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ARYLAMINE *N*-ACETYLTRANSFERASE IN HUMAN RED BLOOD CELLS

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Abstract—*N*-Acetyltransferase activities associated with erythrocytes from 20 individuals have been determined with *p*-aminobenzoic acid as substrate. A three-fold variation in V_{max} is found. The *N*-acetyltransferase genotype of the individuals has been determined and there is no correlation between the extent of acetylation measured in the individuals' erythrocytes and the inheritance of alleles at the polymorphic NAT locus. Folate is confirmed to be an inhibitor of arylamine *N*-acetyltransferase activity measured in erythrocytes. The content of folate in erythrocytes of individuals also varies. The individual with the maximum folate content has the minimum *N*-acetyltransferase activity. The monomorphic *N*-acetyltransferase gene from individuals spanning the range of *N*-acetyltransferase activity have been amplified, using the polymerase chain reaction. The pattern of restriction enzyme digestion of the monomorphic *N*-acetyltransferase gene with a series of eight restriction enzymes is the same for individuals spanning the activity range of arylamine *N*-acetyltransferase in their erythrocytes.

Arylamine *N*-acetyltransferase catalyses the acetylation of a range of arylamine and hydrazine substrates [1]. There are two gene loci in humans encoding different isozymes of *N*-acetyltransferase [2]. One gene locus (*pnat*,† equivalent to *NAT* 2 [2]) is multi-allelic and encodes polymorphic *N*-acetyltransferase. The other gene locus (*mnat*, equivalent to *NAT* 1 [2]) encodes monomorphic *N*-acetyltransferase [3]. The *pnat* locus products can confer either the fast or the slow acetylator pharmacogenetic phenotype [4, 5]. The *pnat* gene products are expressed in liver [3, 6] probably in hepatocytes [7]. The protein product of the allele which confers the fact acetylator phenotype (F1) [5] is able to metabolize sulphamethazine and isoniazid [3]. These substrates were among the first to be recognized as being subject to polymorphic acetylation in humans [8]. The *mnat* gene product is much more wide-spread in tissue distribution. It is expressed in human liver [3, 6] and is also expressed in human leucocytes [9–11] and in human erythrocytes [12]. Monomorphic NAT has a different substrate specificity to polymorphic NAT. Sulphamethazine is a poor substrate both for the expressed product of the *mnat* locus cDNA [3] and the *N*-acetyltransferase activity measured in whole blood [13]. *p*-Aminobenzoic acid is readily acetylated by the expressed monomorphic NAT [3] and is also acetylated by human erythrocytes [12]. So far, the coding region of the *mnat* genes and cDNAs which have been sequenced (less than 10) are invariant [2, 3]. However, we report that the specific activity of *N*-acetyltransferase varies amongst individuals with a three-fold variation in V_{max}. The individuals have been genotyped for the polymorphic *nat* [14] and the distribution of *pnat* alleles does not correlate with the *N*-acetyltransferase activity measured in the red cells of these individuals.

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† Abbreviations: PBS, phosphate-buffered saline (145 mM NaCl, 10 mM sodium phosphate, pH 7.5); *pnat*, genetic locus for human polymorphic arylamine *N*-acetyltransferase; *mnat*, genetic locus for human monomorphic arylamine *N*-acetyltransferase; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Erythrocytes. Samples (20 mL) of blood from individuals were collected in a final concentration of 5 mM EDTA and then centrifuged at 2000 rpm in a Sorvall RT6000 centrifuge at 4°, 20 min. The plasma was removed and the buffy coat was stored in liquid nitrogen for subsequent preparation of DNA. The red cell pellet was washed by centrifugation three times, each wash in a total volume of 50 mL of 10 mM sodium phosphate, 145 mM NaCl, pH 7.5, (PBS) at 4°. The final red cell pellet (5–7 mL) was resuspended in PBS (1 mL) and a sample (100 μL) was used to determine the number of intact erythrocytes after dilution with Trypan blue (0.1% w/v, in PBS) using a haemocytometer. The following reagents were added to the final concentrations indicated: phenylmethylsulphonyl fluoride (50 μM), leupeptin (10 μM) and dithiothreitol (2 mM). The red cells were then mixed by vortexing and stored as aliquots (2 mL) in liquid nitrogen. Before use for determination of *N*-acetyltransferase activity or for determination of folate content, cells were thawed at 20°. For measurement of enzymic activity, dithiothreitol was added again to give an additional 1 mM and the extent of lysis was determined to be greater than 95% by microscopic examination. The lysed red cells were centrifuged (13,000 rpm, 5 min) in a Fisons microfuge at 4° and the supernatant was used for determination of *N*-acetyltransferase activity. The amount of haemoglobin in the red cell lysates was determined after measuring the absorbance at 430 nm of a 1:1000 dilution in water, using 135 M^{-1} cm^{-1} as the extinction coefficient for haemoglobin [15]. The haemoglobin content varied

