The Isolation and Preliminary Characterization of *N*-Acetyl-D-Glucosamine Kinase from Rat Kidney and Liver

M. Bruce ALLEN and Deryck G. WALKER
Department of Biochemistry, University of Birmingham, P.O. Box 363,
Birmingham B15 2TT, U.K.

(Received 26 July 1979)

1. Procedures for the extensive purification in high yield of *N*-acetyl-D-glucosamine kinase from rat liver and kidney are described. The separation of this enzyme from hepatic glucokinase depended primarily on their differing behaviour on an affinity column of Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. 2. This *N*-acetyl-D-glucosamine kinase also catalyses the phosphorylation of *N*-acetyl-D-mannosamine and, at a lower rate, several other sugar analogues, including D-glucose. 3. A comparison of the behaviour of the enzyme during gel filtration and electrophoresis in sodium dodecyl sulphate/polyacrylamide gels suggests that *N*-acetyl-D-glucosamine kinase is a symmetrical dimer of mol.wt. 80 000.

Weinhouse (1976) has given an account of the discovery in rat liver of a unique glucose-phosphorylating enzyme known as glucokinase (EC 2.7.1.2), which has a much higher K_m [about 5 mM (Storer & Cornish-Bowden, 1976; see also Niemeyer *et al.*, 1975)] than other mammalian hexokinases (EC 2.7.1.1) (Walker, 1966; Ureta, 1975). There have been several reports of the presence of this glucokinase in low activity in non-hepatic tissues [see Weinhouse (1976) for a survey]. Evidence has also been presented suggesting that there may be high- K_m glucose-phosphorylating activity in rat liver that cannot be accounted for by the glucokinase activity (Alvares & Nordlie, 1977).

We have reported preliminary evidence, involving electrophoresis in agarose gel, separations by chromatography on DEAE-Sephadex and immunological procedures, that four types of glucokinase may be present in rat liver (Allen & Walker, 1976). One of these, with the slowest electrophoretic mobility and provisionally designated GK_a, was demonstrated electrophoretically to be present in several other rat tissues. Conditions for separating this minor activity from the remaining glucokinase activity in liver had been found during the development of our procedure for the purification of the latter (Holroyde *et al.*, 1976b). Working on evidence (M. B. Allen, J. L. Brocklebank & D. G. Walker, unpublished observations) that isoenzyme GK_a may in fact be *N*-acetyl-D-glucosamine kinase (EC 2.7.1.59), which possesses some reactivity towards glucose as a substrate, a possibility strengthened by a preliminary report by Davagnino & Ureta (1978), we now report on the isolation of

this enzyme from rat liver and kidney tissue, and its characterization.

Leloir *et al.* (1958) first reported the presence of *N*-acetyl-D-glucosamine kinase activity in $(\text{NH}_4)_2\text{SO}_4$ fractions prepared from rat liver and kidney. The enzyme has been purified 110-fold from rat liver and shown to have a K_m for *N*-acetyl-D-glucosamine of 0.06 mM and to be inhibited by ADP (Saeki *et al.*, 1971). The enzyme in pig kidney is also inhibited competitively by ADP (Ghosh & Datta, 1967). More extensive purifications of *N*-acetyl-D-glucosamine kinase from human gastric mucosa (Gindzienski *et al.*, 1971, 1974) and pig spleen (Datta, 1970, 1971) have been described, but in no case was any examination of the purity of the preparations reported. The procedures we describe provide the basis for the isolation of more highly purified enzyme from any mammalian tissue.

Experimental

Materials

The sources of most materials have been given previously (Holroyde *et al.*, 1976a,b). Agarose and Bio-Gel P-100 were purchased from Bio-Rad Laboratories, Bromley, Kent, U.K., and phenyl-Sepharose was from Pharmacia, Hounslow, Middx., U.K. *N*-Acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine 1-phosphate were from Sigma Chemical Co., Poole, Dorset, U.K.

Alkaline phosphatase (grade I, from calf intestine) was from Boehringer Co. (London) Ltd., Lewes, Sussex, U.K. A sample of hexokinase purified from

rat muscle and having a specific activity of 200 units/mg of protein was kindly provided by Mr. B. Connolly of this Department.

