Biochemical Pharmacology, Vol. 39, No. 4, pp. 647–654, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

ARYLAMINE *N*-ACETYLTRANSFERASE FROM FAST (C57BL6) AND SLOW (A/J) N-ACETYLATING STRAINS OF MICE

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(*Received* 20 *June* 1989; *accepted* 15 *September* 1989)

Abstract—Many drugs and xenobiotics which are arylamines or hydrazines are metabolized by *N*-acetyltransferase. The enzyme is polymorphically expressed in humans and inbred strains of laboratory animals can be classified as fast or slow acetylating strains. *N*-Acetyltransferase has been partially purified from livers from a fast acetylator, C57BL6, and a slow acetylator, A/J, strain of mouse. The enzyme has been purified 1900- and 955-fold, respectively from the two strains, but still represents less than 20% of the total protein. These studies show that at least 5000-fold purification is required to isolate mouse liver *N*-acetyltransferase from either strain. During purification, *N*-acetyltransferase from both strains of mice elute identically as a single peak on ion exchange chromatography. Sucrose density gradient centrifugation of *N*-acetyltransferase shows partial separation of the activity from A/J mice into two peaks whilst the enzyme from C57BL6 mice migrates as one peak which is distinct from both the major and minor types of *N*-acetyltransferase in A/J mouse liver. The hydrodynamic parameters of *N*-acetyltransferase from C57BL6 mice and the major peak of *N*-acetyltransferase from A/J mice show that these enzymes are likely to be monomers of apparent molecular weights 33,000 ± 1000 and 30,000 ± 2000, respectively. These results indicate that the *N*-acetyltransferase isozymes in liver of these two strains of mice are not identical.

Arylamine *N*-acetyltransferase has been identified as the activity responsible for acetylation of a range of arylamines including *p*-amino benzoic acid, sulphamethazine, anisidine, aminofluorene and other carcinogenic amines as well as the aromatic hydrazine, isoniazid [1]. *N*-Acetyltransferase activity is widespread and is found in liver [2, 3] but has also been reported in bladder [3], human lymphocytes [4] and rabbit reticuloendothelial cells [5]. The activity in any one species differs amongst individuals and humans have been classified as either fast or slow acetylators on the basis of ability to convert an appropriate orally administered substrate e.g. sulphamethazine [6] or a caffeine constituent [7] to the acetylated form detectable in urine and/or plasma. Strains of laboratory animals have been described as fast or slow N-acetylators on the basis of their ability to acetylate orally-administered substrate [8]. For mice, this N-acetylation status is also reflected in the acetylation activity measured *in vitro* of the livers, blood and bladders derived from these animals [3, 9]. The acetylation of *p*-amino benzoic acid by liver cytosol in the fast acetylating strain C57BL6 has been reported as up to three times quicker than with the slow acetylating strain A/J [9] although it has been suggested that greater discrepancies in *in vitro* acetylating activity may exist between fast and slow acetylating strains with different substrates. The existence of *N*-acetyltransferase isozymes within livers from individuals has been reported in hamsters [10] and humans [11] but not in rabbits [12] or pigeons [13]. Whilst there is good evidence for genetic control of these isozymes in hamster liver the individual genes in the hamster have not yet been identified. The molecular basis of *N*-acetyltransferase polymorphism may differ in different species and so *N*-acetyltransferase has been partially purified from fast and slow N-acetylating strains of mice and the hydrodynamic parameters of the enzyme from the liver of the two strains has been obtained. The results indicate that the enzymes differ in size between the two strains and an additional form of *N*-acetyltransferase is likely to be present in A/J mouse liver.

MATERIALS AND METHODS

Mice

C57BL6 and A/J mice (6 weeks old) were purchased from Charles River and killed by CO_2 asphixiation. Livers were removed immediately and homogenized in 5 vol. 50 mM potassium phosphate, pH 7.5 containing 100 mM KCl, 1 mM dithiothreitol (DTT), 50 μM phenylmethyl suphonylfluoride (PMSF) using a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). The homogenate was centrifuged (10,000 *g*, 20 min) and the supernatant from the first spin was centrifuged again (105,000 *g*, 60 min). All steps were carried out at 4° or on ice.