by less than 5% for different aliquots from each individual used for folate determination and for *N*-acetyltransferase activity measurements.

Determination of N-*acetyltransferase activity.* Routinely, *N*-acetyltransferase activity was determined with *p*-aminobenzoic acid as substrate as described previously [10], using a microtitre plate reader (Titertek Multiscan Plus II, Flow) for detection of the absorbance at 450 nm of the adduct formed between the remaining unacetylated arylamine and dimethylaminobenzaldehyde. For adaptation to erythrocytes, incubation of the lysate and substrates was carried out in conical centrifuge tubes (1.5 mL) in a total volume of 100 μL. All assays were done in triplicate and the red cell lysate was diluted in 20 mM Tris–HCl, pH 7.5 containing 1 mM EDTA and 1 mM dithiothreitol such that the initial rate of acetylation of *p*-aminobenzoic acid was linear for each concentration of *p*-aminobenzoic acid used. The concentration range was 75–800 μM *p*-aminobenzoic acid. All reagents, except acetylCoA, were pre-equilibrated for 5 min at 37°. In experiments with folate and amethopterin as inhibitors, pre-equilibration was carried out for 10 min at 37°. The reaction was started by addition of acetylCoA, in a volume of 20 μL to a final concentration of 0.4 mM. The reaction was stopped by addition of 100 μL of 20% (w/v) trichloracetic acid at 4° and the protein was pelleted by centrifugation for 5 min at 13,000 rpm. No material absorbing at 450 nm remained in the supernatant after precipitation of protein. The initial rate of acetylation of *p*-aminobenzoic acid was determined graphically. In each experiment, blank reactions were performed in parallel without substrate but including potential inhibitors, where appropriate.

In order to determine whether the interindividual variation in arylamine *N*-acetyltransferase activity was due to deacetylation of acetylarylamine occurring during the incubation, *p*-aminobenzoic acid was replaced by *N*-acetyl *p*-aminobenzoic acid (750 μM). Erythrocyte lysates pooled from five individuals were used for these assays.

Measurement of folate content of erythrocyte lysate. Samples of frozen erythrocyte lysates were thawed and diluted 1:20 with 0.1% (w/v) sodium ascorbate prior to determination of folate using growth of folate-requiring *Lactobacillus casei* [16]. The assays were done in duplicate and the method used is a routine clinical haematology test and was performed by J. Darley, Department of Haematology, John Radcliffe Hospital, Headington, Oxford. The accuracy of the method has been determined as ±9% by comparison of the means of 20 individual samples tested on two separate occasions.

Genotyping of polymorphic N-*acetyltransferase.* The genotyping method used is as described previously [14, 17]. It is based on the difference in susceptibility of individual alleles at the *pnat* locus to restriction enzyme digestion [14, 17]. Briefly, genomic DNA was prepared from the buffy coat and the *pnat* alleles were amplified by the polymerase chain reaction (PCR) using specific primers, as described previously [14, 17]. The alleles present were determined by comparison of restriction enzyme digestion patterns with *Kpn*I, *Taq*I, *Bam*HI and *Dde*I, with digestion patterns of samples of known genotype [14, 17]. The method has been validated by phenotyping all genotyped individuals in the present study using sulphamethazine as the probe drug, as described previously [14].

