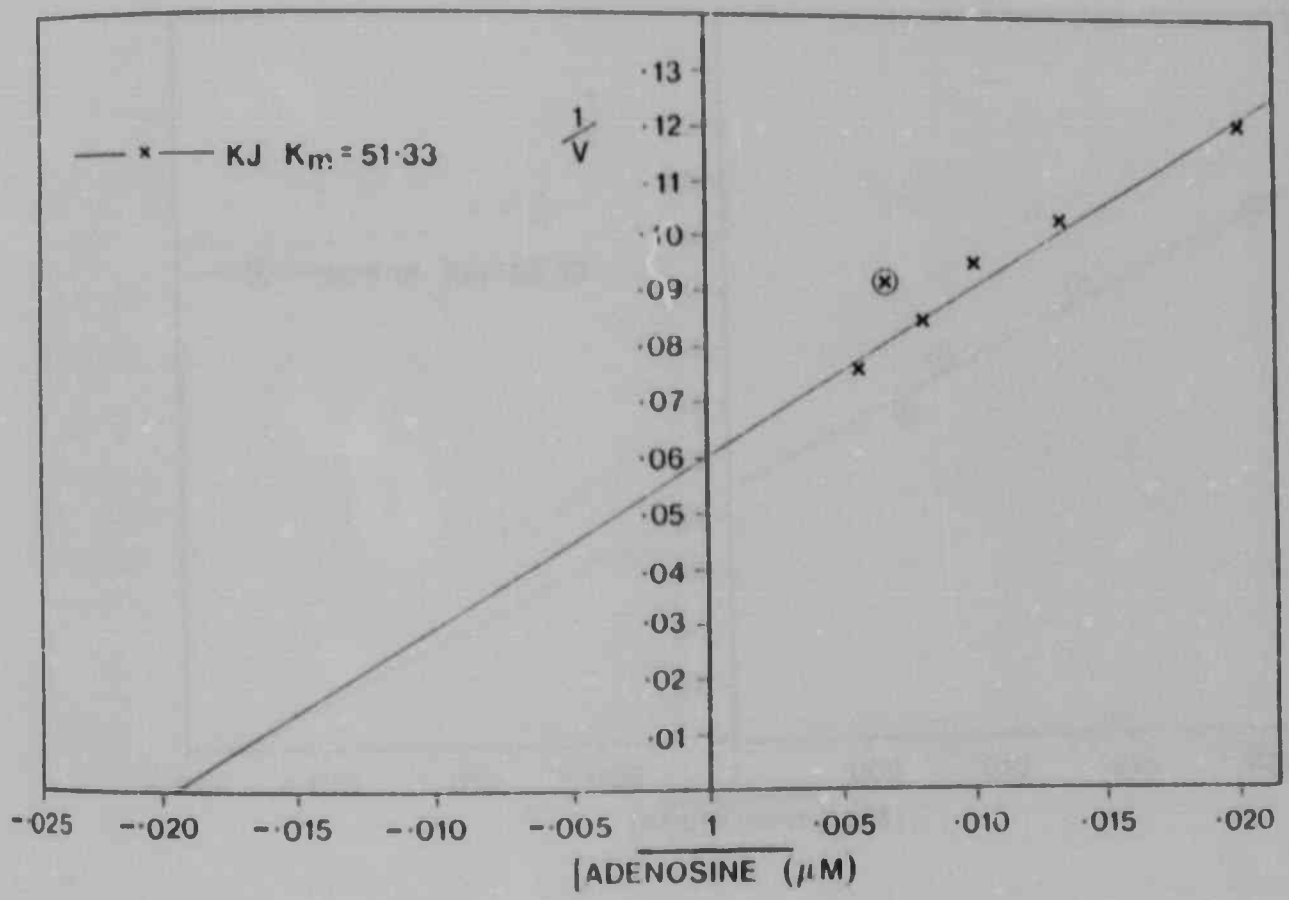



FIGURE 26a : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of the proband with partial ADA deficiency (51.33μM).

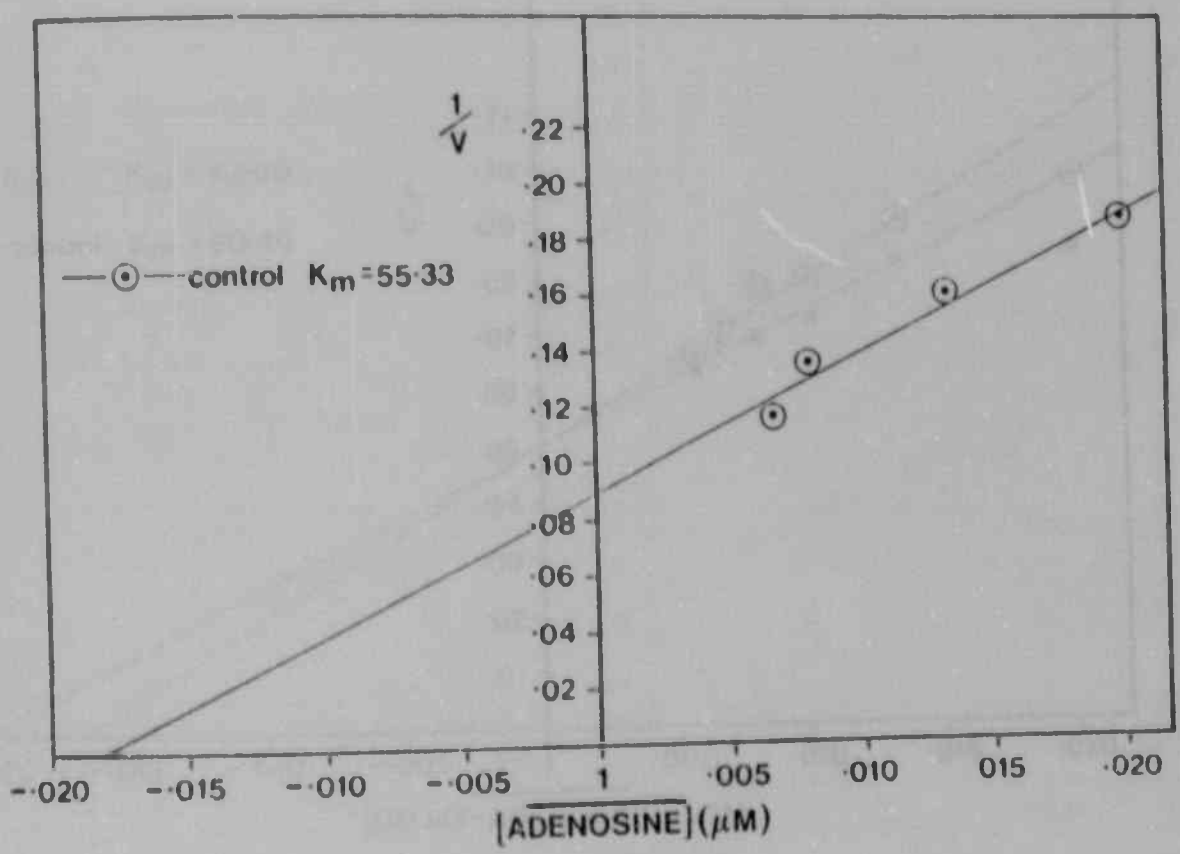



FIGURE 26b : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of a normal individual (55.33μM).