Assay of N-*acetyltransferase*

N-acetyltransferase was assayed with *p*-amino benzoic acid (final concentration 45 μM), as substrate [14]. The activity was measured after 10 min incubation at 37° and the enzyme sample was diluted with 50 mM potassium phosphate pH 7.5 containing

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1 mM DTT such that an absorbance decrease of less than 0.077 occurred at 540 nm which was established as within the linear range. Protein was determined as described by Lowry *et al.* [15] or from the absorbance at 280 nm.

Protein purification

Step 1. Ammonium sulphate precipitation was carried out by addition of solid ammonium sulphate to 40% (w/v), mixing for 30 min and collecting the pellets after centrifugation (10,000 *g*, 10 min). The pellets were discarded and the supernatants were made 70% (w/v) by addition of solid ammonium sulphate. After precipitation and centrifugation, as before, pellets were resuspended in 5 mM Tris–HCl, pH 7.5, containing 2 mM DTT, 1 mM EDTA and were dialysed against the same buffer.

Step 2. Resuspended pellets from step 1 were loaded at 1 mL/min onto a column (12 × 1.5 cm) of DEAE-Trisacryl M equilibrated in 15 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 mM DTT. The column was washed with the same buffer and then developed with a gradient (200 mL total volume) of 0–500 mM NaCl in the starting buffer. The flow rate during the gradient was 0.5 mL/min.

Step 3. The pool of *N*-acetyltransferase was concentrated 20-fold using an Amicon ultrafiltration cell with a PM10 membrane using O_2-free N_2 as the pressurizing gas and was then loaded (via a 0.2 mL loading loop) onto an FPLC Superose HR12 column (Pharmacia) equilibrated in 50 mM potassium phosphate, pH 7.5, containing 1 mM DTT. The column was eluted at 0.5 mL/min.

Step 4. For the final chromatographic step, the pool of *N*-acetyltransferase from gel filtration was diluted with 1 mM Tris–HCl, pH 7.5 containing 1 mM EDTA and 2 mM DTT to the ionic strength of the Mono Q equilibration buffer and loaded onto an FPLC Mono Q column (Pharmacia) equilibrated in 20 mM Tris–HCl pH 7.5, 1 mM DTT, 10% (w/v) glycerol. The column was washed and eluted with a gradient (30 mL) of 0–500 mM KCl at 1 mL/min.

Steps 3 and 4 were carried out at room temperature with fractions kept on ice. Other procedures were carried out at 4°.

SDS–polyacrylamide gel electrophoresis

Samples of column fractions and pools were routinely analysed by SDS–polyacrylamide gel electrophoresis after reduction and alkylation of samples at pH 6.8, as described previously [16]. To assign a band to *N*-acetyltransferase, samples of 50 μL of each fraction across the appropriate region of columns were treated identically and the stained gels [16] were compared with the activity profiles.

Analytical gel filtration

Samples of ammonium sulphate precipitates of mouse liver cytosol [0.5 mL containing 2.0–4.5 mg cytosolic protein and myoglobin (0.5 mg) as standard] were applied to a column (16 mm dia. × 95 cm) of Sephacryl S200 superfine and eluted with 50 mM potassium phosphate pH 7.5 containing 1 mM DTT at a flow rate of 6 mL/hr. The void volume (V_0) was determined from the elution volume of the breakthrough peak of protein (absorbance at 280 nm) on separation of liver cytosol and from the elution volume of protein aggregates on saturation of the column with 0.5 mL of human serum (diluted 1:5 with the same elution buffer). V_0 was the same by these two methods. The total permeable space (V_t) was determined from the elution of [^{3}H]glucose included in a sample of mouse liver cytosol. The column was calibrated using radio-iodinated bovine serum albumin (Stokes' radius, $a = 3.7$ nm) ovalbumin ($a = 2.81$ nm) carbonic anhydrase ($a = 2.43$ nm), soybean trypsin inhibitor ($a = 2.41$ nm) and myoglobin ($a = 1.93$ nm) using the same running conditions as for the experimental separations. ^{125}I-Labelled bovine serum albumin (prepared using Iodobeads as catalyst, as previously described, [17]) was detected by gamma-counting. Elution of other proteins was determined from the absorbance at 280 nm or, for myoglobin, from the absorbance at 420 nm. The Stokes' radii of the standard proteins was obtained from published tables [18] or was determined from published diffusion coefficients [19] using Eqn 1:

$$\text{Stokes' radius } (a) = \frac{kT}{6\,z D_{20,w}} \qquad (1)$$

where k = Boltzmann constant, T = temperature in °K, z = viscosity of water at 20° and $D_{20,w}$ = diffusion coefficient corrected to water at 20°.