Comparison of monomorphic nat *genes of different individuals.* Genomic DNA was prepared from buffy coat and *mnat* was amplified using a pair of primers: monoA (sense) 5′ATCATGGACATTGAAGCATATC3′, which corresponds to nucleotides −19 to −3 of monomorphic *nat* [3]) and monoC (anti-sense) 5′AAATAGACAAGATTGTTTCACTC3′, which corresponds to nucleotides 880–902 of monomorphic *nat* [3]). The amplification conditions were 30 cycles: annealing at 60°, 1 min; extension at 72°, 2 min and denaturation at 94°, 0.5 min. The amplified product was extracted from under the mineral oil used to avoid evaporation in the PCR incubations. The products were digested in a total volume of 20 μL with restriction enzymes using the appropriate buffer. All restriction enzymes, *Taq* polymerase and buffers were purchased from Boehringer Mannheim.

RESULTS

Arylamine N-*acetyltransferase in individuals*

The acetylation of *p*-aminobenzoic acid was determined using erythrocyte lysates of each individual (Fig. 1a) as a source of enzyme. The V_{max} was determined from a Lineweaver–Burk plot (Fig. 1b). From a comparison of the V_{max} values of the 20 individuals tested it is clear that there is a variation in the activities amongst these individuals (Fig. 2). Results on rates of arylamine *N*-acetyltransferase obtained on a group including four of the same individuals whose erythrocyte *N*-acetyltransferase activities were investigated 3 years previously show that the inter-individual variation is consistent over this time period. The inter-individual variation is not due to a difference in the haemoglobin content of the individual erythrocytes, since the specific activities of the earlier samples are expressed relative to the number of red cells.

No acetylarylamine deacetylase activity was detected using a pool of erythrocyte lysates from five individuals. Therefore, these results indicate that the difference in activity measured is not due to deacetylation but is a difference in acetylation activity.

Effect of folate

Folate is an inhibitor of liver and leucocyte arylamine *N*-acetyltransferase [1]. It is an inhibitor of the human erythrocyte *N*-acetyltransferase measured with *p*-aminobenzoic acid as substrate [12]. Amethopterin is also an inhibitor [18] and this is illustrated here (Fig. 3). It was considered a possibility that the amount of folate present in the erythrocyte lysate of an individual could contribute to the difference in activity of *N*-acetyltransferase observed. The folate content of the erythrocytes of six individuals is shown in Table 1. Whilst the folate content of erythrocytes is below concentrations which have been tested as inhibitory (Fig. 3), the individual with the lowest *N*-acetyltransferase activity