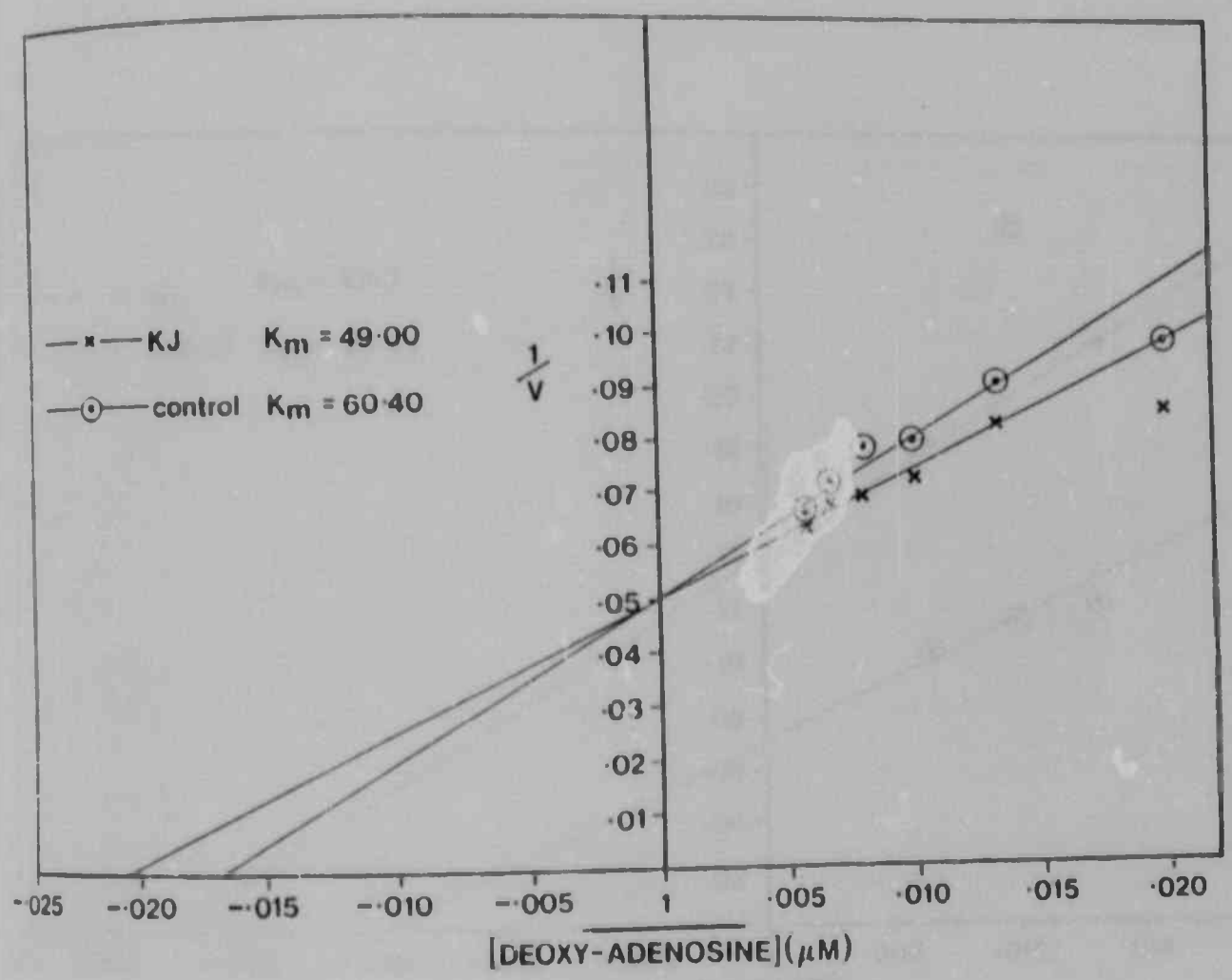



FIGURE 27 : Lineweaver-Burk plot of data used to estimate the $K_m$ for deoxyadenosine of ADA in haemolysates of a normal individual (60.40μM) and the proband with partial ADA deficiency (49.00μM).

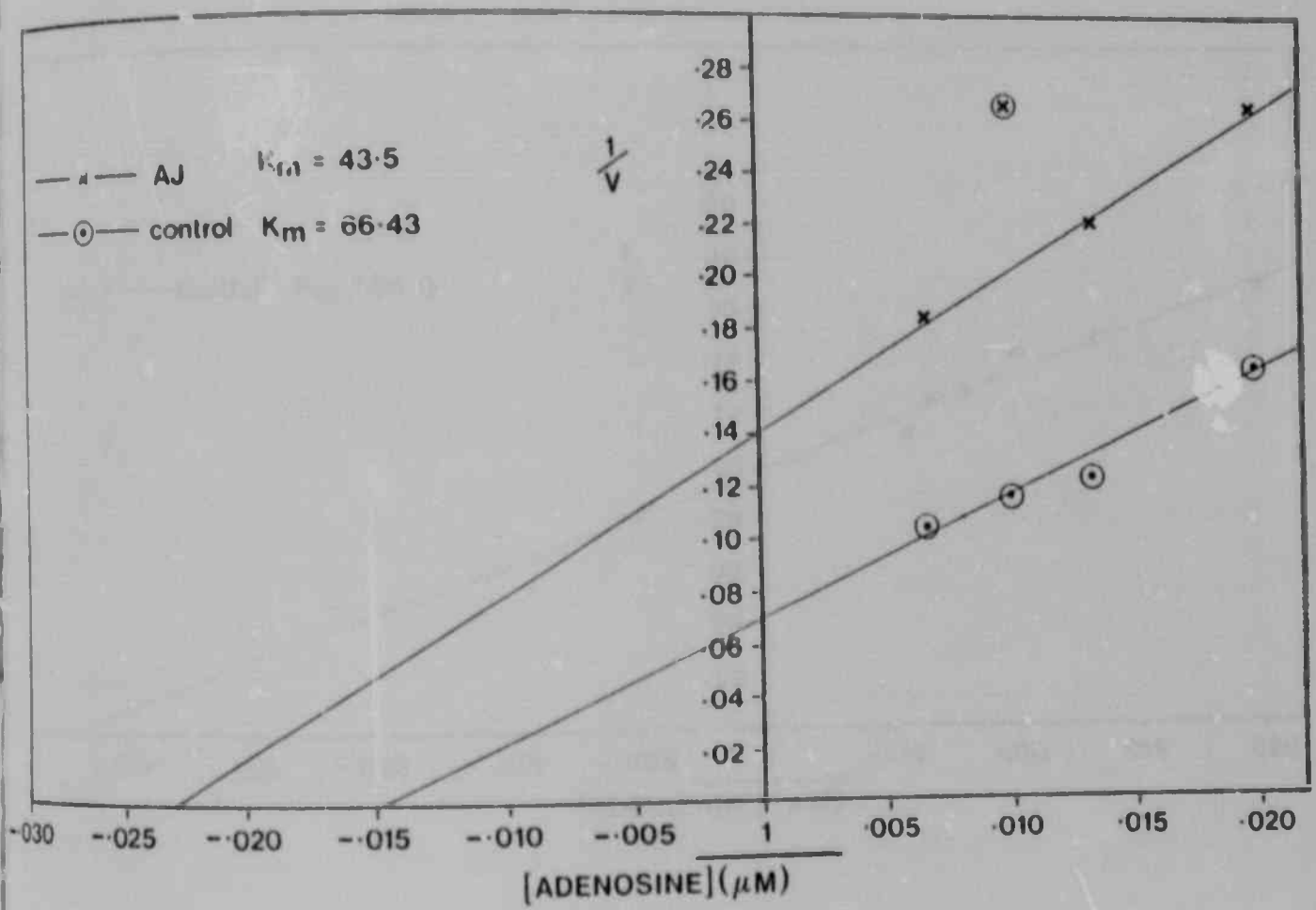



FIGURE 28 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of a normal individual (66.43μM) and the father of the proband (43.50μM).