The Stokes' radius of *N*-acetyltransferase from different sources was calculated using the average pore radius of the column determined from standard proteins by the method of Ackers [20] and was used to calculate the diffusion coefficient using Eqn 1. The sedimentation coefficients, determined from sucrose density gradient centrifugation, and diffusion coefficients of *N*-acetyltransferase were used to calculate the frictional ratios and molecular weight of *N*-acetyltransferase using the method of Siegel and Monty [21].

Sucrose density gradient centrifugation

Linear gradients of 5–20% (w/v) sucrose in 50 mM potassium phosphate pH 7.5, containing 1 mM DTT were prepared on ice and samples (200 μL) of mouse liver *N*-acetyltransferase after ammonium sulphate precipitation and dialysis (step 1) containing 2–6 mg protein were layered over the gradient and centrifuged for 22 hr at 55,000 rpm in an SW 60Ti rotor at 4°. The gradient was fractionated as previously described [22] except that fractions of eight drops or four drops were collected manually at 4°. Myoglobin was included in all tubes as an internal standard. Other protein standards, carbonic anhydrase, bovine serum albumin, and also myoglobin were centrifuged in parallel with experimental centrifugations. The sedimentation coefficient of *N*-acetyltransferase was determined by comparison with the migration positions of standard proteins, as previously described [22].

RESULTS

Protein purification

Ammonium sulphate precipitation of the cytosols of both C57BL6 and A/J mouse livers show that approximately 80% of the activity is recovered in

Table 1. Purification of *N*-acetyltransferase from C57BL6 mouse liver cytosol

Step	Volume (mL)	Total protein (mg)	Specific activity (nmol/mg/min)	Total activity (nmol/min)	Fold purification	Total recovery (%)	Step recovery (%)
Cytosol	50	782	12.1	9430	1	100	100
Ammonium sulphate precipitation	10	375.2	20.5	7680	1.7	81	81
DEAE-Trisacryl M pool	12	21.2	345.6	7326	28.6	78	95
FPLC Superose HR12 pool	8	1.4	3244.5	4542	268	48	62
FPLC Mono Q pool	1	0.1	23,020	2302	1902	24	51

Table 2. Purification of *N*-acetyltransferase from A/J mouse liver cytosol

Step	Volume (mL)	Total protein (mg)	Specific activity (nmol/mg/min)	Total activity (nmol/min)	Fold purification	Total recovery (%)	Step recovery (%)
Cytosol	60	732	15.7	11,520	1	100	100
Ammonium sulphate precipitation	10	351	26.2	9216	1.7	80	80
DEAE-Trisacryl M pool	12	19.4	422.7	8202	27	71	89
FPLC Superose HR12 pool	8	1.2	3346	4015	213	35	49
FPLC Mono Q pool	2	0.15	15,000	2250	955	19	56

both strains between 40% (w/v) and 70% (w/v) ammonium sulphate with a 1.7-fold purification in each case (Tables 1 and 2). The specific activity towards *p*-amino benzoic acid as substrate was lower with C57BL6 than with A/J mouse liver cytosol in the preparations described in Table 1 although values for the specific activity of C57BL6 cytosol ranged from 75 to 217% of the values obtained for A/J cytosol assayed at the same time. Ion exchange chromatography on DEAE-Trisacryl M, of the *N*-acetyltransferase recovered from ammonium sulphate precipitation shows that *N*-acetyltransferase emerges as a single sharp peak at 100 mM NaCl for both C57BL6 and A/J mice (Fig. 1). The purification factor of this step (27–29-fold) (Tables 1 and 2) is the same for both mouse strains as is the elution profile (Fig. 1a and b).