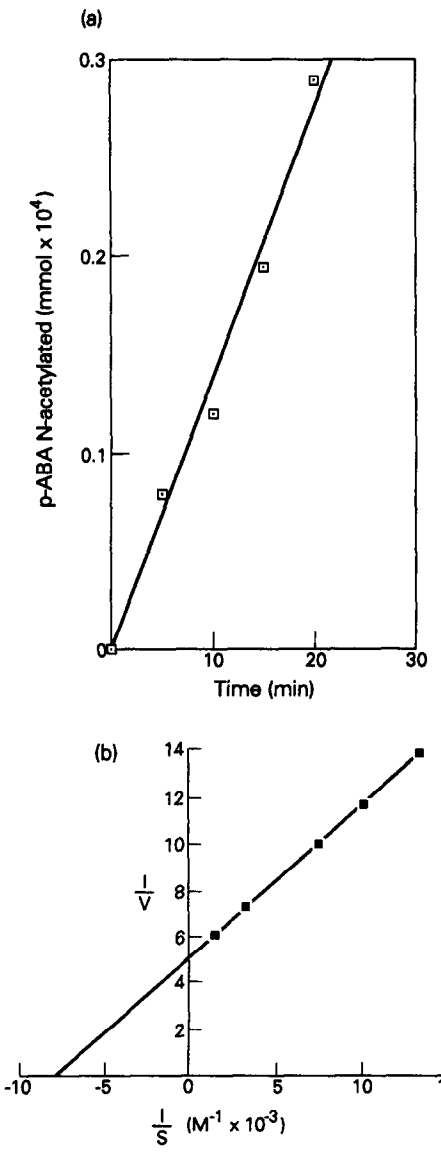

Fig. 1. (a) Rate of acetylation of *p*-aminobenzoic acid (pABA) catalysed by human erythrocyte lysates. Measurement of the acetylation of *p*-aminobenzoic acid was determined as described in Materials and Methods using the erythrocyte lysate from K.X. The concentration of *p*-aminobenzoic acid at the start of the reaction was 0.77 mM and the concentration of haemoglobin in the assay mixture was 101 mM. (b) Lineweaver–Burk plot for K.X. showing determination of V_{max}.

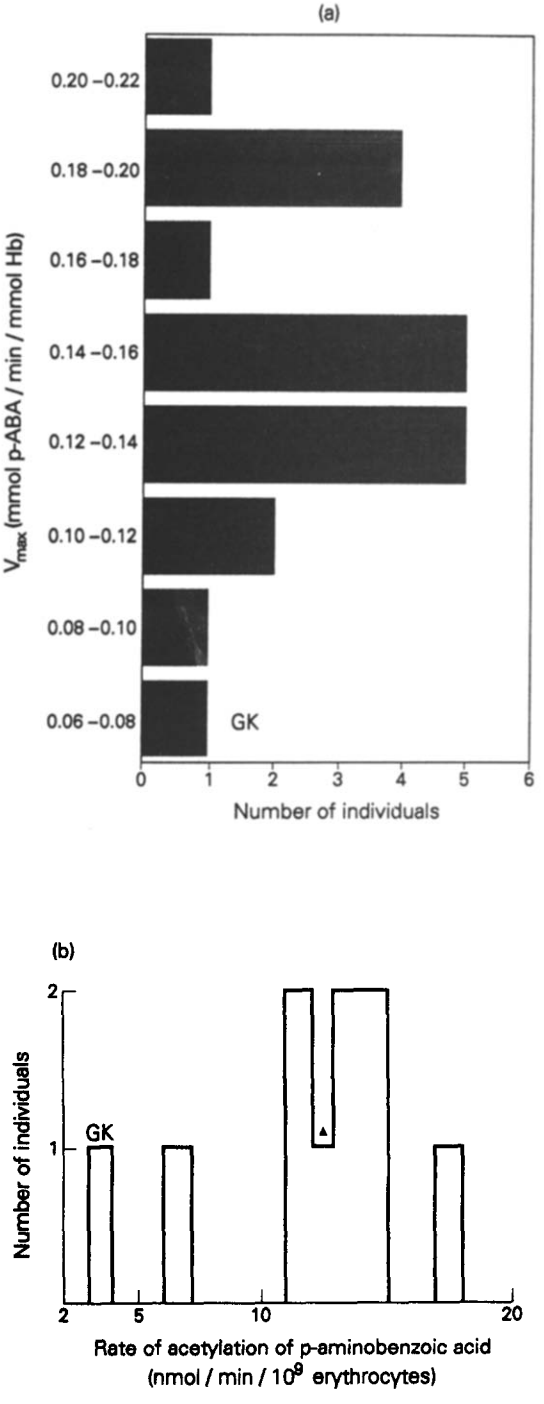

Fig. 2. Inter-individual variation in rate of *N*-acetyltransferase activity associated with human erythrocytes. (a) The V_{max} for the different individuals was determined as described in Materials and Methods as shown in Fig. 1 and are expressed as mmol *p*-aminobenzoic acid (pABA) acetylated per min per mmol Hb. The individual G.K. is indicated. (b) Rates of acetylation were determined as described in Materials and Methods except an initial concentration of *p*-aminobenzoic acid of 0.22 mM was used and an initial concentration of 0.5 mM AcetylCoA was used. The rate is expressed as nmol *p*-aminobenzoic acid acetylated per min per 10^9 erythrocytes. The individual G.K. is indicated. The triangle denotes a homozygous fast acetylator.