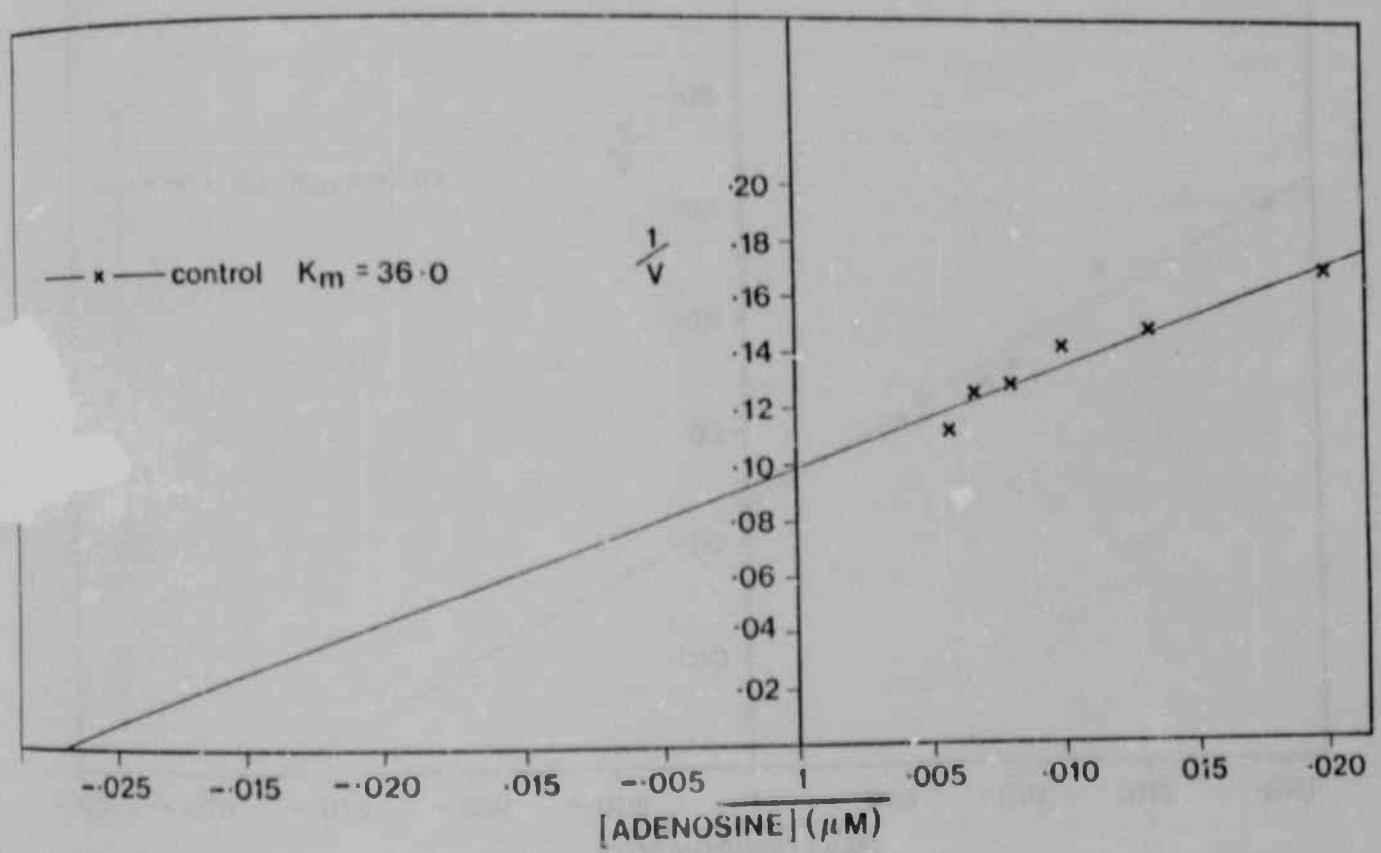



FIGURE 29a : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates of a normal individual (36.0μM).

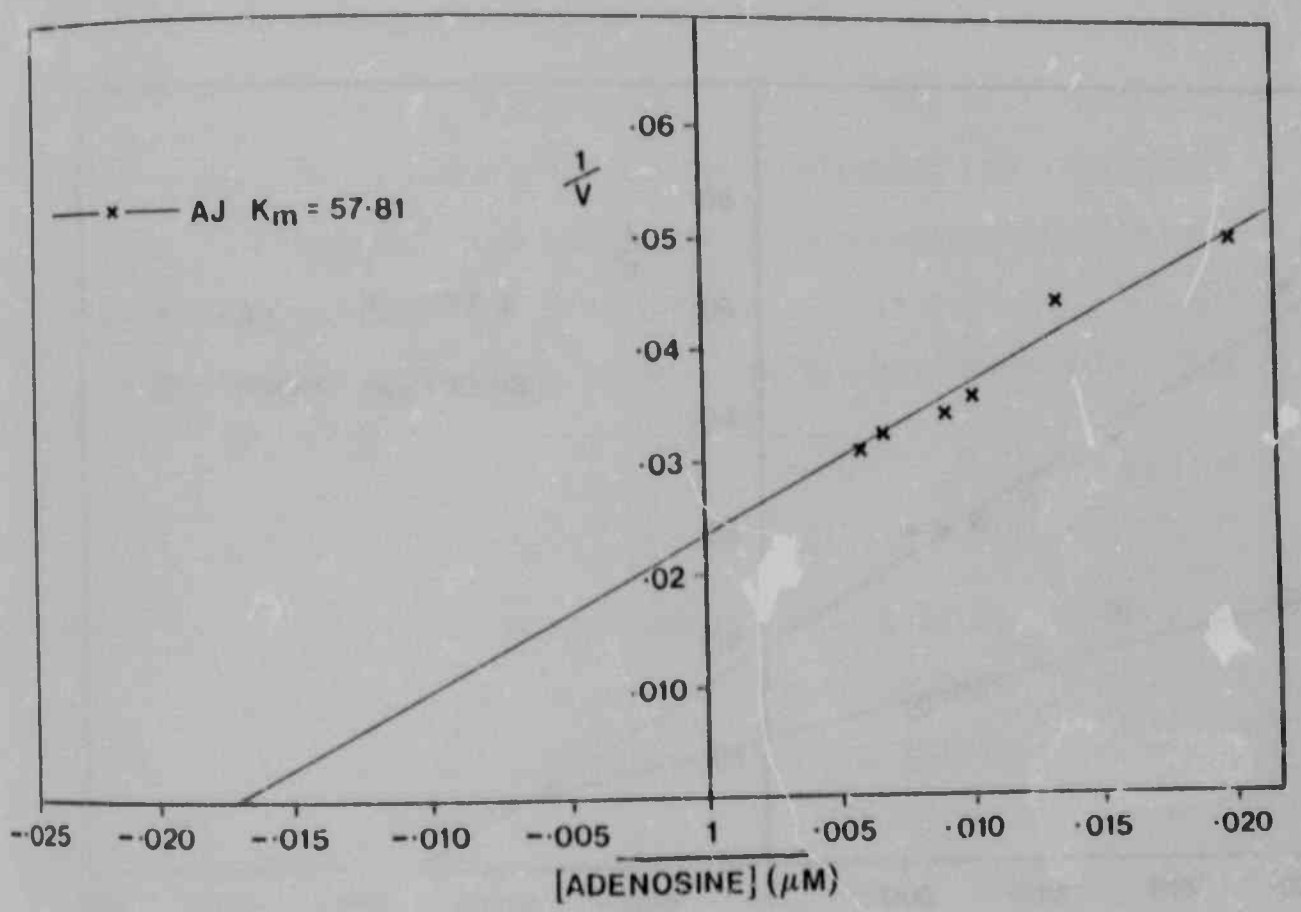



FIGURE 29b : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates from the father of the proband (57.81μM).