Assay of enzymic activities

N-Acetyl-D-glucosamine kinase activity was assayed by coupling the ADP formed in the reaction to the oxidation of NADH by means of pyruvate kinase and lactate dehydrogenase, and measuring absorbance at 340 nm in a Gilford 2400 S multiple-sample recording spectrophotometer. The reaction mixture contained in a total volume of 1 ml: 100 mM-triethanolamine hydrochloride, pH 7.5, 5 mM-ATP, 10 mM-MgCl₂, 2 mM-*N*-acetyl-D-glucosamine, 0.4 mM-NADH, 2.5 mM-phosphoenolpyruvate, 1.25 units of pyruvate kinase, 3.25 units of lactate dehydrogenase and the enzyme sample. One unit of *N*-acetyl-D-glucosamine kinase activity is defined as that which catalyses the formation of 1 μ mol of *N*-acetyl-D-glucosamine 6-phosphate/min at 32°C.

Glucokinase and hexokinase activities were assayed at 32°C as previously described (Holroyde *et al.*, 1976a).

Chromatographic procedures

Sephacrose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was made and then coupled to CNBr-activated Sepharose 4B as previously described (Holroyde *et al.*, 1976a). The concentration of ligand coupled was about 16 μ mol/g wet packed wt. of gel.

Bio-Gel P-100, phenyl-Sepharose and ion-exchange resins were prepared and equilibrated with buffers as recommended by the respective manufacturers. Molecular weights were calculated from gel-filtration rates on Bio-Gel P-100 as described by Andrews (1965).

Electrophoretic procedures

Polyacrylamide-gel electrophoresis in the presence of 0.2% (w/v) sodium dodecyl sulphate was performed in slabs prepared at 7.5% (w/v) polyacrylamide (containing 3% of bisacrylamide), essentially as described by Weber & Osborn (1969). A discontinuous buffer system was used. The cathode compartment contained 0.1 M-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, pH 8; the gel was made in 0.01 M-NaH₂PO₄ buffer, pH 7, and the anode compartment contained 0.1 M-NaH₂PO₄ buffer, pH 7; 0.2% (w/v) sodium dodecyl sulphate was present in each. Samples of the enzyme and standard protein were prepared for electrophoresis by heating for 15 min with an equal volume of 2% (w/v) sodium dodecyl sulphate/5 mM-dithiothreitol solution in 20 mM-NaH₂PO₄ at pH 7.0.

Electrophoresis under non-dissociating conditions was performed with a discontinuous buffer system and agarose as supporting medium. Agarose

(0.8%, w/v) gels were made in 0.01 M-KH₂PO₄/0.05 M-glucose/1 mM-EDTA/10% (w/v) sucrose at pH 6.0 and cast between plastic plates to give a gel thickness of 3 mm and size approx. 10 cm $\times$ 10 cm. The gels resting on a Melinex film were placed on a metal cooling plate maintained at 0–2°C. Three thicknesses of 3 MM Whatman filter paper served as wicks connecting to the anode buffer (0.1 M-maleic acid adjusted to pH 6.0 with KOH) and the cathode buffer [0.1 M-Mops (4-morpholine-propanesulphonic acid) adjusted to pH 7.0 with KOH]. Electrophoresis took about 45 min to complete at 700 V and 50 mA, during which time a Bromophenol Blue marker transversed a distance of nearly 10 cm, i.e. almost completely along the length of gel.

The gels were stained for glucose-phosphorylating activity by flooding the gel with 1.5 ml of a solution containing per ml: 150 μ mol of KCl, 20 μ mol of triethanolamine hydrochloride, pH 7.5, 0.1 μ mol of EDTA, 10 μ mol of MgCl₂, 100 μ mol of glucose, 5 μ mol of ATP, 0.2 μ mol of NADP⁺, 1 unit of glucose 6-phosphate dehydrogenase activity, 2 mg of Nitro Blue Tetrazolium and 0.5 mg of phenazine methosulphate. The gel was then incubated in a humid box at 37°C. *N*-Acetyl-D-glucosamine kinase activity was detected by staining the gel with the assay medium for this enzyme as described above; the positions where oxidation of NADH occurred were observed under u.v. light.