(G.K.) is the individual with the highest folate concentration. In contrast, the individuals with the lowest folate concentrations (K.K. and J.P.) were at the upper end of the range of erythrocyte arylamine *N*-acetyltransferase activity.

Genotype of polymorphic N-*acetyltransferase.* Using a method which correctly predicts the phenotype of greater than 95% of individuals from the alleles of the polymorphic *N*-acetyltransferase present in genomic DNA [14, 17], the genotype of

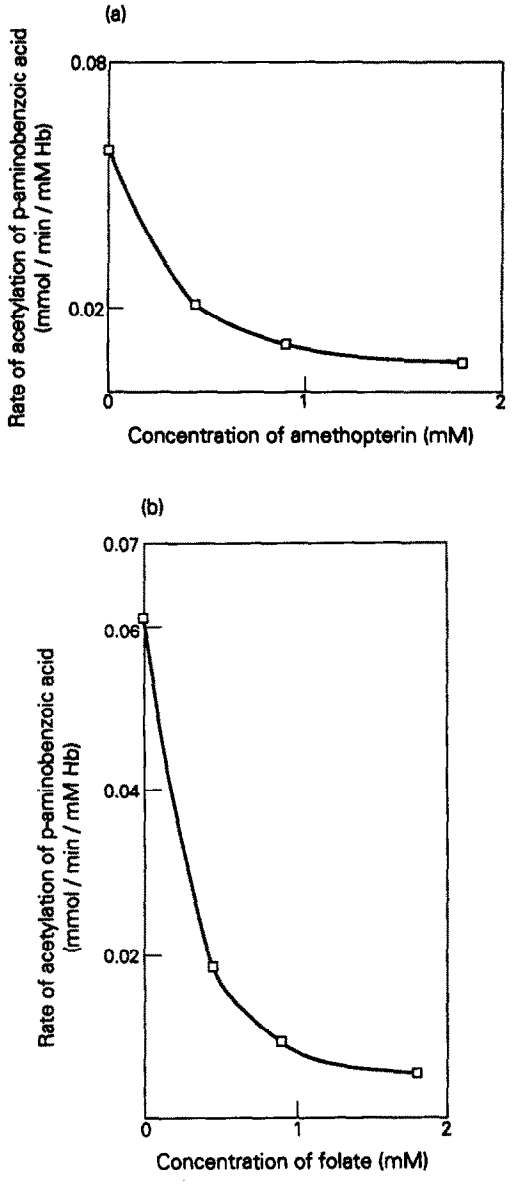

Fig. 3. Inhibition of acetylation of *p*-aminobenzoic acid by (a) amethopterin and (b) folate. (a) The rate of acetylation of *p*-aminobenzoic acid (0.28 mM) was measured as described in Materials and Methods in the presence of erythrocyte lysate (52 mM Hb) with increasing concentrations of amethopterin. (b) The rate of acetylation of *p*-aminobenzoic acid (0.33 mM) in the presence of erythrocyte lysate (57 mM Hb) was measured as described in Materials and Methods with increasing concentrations of folate.

the individuals in this study was determined and is shown in Table 1, together with the values for V_{max}. There is no correlation between the inheritance of any of the alleles and the activity of erythrocyte *N*-acetyltransferase for *p*-aminobenzoic acid. If rapid and slow acetylators are compared they are not significantly different (P = 0.34). In addition, the individual noted with a triangle in Fig. 2b is a homozygous fast acetylator (E.S., Table 1).