Subsequent gel filtration of the *N*-acetyltransferase pool from C57BL6 and A/J mice on Superose HR12 after ion exchange on DEAE-Trisacryl M results in a single peak of activity (Fig. 2). The elution profile of both preparations is similar although the peak is slightly broader for the A/J preparation (Fig. 2a and b). The purification factor is again similar for both

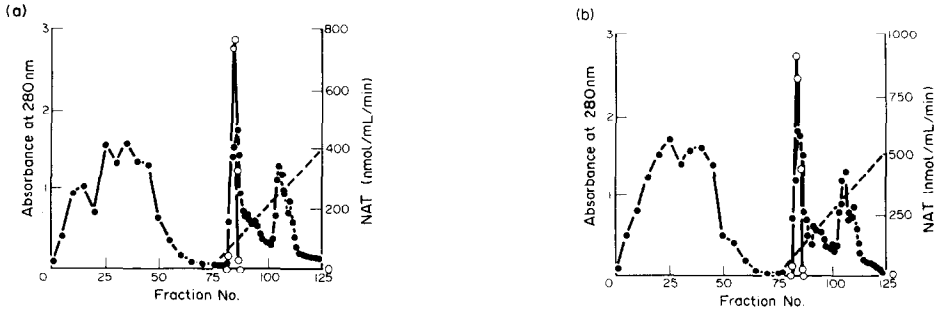

Fig. 1. Ion exchange chromatography of *N*-acetyltransferases from (a) C57BL6 and (b) A/J mouse liver on DEAE-Trisacryl M. The ammonium sulphate precipitates were dialysed into 15 mM Tris–HCl, 1 mM EDTA, 2 mM DTT and separated as described in the text. Fractions of 4 mL were collected. The *N*-acetyltransferase activity was measured (open circles) and the absorbance at 280 nm was determined (closed circles). The gradient of NaCl (0–500 mM) in the same buffer is indicated by the dashed line.

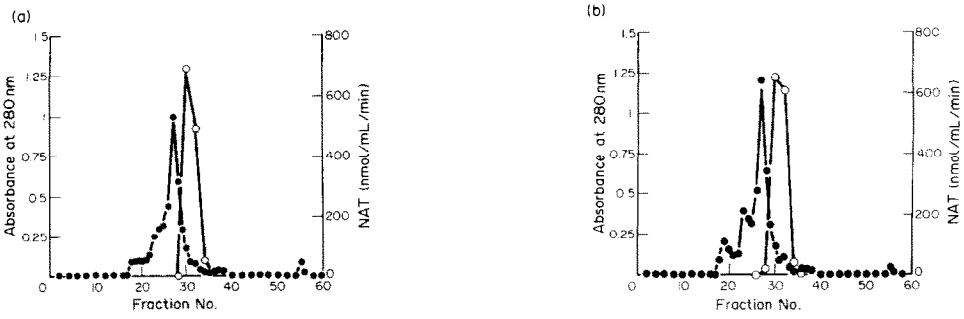

Fig. 2. Gel filtration of *N*-acetyltransferase from (a) C57BL6 and (b) A/J mouse liver on FPLC Superose HR12. The pools of *N*-acetyltransferase from DEAE-Trisacryl M chromatography (Fig. 1) were each concentrated to 0.8 mL and separated, each in 4 batches, on a column of Superose HR12 and eluted with 50 mM potassium phosphate, pH 7.5 containing 1 mM DTT. Fractions of 1 mL were collected and *N*-acetyltransferase activity is shown by the open circles. The absorbance at 280 nm is shown by closed circles.

strains of mice. The recovery of this gel-filtration step was, in this case, 62% for C57BL6 and 49% for A/J mice.

The second ion exchange step on FPLC Mono Q shows a single peak eluting between 150 and 165 mM NaCl for both mouse strains (Fig. 3a and b). The overall recovery of *N*-acetyltransferase from both mouse strains is around 20%. The purification factor for the fast acetylating strain is 1902-fold whilst for the slow acetylating strain, the purification factor is less than 1000-fold.