Identification of product

Preliminary experiments indicated that it was necessary to include an ATP-regenerating system in the incubation mixture containing substrates and enzymes in order to overcome strong inhibition by the product ADP. The incubation mixture therefore contained in a total volume of 50 ml: 20 mM-triethanolamine hydrochloride, pH 7.5, 30 mM-KCl, 2 mM-ATP, 10 mM-MgCl₂, 10 mM-*N*-acetyl-D-glucosamine, 10 mM-phosphoenolpyruvate, 0.5 mM-dithiothreitol, 10 units of pyruvate kinase together with 0.5 unit of *N*-acetyl-D-glucosamine kinase isolated from either liver or kidney as described below and desalted by passing through a Sephadex G-25 column equilibrated with a buffer solution containing 20 mM-triethanolamine hydrochloride, pH 7.5, 30 mM-KCl and 0.5 mM-dithiothreitol. The mixture was incubated at 37°C for 5 h, when the enzyme was recovered from the mixture by ultrafiltration. Most of the nucleotides were removed from the filtrate by shaking vigorously for 5 min with 50 mg of activated charcoal/ml. The charcoal was removed by vacuum filtration and washed with water. The washings were combined with the initial filtrate and made up to a final volume of 150 ml. The reaction product was separated from unchanged *N*-acetyl-D-glucosamine by passing this solution through a

column (7 cm × 2 cm) of Dowex 1-X8 100 anion-exchange resin (dry mesh 50–100; Cl[−] form). The column was washed with 50 ml of water to remove traces of the substrate, and the reaction product was eluted with 20 ml of 0.1 M-HCl. The eluate was neutralized with aq. NH₃ and taken to dryness by rotary evaporation at 45°C. The residue was suspended in 5 ml of water.

Paper chromatography was carried out on Whatman No. 1 paper with butan-1-ol/acetic acid/water (4:1:5, by vol.) as solvent (Crumpton, 1959). Chromatograms were identified with AgNO₃ (Trevelyan *et al.*, 1950), phosphate (Bandurski & Axelrod, 1951) or Reissig (Cardini & Leloir, 1957) spraying reagents.

The reaction product was hydrolysed with alkaline phosphatase. A portion of the solution of product was made up to 20 mM with respect to glycine and 5 mM with respect to MgCl₂ and the pH adjusted to 8.0. Calf-intestinal alkaline phosphatase (10 units) was added and the mixture incubated at 32°C for 30 min. The products were examined directly by paper chromatography as above.

Analytical method

Protein was determined by measurement of absorbance at 280 nm (Layne, 1957).

Results

Purification of rat kidney *N*-acetyl-D-glucosamine kinase

Step 1: extraction. Adult rats (80) were killed by cervical dislocation. The kidneys were excised and immediately placed on ice; connective tissue and suprarenal fat were removed. The 150–200 g of tissue was homogenized for 15 s with 2 vol. of ice-cold 0.15 M-KCl/0.001 M-EDTA/0.01 M-NaHCO₃/0.05 M-glucose in an MSE Ato-Mix blade-type blender at top speed. The soluble fraction was obtained by centrifugation for 90 min at 20 000 *g*, followed by passage of the supernatant through glass wool to remove any fat-particles. This and all subsequent steps were carried out at 4°C.

Step 2: batch DEAE-cellulose (DE-52) chromatography. The ionic strength of the supernatant was lowered and glycerol included by the addition of 0.5 vol. of 0.02 M-KH₂PO₄/0.05 M-glucose/15% (v/v) glycerol, pH 6.5. The diluted supernatant was adjusted to pH 6.5 by the addition of 5% (w/v) phosphoric acid. Enzymic activity was adsorbed on the DEAE-cellulose by stirring for 5 min with 10% (w/v) of the column material, which had previously been equilibrated with 0.1 M-KCl/0.02 M-KH₂PO₄/0.001 M-EDTA/0.05 M-glucose/5% (v/v) glycerol, pH 6.5. The slurry was filtered on a Buchner funnel and washed with 25 bed-vol. of the

equilibration buffer that now also contained 0.5 mM-dithiothreitol. The moist cellulose cake was then packed into a 4.5 cm-diameter glass column with the aid of more equilibration buffer. The enzyme activity was eluted by increasing the KCl concentration in the wash buffer to 0.22 M. The enzyme is very stable at this stage and could be stored for several weeks at −10°C without loss of activity.