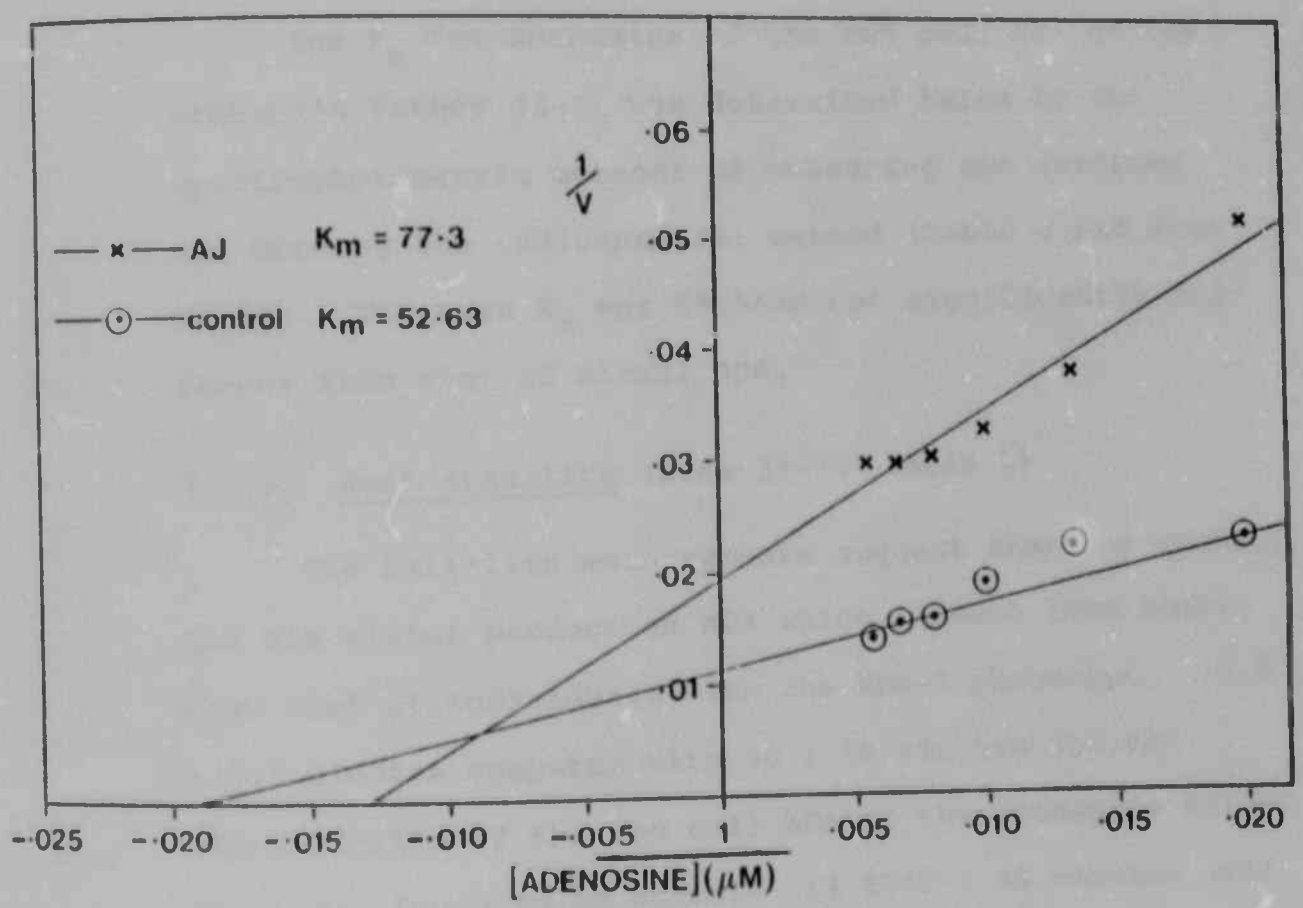



FIGURE 30 : Lineweaver-Burk plot of data used to estimate the $K_m$ for adenosine of ADA in haemolysates from a normal individual (52.63μM) and the father of the proband (77.30μM).

for normal $ADA_1$; 51.7 ± 11.4M (0.80>p>0.70). (Table 4 and Figs 20-30). The $K_m$ for deoxyadenosine was estimated to be 39.9 ± 11.4μM for the proband and 50.7 ± 22.2μM for normal ADA, which were not found to be significantly different (0.70>p>0.60).

The $K_m$ for adenosine of the red cell ADA of the proband's father (I-1) was determined twice by the spectrophotometric methods of measuring ADA activity and once by the radiochemical method (Table 4 and Figs 28-30). The mean $K_m$ was 59.54μM not significantly different from that of normal ADA.

3.2.3. Heat stability (Figs 31-35 Table 5)

The half-life measurements suggest that the proband and his sister produce an ADA which is much less stable than that of individuals with the ADA-1 phenotype: 13.5 ± 3.5 minutes compared with 58 ± 18 minutes (p<.02). The half-life of the red cell ADA of the proband's father (I-1) was found to be normal: $t_{\frac{1}{2}}$ 57°C = 45 minutes (Fig 35) (0.5<p<0.4).

## 3.3. RED CELL DEOXY-ATP LEVELS

The measurement of deoxy-ATP levels in red cells of six normal controls gave a mean value of 2.99 ± 1.19nmoles deoxy-ATP/mℓ packed erythrocytes. Levels of deoxy-ATP in red cells of the proband II-2 were consistently elevated, two to three times higher than the control level, with a *mean* value of 8.50 ± 5.3( nmoles

deoxy-ATP/mℓ packed erythrocytes. The proband's sister, II-1 who has similar ADA activity levels to the proband also appears to have elevated deoxy-ATP levels in her red cells with a mean of 5.24 ± 3.47nmoles deoxy-ATP/mℓ packed erythrocytes although the difference between her levels and the mean normal level is not as great as that of the proband.

| | $t_{\frac{1}{2}}$ |
|---|---|
| Normal | 58 ± 18 (4) |
| KJ | 13.5 |
| VJ | 13.5 |
| AJ | 45.0 |

TABLE 5 : Heat Stability.

Half-life at 57°C in minutes. Number in brackets refers to the number of observations in different normal individuals.

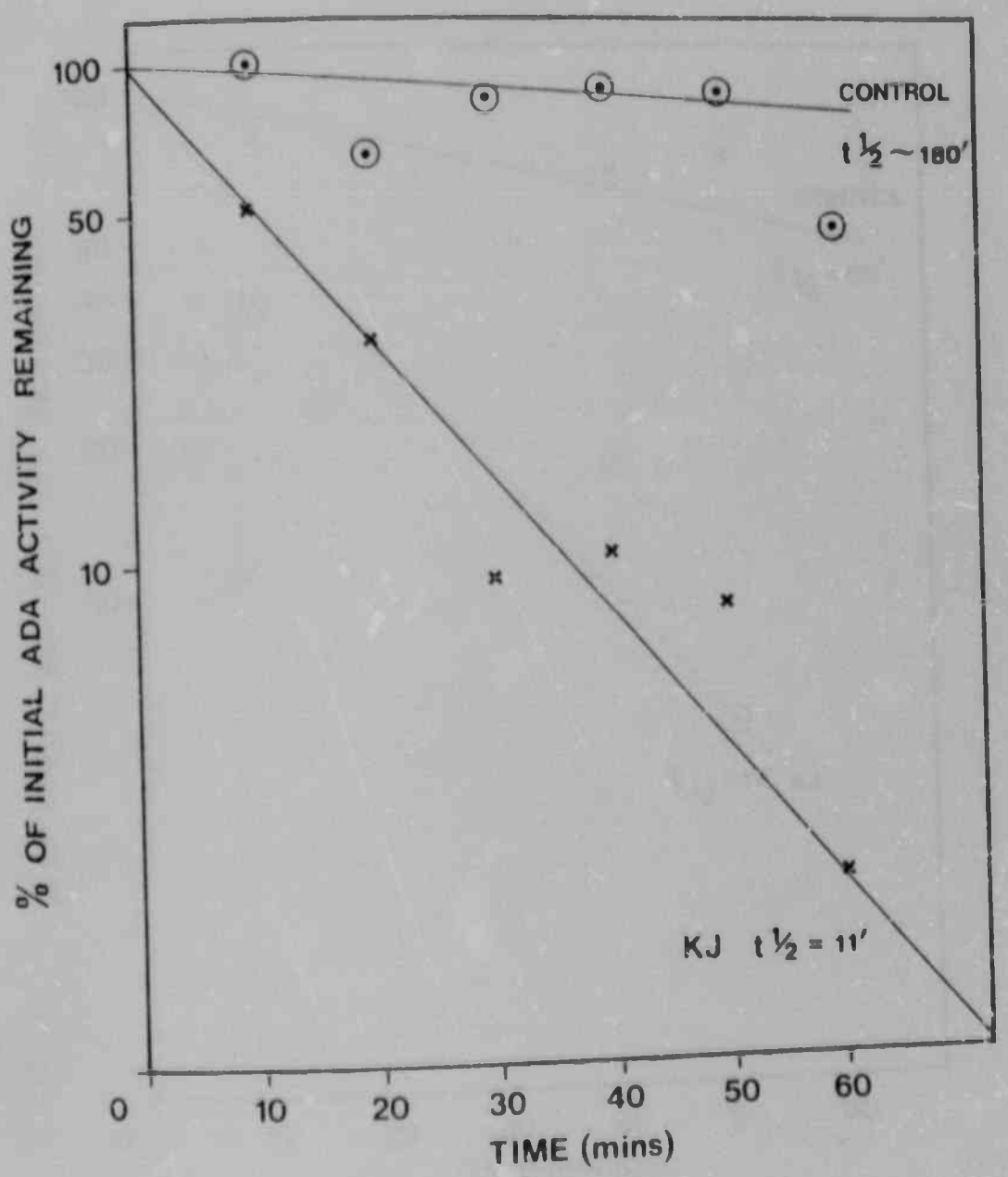



FIGURE 31 : Logarithmic plot of data used to estimate the half-life of ADA in haemolysates of a normal individual (about 180') and the proband with partial ADA deficiency (11').