Monomorphic N-*acetyltransferase*

Using the primers monoA and monoC, the gene encoding monomorphic NAT only is amplified in the PCR. The product in each case is the same size and is resistant to digestion with *Hinc*II but is digested by *Hin*dIII into a major fragment of 850 bp and a small fragment which is not detected (Table 2). These are diagnostic tests for confirming the specificity of the amplification reaction, since the polymorphic NAT gene is resistant to digestion with *Hinc*II but is cleaved by *Hin*dIII [3]. The amplified monomorphic product from each of three individuals was digested with a series of restriction enzymes. The restriction enzymes digested the amplified product from each individual to produce the expected pattern of fragments. This is illustrated for digestion with the enzyme *Dde*I in Fig. 4. The sizes of the fragments produced with the other enzymes are illustrated in Table 2.

DISCUSSION

It has been shown that the *N*-acetyltransferase activity associated with erythrocytes does apparently vary amongst individuals using *p*-aminobenzoic acid as substrate. An inter-individual variation in erythrocyte *N*-acetyltransferase has been suggested previously using sulphamethazine as substrate and whole blood as the source of enzyme [19]. However, in these experiments the incubation period for detection of enzymic activity was 24 hr. Nevertheless, the interindividual variation was found to be reproducible over a period of 24 months and the variation in enzymic activity between individuals was found to be three-fold. In a recent report on *N*-acetyltransferase activity in blood leucocytes, *N*-acetyltransferase activity was measured with both *p*-aminobenzoic acid and sulphamethazine as substrates [11]. Cribb *et al.* [11] found that there was a 200-fold higher K_m of *N*-acetyltransferase from human mononuclear leucocytes for sulphamethazine and that the V_{max} with sulphamethazine was approximately 20-fold lower than with *p*-aminobenzoic acid. However, using *p*-aminobenzoic acid Cribb *et al.* observed a variation in acetylation of *N*-acetyltransferase of seven-fold.

In the present study, we have demonstrated that there is a variation in the capacity of individuals to acetylate *p*-aminobenzoic acid. The genotype of these individuals has been demonstrated to be independent of the extent to which acetylation is carried out. Indeed, the individual with the lowest level of acetylation was found to be a heterozygous fast acetylator and a homozygous fast acetylator fell in the middle of the range of individuals tested for the rate of acetylation of *p*-aminobenzoic acid.

Under the conditions of measurement of *N*-acetyltransferase, no deacetylation could be detected with pooled erythrocyte lysates and therefore variation in deacetylation is unlikely to contribute to the observed inter-individual variation in *N*-acetyltransferase activity. Deacetylase activity in liver has been associated with microsomes [20] and

Table 1. Comparison of genotype of *pnat* and activity of NAT of red blood cells

Individual	Genotype	Phenotype	V_{max} (mmol/min/mmol Hb)	Folate (μM)
J.P.	S1a, S1a	Slow	0.20	0.8
J.W.	S1a, S1a	Slow	0.13	—
Ja.W.	S1a, S1a	Slow	0.14	—
T.A.	S1c, S2	Slow	0.10	—
C.G.	S1a, S1a	Slow	0.14	—
E.C.	S1a, S1c	Slow	0.12	—
K.X.	S2, S3	Slow	0.19	1.5
A.C.	S1a, S2	Slow	0.12	1.4
J.H.	S1b, S2	Slow	0.15	—
D.H.	S1a, F1	h Fast	0.18	—
G.K.	S1a, F1	h Fast	0.08	2.1
T.D.	S1a, F1	h Fast	0.15	—
A.S.	S1a, F1	h Fast	0.20	—
L.C.	S2, F1	h Fast	0.17	—
K.K.	S2, F1	h Fast	0.19	0.8
P.J.	S2, F1	h Fast	0.15	1.2
E.S.	F1, F1	Fast	0.16	—

The genotype of individuals was determined from genomic DNA as described, and the V_{max} values were determined as illustrated in Fig. 1. "h" denotes a heterozygote. The concentration of folate in erythrocytes was determined as described in Materials and Methods and is expressed as the concentration of folate in lysed erythrocytes. A dash indicates the folate content was not determined.