Step 3: gradient elution from DEAE-Sephadex. DEAE-Sephadex (A50) was equilibrated in 0.2 M-KH₂PO₄/0.001 M-EDTA/0.05 M-glucose/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 7.0. A column (4.5 cm × 5 cm) was prepared and two batches of enzyme from Step 2, i.e. the enzyme activity from about 150 rats or 300 g of kidney, was applied. Enzyme activity was eluted by a linear gradient formed from 250 ml of 0.2 M-KH₂PO₄/0.001 M-EDTA/0.05 M-glucose/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 7.0, and 250 ml of the same buffer but containing additionally 0.06 M-KH₂PO₄. A typical elution profile is shown in Fig. 1(a). The first small peak of phosphorylating activity towards *N*-acetyl-D-glucosamine to be eluted was found by electrophoresis in agarose to coincide with hexokinase isoenzyme II and represents about 20% of the apparent *N*-acetyl-D-glucosamine kinase activity applied to the column. The major peak of activity was pooled as indicated (Fig. 1a).

Step 4: hydrophobic chromatography. Preliminary experiments had shown that the latter *N*-acetyl-D-glucosamine kinase activity interacts relatively strongly with phenyl-Sepharose under conditions of comparatively low ionic strength, which are not particularly favourable to hydrophobic interactions. The pool of activity from Step 3 was applied therefore to a small column of phenyl-Sepharose (Fig. 1b) previously equilibrated with 0.22 M-KCl/20 mM-KH₂PO₄/1 mM-EDTA/50 mM-glucose/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 6.5. Most of the protein applied passed directly through the column. The column was washed with 1 bed-vol. of this buffer (applied at point A), and activity was eluted at point B with the same buffer, except that the concentration of KCl was 0.03 M.

Step 5. A column (2.5 cm × 4.5 cm) of Sepharose-*N*-(6-aminohexanoyl)-2-deoxy-D-glucopyranose, prepared as described in the Experimental section was equilibrated with 0.1 M-KCl/0.02 M-triethanolamine hydrochloride/0.001 M-EDTA/5% (v/v) glycerol/0.05 mM-dithiothreitol, pH 7.2. Active fractions from Step 4, pooled as indicated (Fig. 1b), were dialysed overnight against 10 vol. of this equilibration buffer, with no loss of activity, and applied to the column, which was then washed with equilibration buffer until no protein was detectable in the eluate by *A*₂₈₀ measurements. Some enzyme leaked from the column during the washing procedure as shown in Fig. 1(c), but most of the enzyme was

Fig. 1. Chromatography of *N*-acetyl-D-glucosamine kinase from rat kidney on (a) DEAE-Sephadex (Step 3), (b) phenyl-Sepharose (Step 4) and (c) an affinity column (Step 5)

(a) Pooled fractions from Step 1 were applied to a DEAE-Sephadex column ($4.5 \text{ cm} \times 5 \text{ cm}$) equilibrated with a buffer containing $0.20 \text{ M-KH}_2\text{PO}_4$, 0.001 M-EDTA , 0.05 M-glucose , $5\% \text{ (v/v) glycerol}$ and $0.5 \text{ mM-dithiothreitol}$, pH 7.0. When all the activity had been adsorbed, a linear gradient was applied consisting of 250 ml of the buffer and 250 ml of the same buffer except that the KH_2PO_4 concentration was 0.26 M . Fractions (9 ml) were collected. (b) A column ($3 \text{ cm} \times 4.5 \text{ cm}$) of phenyl-Sepharose was equilibrated with the above buffer containing 0.22 M-KCl instead of the KH_2PO_4 . At point A, when all the enzyme activity had been applied, a further 50 ml of the buffer was applied to the column, followed at point B by the same buffer except that the KCl concentration was 0.03 M . Fractions (9 ml) were collected. (c) A column ($2.5 \text{ cm} \times 4.5 \text{ cm}$) of Sepharose-*N*-(6-aminohexanoyl)-2-deoxy-D-glucosamine was equilibrated with $0.1 \text{ M-KCl}/0.02 \text{ M-}$

eluted from the column when 0.4 M-glucose was included in the equilibration buffer at point C.

Step 6. enzyme concentration. The active fractions, as indicated (Fig. 1c), were concentrated by ultrafiltration in an Amicon Diaflo cell with a PM-10 membrane. No activity was lost, and the protein content was determined by the difference in A_{280} between the non-diffusible material and the filtration eluate.