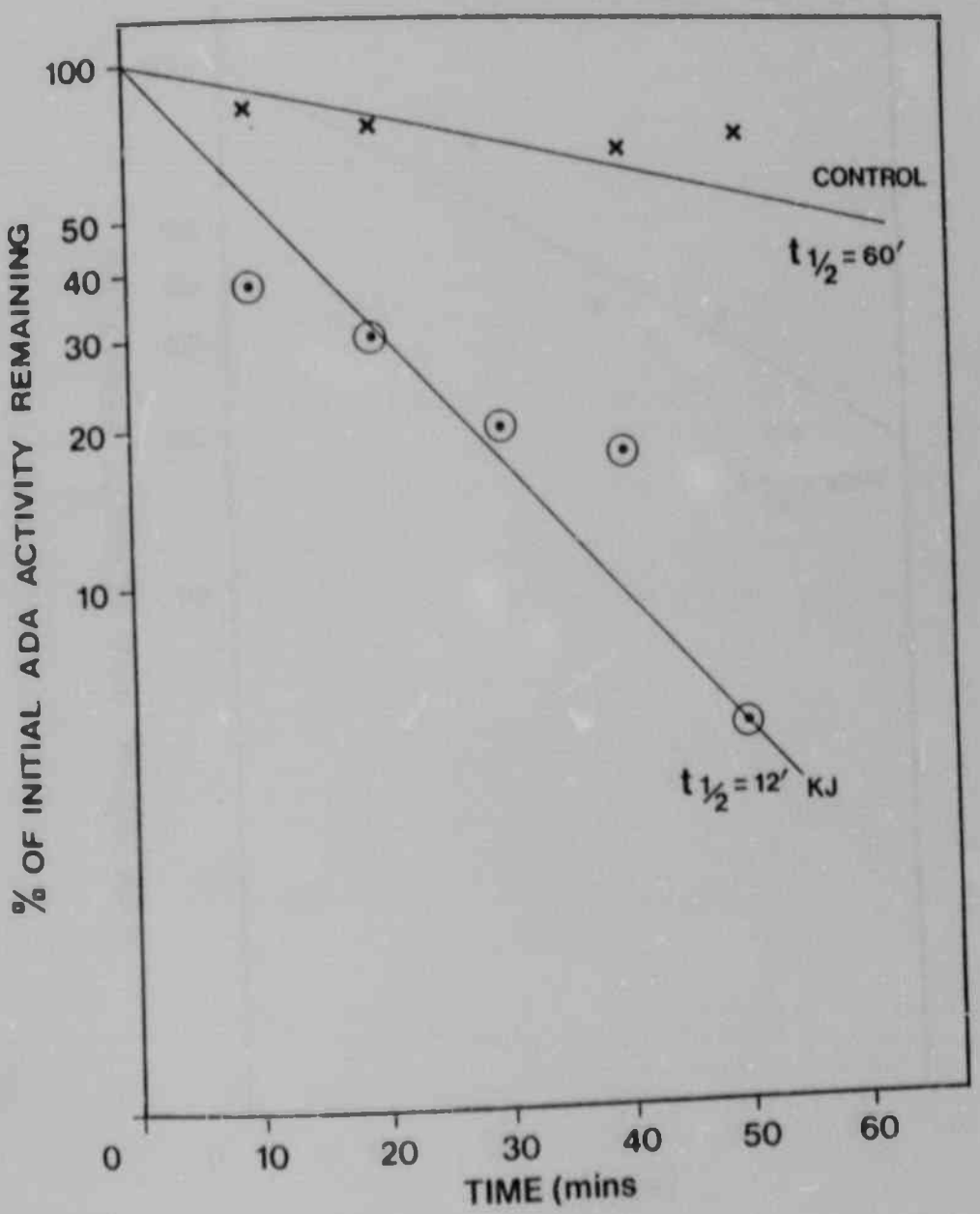



FIGURE 32 : Logarithmic plot of data used to estimate the half-life of ADA in haemolysates of a normal individual (60') and the proband with partial ADA deficiency (12')

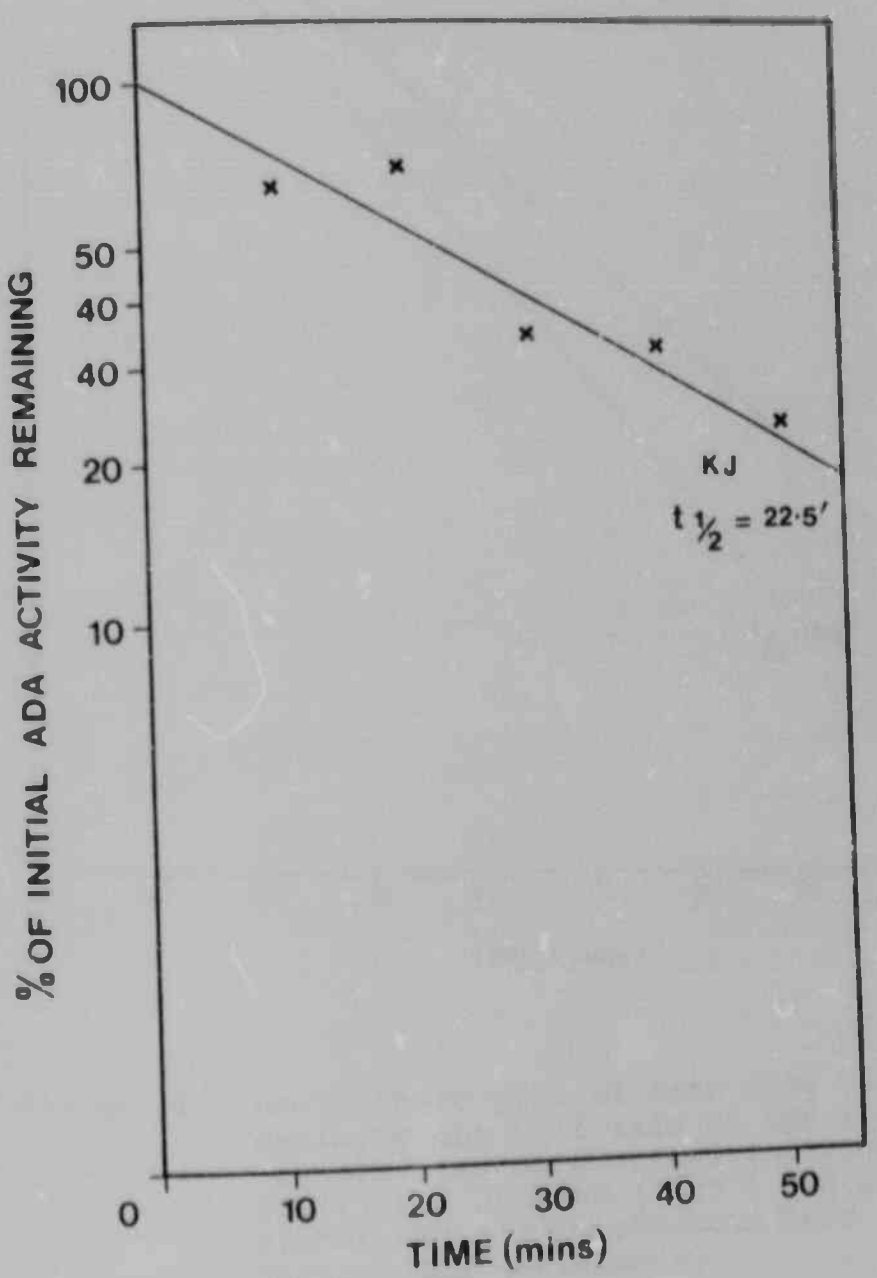



FIGURE 33 : Logarithmic plot of data used to estimate the half-life of ADA in haemolysates of the proband with partial ADA deficiency (22.5').