SDS–polyacrylamide gel electrophoresis shows that there are still at least 10 different proteins after this purification for both strains. The band on SDS–polyacrylamide gels corresponding to *N*-acetyltransferase has an apparent molecular weight of 33,000 and has been identified from SDS–polyacrylamide gel electrophoresis of all chromatographic fractions. A protein band in the molecular weight 30,000–33,000 has been observed in purifications of *N*-acetyltransferase from C57BL6 and A/J mouse liver to have a peak of staining intensity coincident with the peak of *N*-acetyltransferase activity in the final three stages of purification (Tables 1 and 2). The assignment of a protein band of apparent molecular weight 33,000 to *N*-acetyltransferase has also been confirmed from large-scale purifications of *N*-acetyltransferase using 15 g of liver cytosolic protein as starting material.

Determination of hydrodynamic parameters

Fractionation of ammonium sulphate precipitate pools of C57BL6 and A/J mouse liver cytosolic fractions on Sephacryl S200 shows that the peaks of *N*-acetyltransferase from fast and slow N-acetylating strains are superimposable (Fig. 5) although the elution of the enzyme from A/J mouse liver was found to have a slightly longer trailing edge. The Stokes' radii of the enzymes of both strains from this gel filtration column are calculated to be the same (Table 3).

After sucrose density gradient centrifugation *N*-acetyltransferase activity from C57BL6 strains was reproducibly found to migrate faster in the gradient (Fig. 6). In addition, a shoulder of activity was consistently found associated with the leading edge of *N*-acetyltransferase from the slow acetylating (A/J) strain of mice. From these centrifugation and gel

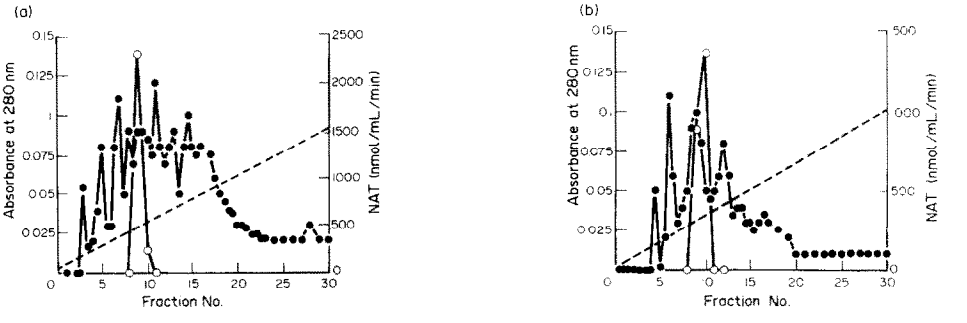

Fig. 3. Ion exchange chromatography of *N*-acetyltransferase from (a) C57BL6 and (b) A/J mouse liver on FPLC Mono Q. Pools of *N*-acetyltransferase from the previous gel filtration step (Fig. 2) were diluted and applied to a Mono Q column equilibrated in 20 mM Tris–HCl, pH 7.5 containing 10% (w/v) glycerol and 1 mM DTT. A gradient of 0–500 mM KCl was developed (dashed line) as described in the text. Fractions of 1 mL were collected over the gradient. *N*-Acetyltransferase activity (open circles) and absorbance at 280 nm (closed circles) were determined.