Overall purification and yield from kidney tissue

Table 1 summarizes the purification scheme: 35% of the *N*-acetyl-D-glucosamine kinase activity was lost at the DEAE-Sephadex stage, but approx. 20% may be accounted for as being due to hexokinase activity. A further 12% was lost on the phenyl-Sepharose column, and the 22% apparently lost on the affinity column could be accounted for by the leakage of activity before elution by glucose. The specific activity of the final product was 42 units/mg of protein, this being 320 times higher than the preparation after Step 2.

It was not possible to obtain a direct estimate of the *N*-acetyl-D-glucosamine kinase activity in the starting material, because of the presence of NADH oxidase and ATPase activities, which interfered with the ADP-formation assay. These contaminating activities were not prevented by desalting the extracts on a Sephadex G-25 column or by the addition of 30 mM-NaF or 2 mM-KCN or $10 \mu\text{g}$ of oligomycin/ml in the standard assay. Strong inhibition of the kinase activity by ADP (see below) would largely invalidate any assay involving direct estimation of *N*-acetyl-D-glucosamine disappearance or *N*-acetyl-D-glucosamine 6-phosphate formation. Assuming that there had been quantitative recovery of the enzyme after Step 2, the specific activity of the enzyme in the original supernatant (Step 1) was 0.001 unit/mg of protein, giving a 93 -fold increase in specific activity at Step 2 and hence an overall purification of 30000 -fold.

Purification of rat liver *N*-acetyl-D-glucosamine kinase

The basic difference of liver from kidney tissue from the viewpoint of purification of this enzyme is

triethanolamine hydrochloride/ $0.001 \text{ M-EDTA}/5\% \text{ (v/v) glycerol}/0.5 \text{ mM-dithiothreitol}$, pH 7.2. Active fractions from (b) were dialysed overnight against 10 vol. of this buffer and applied to the column. The column was then washed with the same buffer until no more protein was eluted. At point C, 0.4 M-glucose was included in the buffer. Fractions (8 ml) were collected. ●, A_{280} ; ○, *N*-acetyl-D-glucosamine kinase activity; △, hexokinase activity. The horizontal bars indicate those fractions that were pooled and taken to the next step.

Table 1. Purification scheme for rat kidney N-acetyl-D-glucosamine kinase

The results presented are for a preparation starting with kidneys (weighing 300g) from about 150 adult rats. The procedures used are described in the text. No starting activity can be quoted for reasons given in the text, and yields and purification are based upon the estimate of the activity in the initial eluate from DEAE-cellulose (Step 2); this estimate will include some hexokinase activity.

Step	Description	Volume (ml)	Protein concentration (mg/ml)	Activity (unit/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
2	Batchwise DEAE-cellulose	278	2.0	0.25	70	100	0.13	1
3	DEAE-Sephadex	224	0.63	0.20	45	65	0.33	2.5
4	Phenyl-Sepharose	102	0.16	0.36	37	53	2.2	17
5	Affinity chromatography	106	0.005	0.21	22	32	42	320

Fig. 2. Chromatography of N-acetyl-D-glucosamine kinase from rat liver on DEAE-cellulose and phenyl-Sepharose columns coupled in series (Step 2)

Columns of DEAE-cellulose and phenyl-Sepharose were connected in series as described in detail in the text. This Figure illustrates the composition of the fractions eluted from the second column. In the early stages illustrated this phenyl-Sepharose column alone was being washed with buffer containing 0.22 M-KCl. At point A the eluting buffer was changed to one containing only 0.03 M-KCl. Fractions (9 ml) were collected. O, N-Acetyl-D-glucosamine kinase activity; Δ, glucokinase activity.

the added presence of glucokinase, which behaves very similarly during the various fractionation procedures.

Step 1: extraction. The procedure was similar to that used for kidneys. The livers from 75 adult rats yielded 750–800g of tissue.

Step 2: batch chromatography on DEAE-cellulose and hydrophobic chromatography. The purification of N-acetyl-D-glucosamine kinase from liver was performed in conjunction with the initial stages of the purification of glucokinase (Holroyde *et al.*, 1976b). The batchwise chromatography on DEAE-cellulose was as described for the kidney enzyme above, except that 8% (w/v) DEAE-cellulose was added as adsorbant. The eluate was monitored during the elution phase for glucokinase activity. When this appeared the outlet tube was attached directly to the phenyl-Sepharose column (10cm × 4.5cm), and the two columns were run in

series. When glucokinase was no longer present in the eluate from the DEAE-cellulose column (measured in occasional samples), this column was detached and the washing of the phenyl-Sepharose column alone was continued. Extensive washing with approx. 6 bed-vol. was sufficient to elute most of the glucokinase activity, as shown in Fig. 2. The N-acetyl-D-glucosamine kinase activity was then eluted by lowering the ionic strength at point A, as described above for the kidney preparation and in Fig. 2.