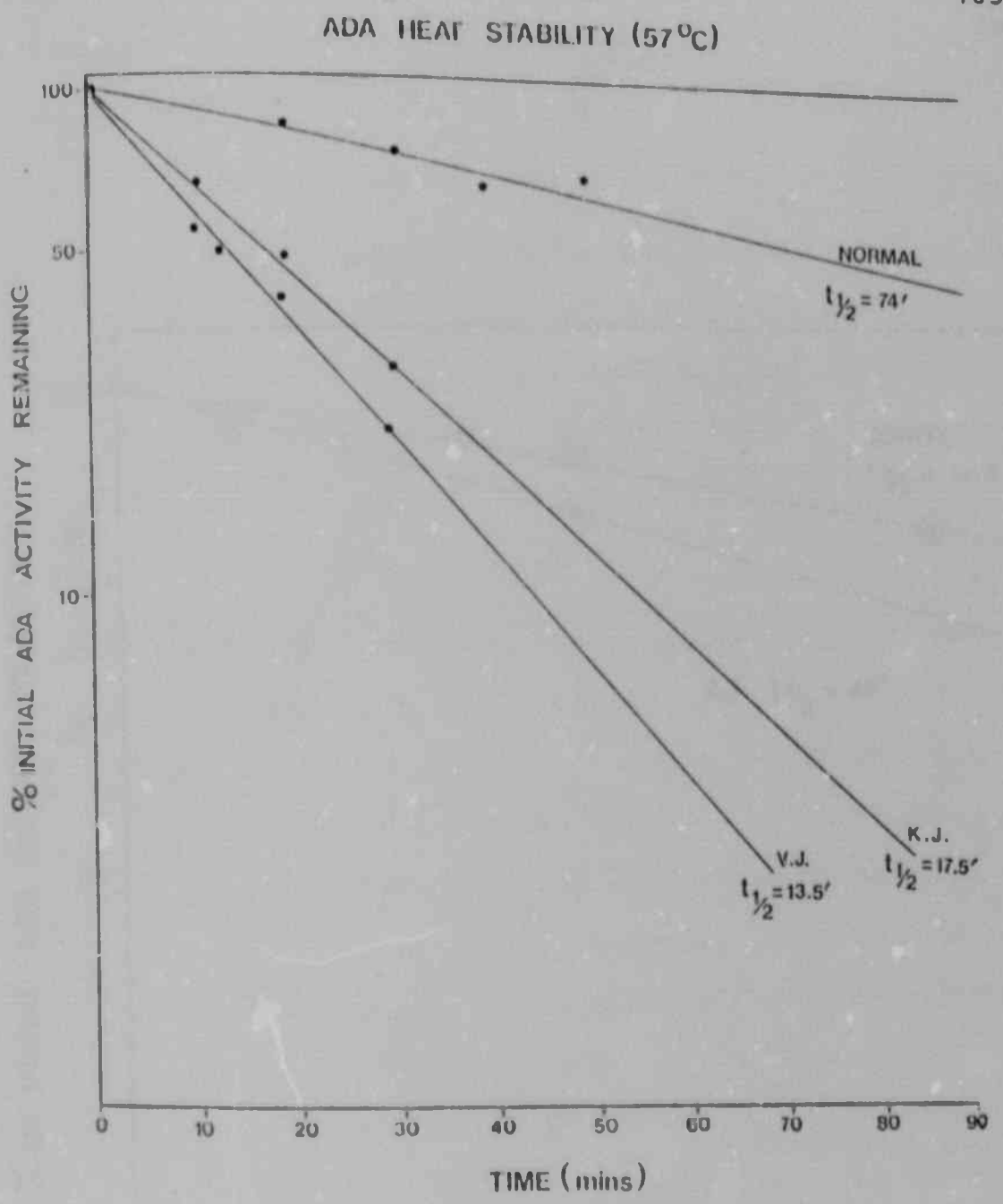



FIGURE 34 : Logarithmic plot of data used to estimate the half-life of ADA in haemolysates of a normal individual (74'), the proband (17.5') and his sister, VJ (13.5') who both have partial ADA deficiency.

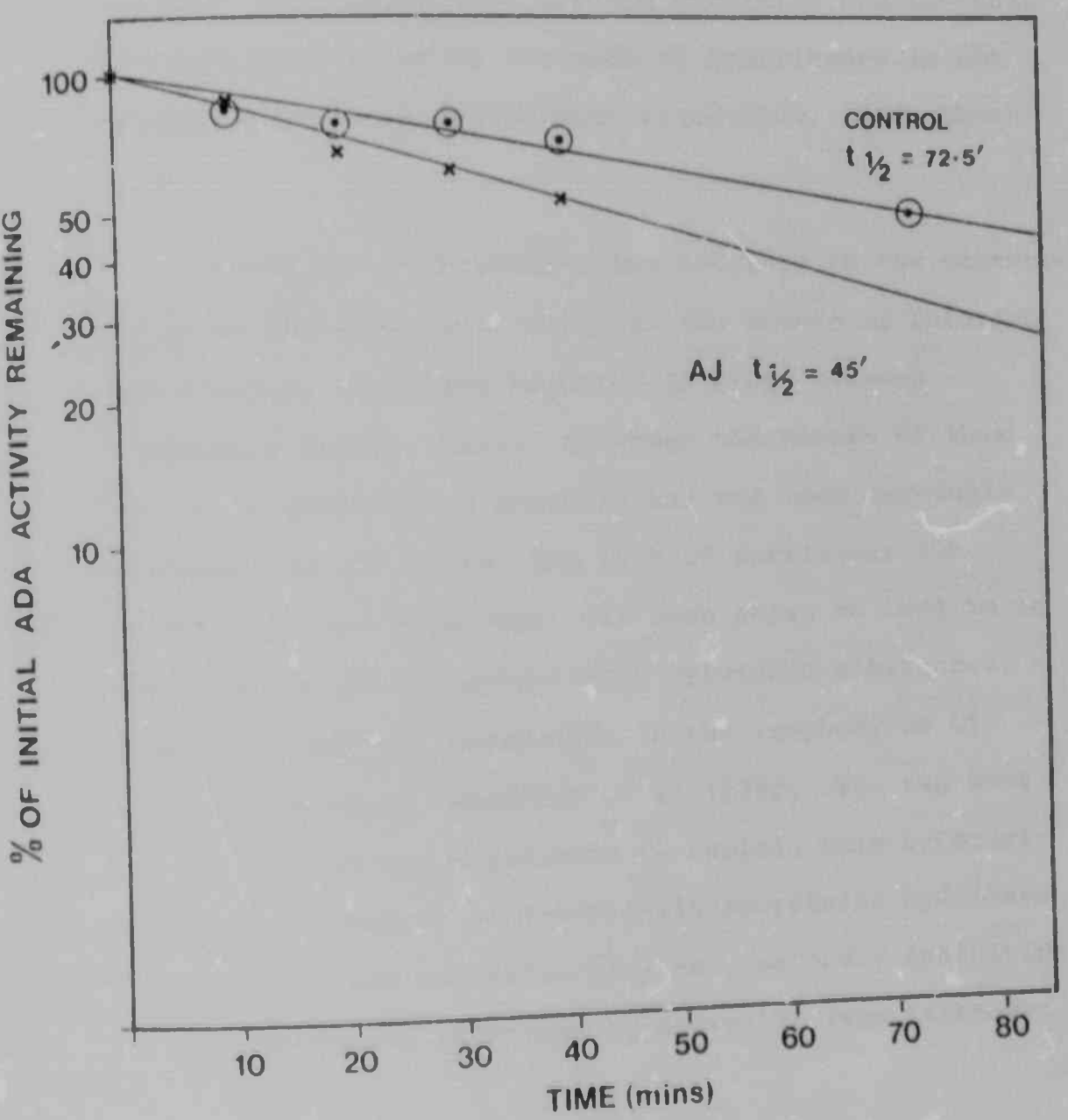



FIGURE 35 : Logarithmic plot of data used to estimate the half-life of ADA in haemolysates of a normal individual (72.5') and the father of the proband with partial ADA deficiency (45').