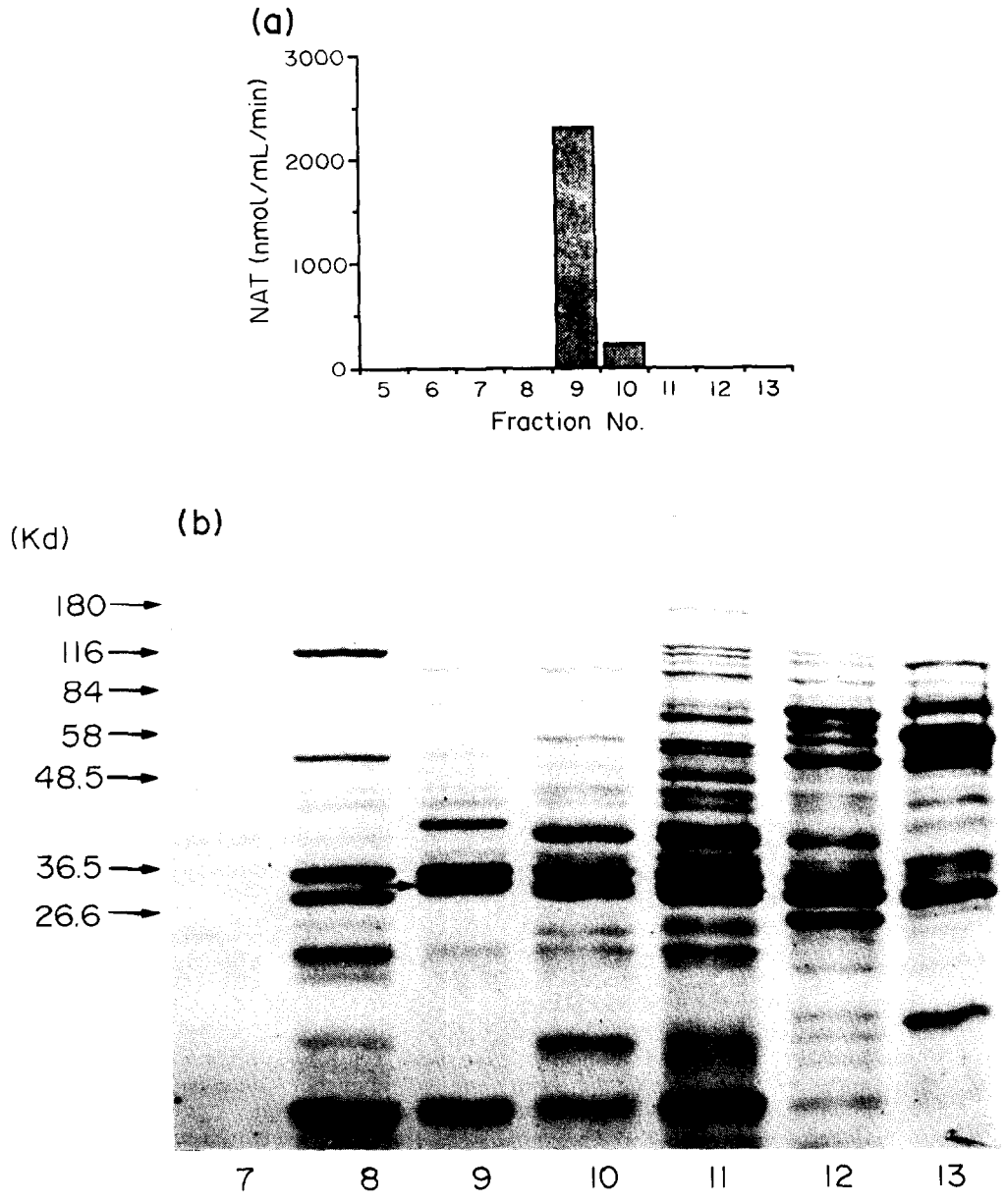

Fig. 4. SDS–polyacrylamide gel electrophoresis of fractions from the peak region of *N*-acetyltransferase of separated on FPLC Mono Q. The activity profile (a) is shown for a preparation from C57BL6 mouse liver. The gel is 12% (w/v) acrylamide and the equivalent of 25 μL of each fraction was electrophoresed in each track shown in (b). The migration positions of pre-stained molecular weight markers are shown. The assigned *N*-acetyltransferase band is indicated with an arrow.

filtration experiments, the molecular weights of the major peak of *N*-acetyltransferase from the two strains was found to differ slightly (Table 3). However, these data and the information from SDS–polyacrylamide gel electrophoresis shows that, like the *N*-acetyltransferases which have been identified from other sources, the enzyme appears to be a monomer of approximately 30,000 molecular weight. The sedimentation coefficient of the minor peak of *N*-acetyltransferase (Fig. 6) cannot be calculated accurately but it is less than 3.3S. Therefore the minor peak does not represent a multimer of *N*-acetyltransferase.

The frictional ratio of *N*-acetyltransferase from both strains suggests that the molecule is spherical as has been observed for the pigeon liver enzyme [13].

Inhibition studies. The enzymes from fast and slow N-acetylating strains could not be distinguished on the basis of inhibition by iodoacetamide (Fig. 7) or increasing salt strength. KCl and NaCl were found to inhibit the enzymes to 50% at around 250 mM.