Agarose-gel electrophoresis of the fractions confirmed that there was still a significant amount of glucokinase activity along with the N-acetyl-D-glucosamine kinase activity. The separation of these was the paramount problem and preliminary experiments revealed conditions under which affinity chromatography could be used to achieve this, as follows.

Fig. 3. Affinity chromatography (Step 3) of *N*-acetyl-D-glucosamine kinase from rat liver on Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose

Enzyme from Step 2 was applied to the column (5 cm $\times$ 4.5 cm) previously equilibrated as described in the text. The column was washed with more buffer until the protein content of the eluate was very low and *N*-acetyl-D-glucosamine activity had begun to leak from the column. At point A 0.4 M-glucose was included in the buffer. Fractions (9 ml) were collected. ●, A_{280} ; ○, *N*-acetyl-D-glucosamine kinase activity; △, glucokinase activity.

Step 3: first affinity column. A column (5 cm $\times$ 4.5 cm) containing the same ligand-matrix as described for the kidney enzyme was equilibrated with 0.1 M-KCl/0.02 M-triethanolamine hydrochloride/0.001 M-EDTA/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 7.2. The pooled enzyme fraction from Step 2 was dialysed against 10 vol. of the equilibration buffer without loss of activity, and the enzyme applied to the column. The column was then washed until enzyme activity began to leak from it, as shown in Fig. 3, and the bulk of the enzyme was then eluted by the addition of 0.4 M-glucose. Fig. 3 shows that some more glucokinase was removed during this step.

Step 4: gradient elution from DEAE-Sephadex (A50). The conditions were similar to those described for the kidney enzyme (Step 3), but a smaller column (2 cm $\times$ 4.5 cm) was employed. Fig. 4(a) shows a typical elution profile. Some protein came through the column before application of the gradient (not illustrated). Further glucokinase was removed at this stage, as shown, but complete separation of the *N*-acetyl-D-glucosamine kinase was not achieved. The reason for the double peak in the *N*-acetyl-D-glucosamine kinase activity profile has not been further investigated, because agarose-gel electrophoresis indicated that the *N*-acetyl-D-glucosamine kinase appeared to be the same in both peaks, which were therefore recombined. The ability of the enzyme to dissociate, discussed below, may have some bearing on these observations.

Step 5: second affinity column. This column was required to remove the last traces of glucokinase activity from the preparation and again utilized the different kinetic properties of glucokinase and *N*-acetyl-D-glucosamine kinase with respect to affinity for glucose. A 5 cm $\times$ 4.5 cm column of affinity matrix, as used in Step 3, was equilibrated with 0.03 M-KCl/0.02 M-triethanolamine hydrochloride/0.001 M-EDTA/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 7.2. The pool of fractions from Step 4, as indicated, was dialysed against 10 vol. of the equilibration buffer and then applied to the column. The remaining traces of glucokinase were eluted from the column with 0.05 M-glucose in equilibration buffer (applied at point A), as shown in Fig. 4(b). Some *N*-acetyl-D-glucosamine kinase activity was also eluted by this concentration of glucose, but most was eluted when 0.005 M-*N*-acetyl-D-glucosamine was applied at point B. The *N*-acetyl-D-glucosamine kinases eluted by both the glucose and the *N*-acetyl-D-glucosamine were found to be identical by both agarose-gel electrophoresis followed by staining for enzyme activity and on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms stained for protein. Fractions were therefore recombined (Fig. 4b).

Step 6: concentration of enzyme. This was achieved as described for the kidney enzyme.

Overall purification and yield from liver tissue

Table 2 summarizes the purification scheme.

Quantitative recovery of activity was obtained on the first affinity column, 37% was lost on the DEAE-Sephadex column and a further 6% was lost on the

second affinity column. Overall a recovery of 57% of the activity recovered from the initial combined DEAE-cellulose/phenyl-Sepharose treatment (Step 2) was obtained.