# 4. DISCUSSION

## 4.1. Overview

An inherited, marked deficiency of ADA has been causally associated with between one-third and one-half of SCID cases in which the mode of inheritance is not obviously X-linked (Hirschhorn *et al* 1979; Hirschhorn 1978).

There is no detectable ADA activity in the erythrocytes of these patients although low levels of putative ADA activity have been reported in other tissues (Dadonna & Kelley 1980). Although the nature of this adenosine deaminating activity has not been carefully evaluated in all cases, the lack of sufficient ADA activity in these patients has been shown to lead to an accumulation of two potentially cytotoxic substances, deoxyadenosine and deoxy-ATP, in the lymphocytes of affected children (Donofrio *et al* 1978). The two most popular mechanisms postulated to explain this cytotoxicity are inhibition of S-adenosylhomocysteine hydrolase by adenosine and deoxyadenosine and, secondly inhibition of ribonucleotide reductase by deoxy-ATP (van Laarhoven *et al* 1983).

Some confusion about the direct causal relationship of ADA deficiency and SCID arose soon after the early reports, when a twelve year old !Kung ('Bushman') boy was discovered who, at first sight, was deficient

in adenosine deaminase activity but, in stark contrast to the ADA-deficient SCID patients, was perfectly healthy (Jenkins 1973). Subsequent studies revealed, however, that the deficiency in the !Kung was not as severe as that observed in SCID patients. He had 2-3% of normal enzyme activity in his red cells and 20-30% of normal activity in other tissues, levels which appeared to be adequate for efficient functioning of the immune system. This form of partial ADA deficiency was considered to constitute a recessively inherited entity mediated by an allele different from those associated with SCID. Population studies on the !Kung of the Kalahari suggested that this allele, designated $ADA^{8}$ occurred in that population at polymorphic frequencies (Jenkins *et al* 1976, 1979). Several other cases of partial ADA deficiency have subsequently been described and their residual enzyme characterised (Hirschhorn *et al* 1983; Dadonna *et al* 1983). Enzyme, DNA and mRNA studies of Epstein-Barr Virus transformed B-lymphocyte cell lines derived from several partially ADA-deficient individuals as well as from ADA-deficient SCID patients have revealed a great deal of genetic heterogeneity (Hirschhorn *et al* 1983; Adrian *et al* 1984; Dadonna *et al* 1985; Valerio *et al* 1984). Adenosine deaminase-deficient SCID cell lines have less than one percent of enzyme activity whereas the ADA activity of partially

ADA-deficient cells vary from about 5-50% of normal (Hirschhorn *et al* 1983).

## 4.2. A comparison with previous examples of partial ADA deficiency

The cell line derived from the !Kung boy, GM3043, had adenosine deaminase with a greatly reduced half-life, a normal electrophoretic mobility and a normal isoelectric point (pI 4.9) (Hirschhorn *et al* 1983). Several DNA probes encoding the gene or parts of the gene for adenosine deaminase have become available (Valerio *et al* 1981; Wigin[illegible]on *et al* 1983; Dadonna *et al* 1984) and studies of hybridization between a cDNA probe and adenosine deaminase mRNA revealed that the ADA mRNA concentration was elevated two-fold in the cell line from the !Kung boy which may explain why his enzyme activity level was so much higher (47.5%) than was found in other partially ADA-deficient cell lines. Cell line GM2294 was derived from a black American individual. Despite their common African origin, the properties of adenosine deaminase from this cell line varied considerably from the !Kung cell line, GM3043. Enzyme activity was lower, the t½ was lower and the isoelectric point was higher (pI 5.01) in GM2294 than in cell line GM3043. The hybridizable mRNA in GM2294 was present at about 85% of normal compared with 102% in GM3043 (Dadonna *et al* 1985).

For the purposes of measuring adenosine deaminase

activity the spectrophotometric method was found to be sufficiently sensitive in this case. By also using the radiochemical method, however, it was possible to measure the amount of deaminase activity occurring in the presence of the inhibitor of the major adenosine deaminase, EHNA. This allows for the estimation of the contribution from a minor isozyme known as $ADA_2$ which may or may not make a more important contribution to the overall deaminating activity occurring in the cells of individuals with partial adenosine deaminase deficiency than normal. It was shown in these experiments that the residual adenosine deaminating activity in the two individuals with partial adenosine deaminase deficiency was almost all EHNA-inhibitible ie. not the product of $ADA_2$ but rather the major gene for adenosine deaminase.

The differential activity seen in the red and white cells in the case of the proband is characteristic of cases of partial deficiency for adenosine deaminase. The two most likely explanations of this are either a mutation at a regulatory locus or one at the structural locus coding for an unstable form of the enzyme, as explained in the introduction.

Analysis of the family pedigree data on adenosine deaminase activity shows that the deficiency is probably inherited in a simple Mendelian fashion.

The heat stability experiment suggests that, under

the specified experimental conditions, the adenosine deaminase of the two partially deficient (II-2 and II-3) individuals is reduced by a factor of about four. The *in vivo* conditions, however, are much different from the experimental conditions and so one must be cautious not to extrapolate too readily from the experimental results. Certainly, the results suggest that the residual enzyme of the two partially deficient individuals is less stable than normal. This would help to explain the low red cell adenosine deaminase activity and also the differential tissue activity when comparing red and white cells of the same individuals. The anucleate red cells lose adenosine deaminase as the molecule degrades, and are unable to replace it. The white cells, being nucleated are able to synthesize new enzyme, replacing the loss, to a certain extent. The rate of replacement, however, is not sufficient to fully compensate the rate of loss and therefore the white cell activity, at about 20% of normal, is still much lower than normal.

Adenosine deaminase derived from red cells and white cells of the Xhosa proband reported here appears to resemble the enzyme from GM2294 rather than that from GM3043 and, like GM2294, our proband is black. His white cell activity level of 27% of normal compares well with the 20% of normal ADA activity of the GM2294 cell line. They both have reduced heat stability, normal electro-

phoretic mobility and an increased isoelectric point. Cell line GM4396 from a white, partially ADA-deficient individual had the lowest ADA activity seen for such a cell line at 4.8% of normal. The heat stability of ADA from this cell line was not as low as in GM2294 and GM3043, the electrophoretic mobility was increased, resembling the ADA-5 phenotype on starch gel electropherogrames, and the isoelectric point (pI 4.8) was slightly lower than normal (pI 4.9). Hybridizable mRNA levels were normal. Cell line K.S (Perignon *et al* 1979; Hirschhorn *et al* 1983) from a Mediterranean, partially ADA-deficient individual had properties which distinguished it from all of the other cell lines so that, in fact, no two enzymes from these four cell lines possessed comparable physical properties apart from differential tissue distribution ie. erythrocytes were more severely ADA-deficient than white cells in all cases.

It has been seen in several other rare, autosomal-recessive disorders, such as the haemoglobinopathies and lysosomal enzyme deficiencies that different cases represent different mutations at the same locus as part of an allelicseries (McKusick 1973). In a non-consanguineous family, a given affected individual may be a 'compound heterozygote' carrying two different 'deficiency' alleles. Evidence from the four partially-deficient cell lines is suggestive of at least four different structural mutations. Adenosine deaminase deficiency

with SCID and partial ADA deficiency may form such an allele series.