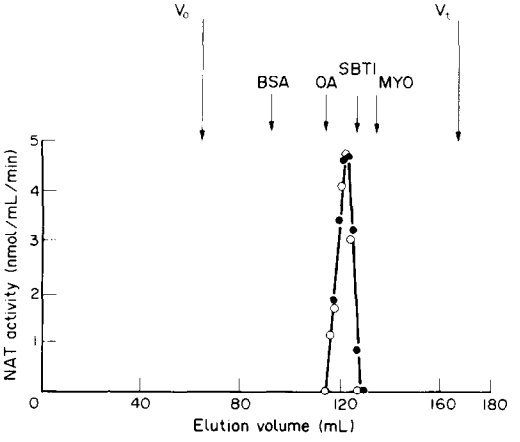

Fig. 5. Analytical gel filtration of *N*-acetyltransferase. C57BL6 and A/J mouse liver. Samples (0.5 mL) of ammonium sulphate precipitates from liver cytosol of C57BL6 (open circles) and A/J (closed circles) mice were separated on a calibrated column (16 mm × 95 cm) of Sephacryl S200. The column was calibrated as described in the text. The elution positions of bovine serum albumin (BSA), ovalbumin (OA), soybean trypsin inhibitor (SBTI) and myoglobin (MYO) are shown. The void volume (V_0) and the total permeable space (V_t) are shown.

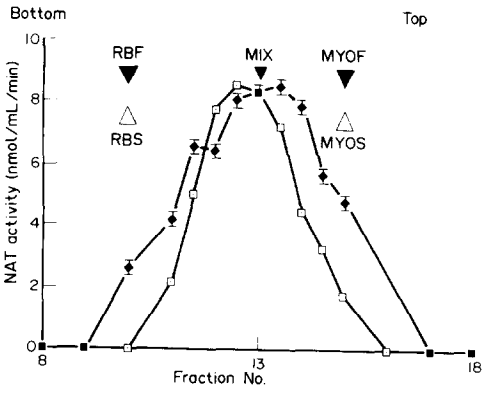

Fig. 6. Sucrose density gradient centrifugation of *N*-acetyltransferase. Samples (200 μL) of mouse liver cytosol containing 2.4 mg cytosolic protein (C57BL6) and 3.1 mg cytosolic protein (A/J) after ammonium sulphate precipitation or a mixture of both (Mix) and each also containing 0.2 mg myoglobin as internal standard were layered over a 5–20% (w/v) sucrose gradient in 50 mM potassium phosphate pH 7.5, containing 1 mM DTT, as described in the text, and centrifuged (22 hr, 55,000 rpm at 4°) in an SW 60Ti Beckman rotor. Nine fractions of 8 drops were collected from the bottom of the gradient and then half-sized fractions of four drops were collected until fraction 15. *N*-Acetyltransferase activity is shown for C57BL6 (open squares) and A/J (closed symbols) is shown across the region of collection of half-size fractions. The position in the gradient of myoglobin (MYO) and red coloured proteins (RB) in the cytosolic fraction was determined by the absorbance at 420 nm. Their positions in C57BL6 and A/J gradients are indicated by F and S, respectively. Mix indicates the peak fraction in a gradient loaded with a mixture of C57BL6 and A/J cytosol. In gradients run in parallel, peaks of bovine serum albumin, carbonic anhydrase and myoglobin were found in fractions 9, 12.5 and 15, respectively. The direction of the gradient is indicated.

DISCUSSION

It is clear that at 2000 and 1000-fold purification, respectively, *N*-acetyltransferase from C57BL6 and A/J strains of mice is estimated to be only 10–20% pure. This is in agreement with a recent study [23] which was reported during preparation of this manuscript. In the study of Mattano *et al.* [23] 10,000-fold purification of *N*-acetyltransferase from C57BL6 mouse liver is shown to be required to effect isolation of the enzyme. *N*-Acetyltransferase from A/J mouse liver was still impure after 20,000-fold purification. Approximately 4000-fold purification has been reported for isolation of the pigeon liver *N*-acetyltransferase [13] and for the chick liver *N*-acetyltransferase at least 3000-fold purification is required [24]. The rabbit liver *N*-acetyltransferase is pure after 1500-fold purification [12]. For the human liver enzyme, one of the isozymes is reported as being only 10% pure after 1000-fold purification [11]. Therefore the *N*-acetyltransferase family represents a minor cytosolic component in all species investigated although it is relatively more abundant in the fast-acetylating rabbit.