Table 2. Restriction enzyme digestion of *mnat* from individuals

Enzyme	Sizes of fragments (bp)
*Hin*dIII	850
*Hinc*II	920
*Alu*I	730, 120
*Dde*I	340, 280, 160, 130
*Kpn*I	920
*Bam*HI	920
*Ban*HI	460, 350
*Taq*HI	380, 300, 230
*Rsa*I	400, 280, 230
*Sau*IIIA	260, 220, 150

The digestion products of amplification of genomic DNA from G.K., D.H. and J.P. with monoA and monoC as primers, as described in the text were identified after agarose gel electrophoresis and the sizes were determined by comparison with molecular mass markers, as in Fig. 5. The original product is 920 bp. The sizes of bands less than 100 bp cannot be determined accurately. The digestion pattern was the same for each of the three individuals. These individuals include the extremes of the range of erythrocyte arylamine *N*-acetyltransferase activity (see Table 1).

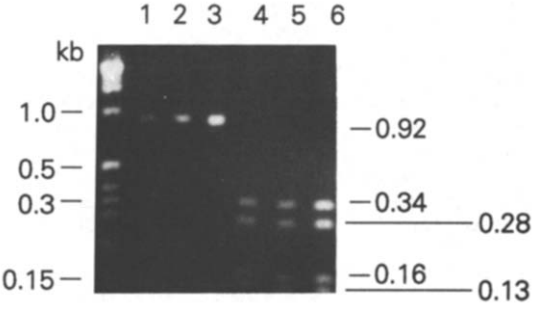

Fig. 4. Amplification of *mnat* using PCR and digestion with *Dde*I. Genomic DNA from G.K. (track 1), D.H. (track 2) and J.P. (track 3) was amplified with primers monoA and monoC as described in Materials and Methods. The amplified product is shown at 920 bp. The amplified product (approx. 1 μg) from G.K. (track 4), D.H. (track 5) and J.P. (track 6) was then digested with *Dde*I for 2 hr at 37°. The digestion products are shown and the sizes in kilobases are indicated on the right. The left hand track shows a 1 kb molecular mass ladder, and the sizes are denoted on the left.

this present study does not exclude the possibility of a deacetylase being associated with the membranous fraction of erythrocytes.

Folate is an inhibitor of *N*-acetyltransferase of erythrocytes, in agreement with previous studies [12]. We have, in addition, observed that there is a variation in the folate content of the erythrocyte samples of individuals which were used to determine *N*-acetyltransferase activity. The levels of folate detected in these erythrocyte samples is within the normal range [16]. Preliminary experiments have shown that washing intact erythrocytes after storage overnight has resulted in an increase in *N*-acetyltransferase activity with *p*-aminobenzoic acid as substrate (J. Gordon and E. Sim, unpublished) and this also suggests that there may be an endogenous inhibitor present. However, the amount

of folate which is detected in the erythrocyte lysates is less than has been shown to be inhibitory. The significance of these results is not entirely clear but it suggests that there may be an interaction between *N*-acetyltransferase and metabolism of folate. This is an interesting question because monomorphic *N*-acetyltransferase has a wide spread tissue distribution [1, 21]. It may therefore have another role in addition to metabolism of xenobiotics.

From the limited comparison carried out on *mnat* from three individuals across the erythrocyte *N*-acetyltransferase range, there appear to be no restriction fragment length polymorphisms with eight restriction enzymes. However, the restriction enzymes which were used to compare *mnat* from different individuals cleave *mnat* amongst them at a total of only 18 different sites in the *mnat* gene. It is therefore possible that there is a mutation in *mnat* which could change the activity of the isozyme in different individuals. There are only 13 amino acids different in rabbit polymorphic *N*-acetyltransferase compared with monomorphic *N*-acetyltransferase [22] and the monomorphic enzyme will not catalyse acetylation of sulphamethazine, whilst the polymorphic enzyme will acetylate both sulphamethazine and *p*-aminobenzoic acid. To be certain that there are no changes in human *mnat* which could give rise to the differences observed in activity with *p*-aminobenzoic acid as substrate it will be necessary to sequence across the *mnat* gene from different individuals.

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