An indirect comparison of the proband's adenosine deaminase with that of the cell line, GM2294, suggests that the same mutation may be involved in the two cases. The isoelectric focusing study of the proband's family suggests, however, that two different ADA deficiency alleles may be segragating in this family. Both father (I-1) and mother (I-2) of the proband are considered to be obligate heterozygotes for partial adenosine deaminase deficiency and, indeed, enzyme activity levels support this hypothesis. Isoelectric focusing of red cell adenosine deaminase of I-1 reveals a major band of activity with a lower pI than normal ADA-1 corresponding to his ADA-5 phenotype which was seen on starch gel electrophoresis. In addition, a weakly staining band was seen with a more basic isoelectric point than ADA-1 although not as high as that of ADA-2. This band corresponds to enzyme coded by his ADA deficiency allele which has been inherited through two successive generations. The proband and his sister, II-1, demonstrate this band as does the proband's son (III-1) whose activity levels support the observations from isoelectric focusing and electrophoresis, that they are heterozygous for $ADA^1$ and an ADA deficiency allele. The proband's mother, however, does not demonstrate this weakly staining band, and only the major band of activity corresponding to enzyme coded for

by her *ADA*$^1$ allele is evident. If any enzyme is coded for by the deficiency allele of 1-2, it must be present at levels much lower than that coded for by the deficiency allele of her husband. This does not rule out, however, the possibility that it does have the same isoelectric point but the probable difference in activity is itself evidence of further genetic heterogeneity.

The two partially ADA-deficient individuals, II-2 and II-3, are probably 'compound heterozygotes' and possess one of three possible genotypes: (a) both alleles code for altered proteins, (b) one allele codes for an altered protein and the other is inactive or (c) one allele codes for an altered protein and the other codes for a very small amount because of an alteration in the regulation of ADA expression. Data from the isoelectric focusing experiments suggest that there are two mutant alleles present but this together with the results of the other experiments reported here do not permit definitive choices from among these possible genotypes.

There is evidence to support the existence of a regulatory locus controlling the expression of ADA. For example, there is an autosomal dominantly inherited 50-fold increase in ADA activity of erythrocytes but with normal lymphocyte and fibroblast activity (Valentine *et al* 1977). There is also the demonstration of the re-expression of human ADA in hybrids between a human tumour line not expressing ADA and a normal murine

cell line (Siciliano *et al* 1978) and the reported reversion to normality of an ADA-deficient lymphoblast cell line (Uberti *et al* 1983). A mutation at the postulated ADA-regulatory locus has also been invoked to explain the difference in ADA activity between erythrocytes and white cells although the commonly observed, reduced heat stability of the protein itself provides a more likely explanation. Reduced heat stability *in vitro* has been shown to reflect *in vivo* molecular stability. The anucleate red cells are unable to synthesize protein, so unstable ADA molecules are more quickly depleted than they would be in white cells which are capable of protein synthesis.

4.3. *ADA*$^5$ in association with partial ADA deficiency

Starch gel electrophoretic studies suggest that, as well as two rare ADA deficiency alleles, a rare structural allele, probably *ADA*$^5$, is segregating within this family. The sister (II-1) of the proband has the phenotype ADA5-1,having inherited the *ADA*$^5$ from her father and *ADA*$^1$ from her mother. The rare ADA5-1 phenotype was first described in 1970 in two unrelated Afro-Americans (Detter *et al* 1970). A photograph of an ADA isoenzyme pattern, published elsewhere (Renninger & Bimboese 1970), also closely resembled the ADA5-1 phenotype; the individual involved was again African, being a member of the Macua tribe of Mozambique.

The isozyme pattern of ADA from the proband's sister

(II-1) resembles very closely that first described as ADA5-1 as well as that of the Macua tribesman. This family is also African, being members of the Xhosa tribe, and would support the suggestion that the *ADA*$^5$ allele has an African origin. The father of II-1 is very unusual because he is heterozygous for the deficient allele of ADA and *ADA*$^5$. His ADA might be considered to have the kinetic properties of ADA 5.

As mentioned earlier, ADA from the transformed partially ADA-deficient cell line GM4396 has increased electrophoretic mobility resembling ADA 5 (Hirschorn *et al* 1979). The finding of an *ADA*$^5$ allele in the family of the proband in association with partial ADA deficiency allele(s) is therefore of great interest although the possible significance of this finding is not easy to define.

## 4.4. Michaelis constants

The residual ADA of the proband has Michaelis constants for adenosine and deoxyadenosine which are very similar to the corresponding values of normal ADA. These findings suggest that the mutational event which results in low red cell ADA activity does not affect the active site of the enzyme. Thus the apparently low activity of the enzyme in red cells and to a lesser degree, white cells of the proband, would appear to be due to a reduced amount of enzyme rather than an inefficient enzyme. This could be due to a faster rate of enzyme breakdown or a slower rate of synthesis.

4.5. Deoxy-ATP levels

It has previously been reported (Hirschhorn *et al* 1979; Cohen *et al* 1978) that ADA-deficient SCID individuals have red cell deoxy-ATP levels which are several hundred-fold higher than those in normals. Red cells of partially ADA-deficient individuals have been shown, however, to have only slightly elevated deoxy-ATP levels (Hirschhorn *et al* 1979). In the present study, the proband's red cells were found to have 2-3 times the normal level of deoxy-ATP and the red cells of his sister, II-2, who has the same ADA phenotype, also showed a slight, though less marked, elevation in the level of this metabolite. These two partially ADA-deficient individuals apparently have sufficient residual ADA activity to prevent the *in vivo* accumulation of adenosine and deoxyadenosine to toxic levels. It has been suggested (Hirschhorn 1983) that the red cell deoxy-ATP levels as well as ADA activity levels should be determined in order to assess the susceptibility of the individual to immune system failure. Adenosine deaminase activities in red and white cells from partially deficient individuals show a very wide range which, at the lower end of the range, may overlap with activity levels found in ADA-deficient SCID patients. Elevated deoxy-ATP levels which, for at least one mechanism, provide the causal link between ADA deficiency and immunodeficiency are an indication of possible stress on the immune system, and should probably be monitored in ADA-deficient newborns as an aid to early detection of immunodeficiency.

Population Study

A population sample, drawn from the Xhosa chiefdom to which the proband and his family belong, was screened for individuals with low red cell ADA activity in order to investigate the possible prevalence of the gene(s) for partial ADA deficiency in this group. Two out of the 68 individuals tested had levels which were similar to those of the obligatory heterozygotes for partial ADA deficiency in the family of the proband and were more than two standard deviations below the sample mean. If these two individuals are carriers of the partial ADA deficiency state then the combined frequency of such alleles in this population sample is about 0.015±0.010, a frequency at which a polymorphism is deemed to be present. A partial ADA deficiency allele (called $ADA^{8}$) occurs, in fact, at a polymorphic frequency of about 0.11 in the !Kung San ("Bushmen") of the Kalahari (Jenkins *et al* 1979).

Until quite recently the !Kung lived in small family groups, hunting game and gathering plant foods. The Bantu-speaking Negro peoples are agriculturalists known to occasionally take San wives, with the result that gene flow between these two groups has occurred, mainly from the hunter-gatherers to the agriculturalists. It is, therefore, possible that an allele such as one resulting in partial ADA deficiency could have attained polymorphic frequencies in the San due to genetic drift and from them have been transmitted to the Bantu-speaking tribesmen.

There is no evidence, however, that the same ADA deficiency alleles are present in these two groups. There is, in fact, phenotypic evidence from studies on eleven other partially ADA deficient individuals that at least eight different partial ADA deficiency alleles exist in the nine individuals of African origin. Such variety does not support the genetic drift-gene flow hypothesis but would be in favour of selection by an environmental agent acting over a large area and malaria would be a possible agent but other intraerythrocytic parasites could be responsible. It is also possible that some of the differences observed between the products of some of the partial ADA deficiency alleles may reflect experimental error.

Four of the six previously described partially ADA-deficient individuals are of African origin (Hirschhorn *et al* 1983) providing circumstantial evidence that natural selection operating in a tropical environment might be responsible for the apparent high frequency of the alleles. It is hoped that DNA sequencing data will elucidate the problem by revealing the true degree of genotypic diversity in the gene for ADA as well as the relationship between mutation at the DNA level and structural effects on the enzyme.

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