Mouse liver *N*-acetyltransferase appears to behave as a single isozyme for both fast and slow N-acetylating strains on ion exchange chromatography, in agreement with the work of Mattano *et al.* [23]. This is different from human [11] and hamster [10] liver *N*-acetyltransferase in which two isozymes, separable by ion exchange chromatography, have been found.

Table 3. Hydrodynamic parameters of *N*-acetyltransferase from C57BL6 and the major form of *N*-acetyltransferase from A/J mouse liver

	Source of *N*-acetyltransferase	
	C57BL6	A/J
Sedimentation coefficient S_{20^w} (S)	3.23 ± 0.13 (6)	2.97 ± 0.2 (6)
Stokes' radius (nm)	2.34 (2)	2.29 (2)
Molecular weight (M_r)	33,000 ± 1000	30,000 ± 2000
Frictional ratio (f/f_0)	1.01	1.05

Values are presented as the mean ± SD and the number of determinations is shown in brackets. For two determinations values are presented as the average. Molecular weights and frictional ratios were determined after the method of Siegel and Monty [21].

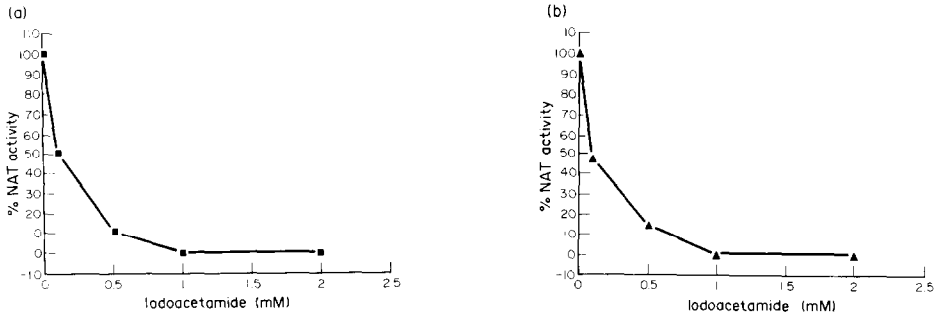

Fig. 7. Inhibition of *N*-acetyltransferase from (a) C57BL6 and (b) A/J mouse liver by iodoacetamide. Samples of *N*-acetyltransferase purified to around 200-fold after Sepharose HR12 chromatography (Table 1) were desalted into 50 mM potassium phosphate, pH 7.5, on a Pharmacia PD 10 column to remove DTT. Samples (25 μL) were diluted (1:20) in the presence of various concentrations of iodoacetamide added from a 4 mM stock solution, in the same buffer. Activity with *p*-amino benzoic acid was determined, as described in the text. The percentage activity remaining compared with a control sample with no iodoacetamide is shown.

For the A/J mouse strain our results suggest that there is a minor form of *N*-acetyltransferase which can be partially resolved by sucrose density gradient centrifugation. Although the major forms of *N*-acetyltransferase from A/J and C57BL6 mice are very similar, in agreement with the study of Mattano *et al.* [23] our results, using sucrose density gradient centrifugation, indicate that they are not identical. It is clear that the mechanism of generation of *N*-acetyltransferase polymorphism differs in different species. In rabbit, *N*-acetyltransferase has recently been cloned and sequenced [25] and in the slow acetylator rabbit it appears that the gene is deleted.* This is certainly not the case in mice.

Pigeon [13], rabbit [12] and chicken [24] liver all appear to express a single *N*-acetyltransferase isozyme. For all *N*-acetyltransferases which have been identified so far the molecular weight appears to be around 30,000. Forty-six per cent protein sequence identity exists between the chick [25] and rabbit [26] *N*-acetyltransferase but sequence comparison is required between *N*-acetyltransferase from fast and slow acetylating strains of mice to provide more information on the molecular nature of the polymorphism in this species.

Acknowledgements—We thank Bob Sim, Denis Grant and Urs Meyer for helpful discussions, and Urs Meyer for communicating data prior to publication. Janet Wood provided superb technical assistance. The studies were supported by the Medical Research Council and by the Wellcome Trust.

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