As described for the kidney, assuming quantitative recovery of activity at Step 2, a specific activity for the enzyme in the original supernatant of Step 1 may be estimated as 0.005 unit/mg of protein. Using this value, a purification of 1280-fold is estimated for Step 2, and the overall purification was 79 000-fold.

Purity of the N-acetyl-D-glucosamine kinase preparations

The specific activities of the purified kidney and liver enzymes were similar, as shown in Tables 1 and 2. Agarose-gel electrophoretograms, with the detection of the glucose-phosphorylating activity of the enzymes, are shown in Fig. 5. The preparations from both tissues showed a single band of activity, which was coincident with the non-fluorescent bands found when other gels were stained for *N*-acetyl-D-glucosamine kinase activity by the ADP assay (Susor & Rutter, 1971). Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels revealed two protein components in both liver and kidney preparations. Scans of these gels are shown in Fig. 6; comparison with standard proteins permitted mol.wts. of 90 000 and 39 000 to be calculated for the major bands. For the kidney the 90 000-dalton protein represents 60% and the 39 000-dalton protein 37% of the total protein. For the liver enzyme, the 90 000-dalton protein is 38% and the 39 000-dalton component 54% of the total protein. The question arose as to which of these proteins corresponded to active enzyme. Gel filtration on Bio-Gel P-100 was performed and the elution profile for the liver *N*-acetyl-D-glucosamine kinase preparation is shown in Fig. 7. The molecular weight for the active protein was estimated as 80 000 from the peak of the profile shown (Fig. 7b), but the elution profile was not symmetrical. Enzyme activity was detectable in fractions overlapping the region where the elution of glucokinase, with a mol.wt. of 50 000, would occur.

Fig. 4. (a) Chromatography of *N*-acetyl-D-glucosamine kinase from rat liver on DEAE-Sephadex (Step 4) and (b) second affinity chromatography of the enzyme on Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose (Step 5)

(a) The conditions under which the column (2 cm × 4.5 cm) was used were the same as described in the legend to Fig. 1(a). (b) The column (2 cm × 4.5 cm), as used in Step 3, was equilibrated with 0.03 M-KCl/0.02 M-triethanolamine hydrochloride/0.001 M-EDTA/5% (v/v) glycerol/0.5 mM-dithiothreitol, pH 7.2. The pooled fractions from Step 4, as indicated on (a), were dialysed overnight against 10 vol. of the buffer and then applied to the column. At point A, 0.05 M-glucose was added to the buffer. At point B the glucose was replaced by 0.005 M-*N*-acetyl-D-glucosamine. Fractions (6 ml) were collected. O, *N*-Acetyl-D-glucosamine kinase activity; Δ, glucokinase activity.

Table 2. Purification scheme for rat liver *N*-acetyl-D-glucosamine kinase

The results presented are for a preparation starting with 750 g of rat liver. All details are as in the legend to Table 1. Yields and purification are based upon the estimate of the activity in the initial eluate from DEAE-cellulose and phenyl-Sepharose (Step 2).

Step	Description	Volume (ml)	Protein concentration (mg/ml)	Activity (unit/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (Fold)
2	DEAE-cellulose and phenyl-Sepharose	120	0.72	0.46	97	100	0.64	1
3	First affinity chromatography	420	0.024	0.24	101	104	10	15
4	DEAE-Sephadex	170	0.008	0.36	61	64	45	70
5	Second affinity chromatography	105	0.014	0.53	55	57	39	61

Fig. 5. Agarose-gel electrophoresis of liver and kidney *N*-acetyl-D-glucosamine kinase

Enzyme samples were applied to agarose slab gels at the origin and electrophoresis was performed as described in the Experimental section. Electrophoretograms were stained for glucose-phosphorylating activity with 100 mM-glucose as described in the text. GK_a , GK_b and GK_c refer to the positions of migration of three forms of high- K_m glucose-phosphorylating activity previously described (Allen & Walker, 1976). The band corresponding to GK_a also reacts strongly with *N*-acetyl-D-glucosamine as substrate, as described in the Results section, and is what we now designate *N*-acetyl-D-glucosamine kinase. (a) Kidney *N*-acetyl-D-glucosamine kinase purified through to Step 6; (b) liver *N*-acetyl-D-glucosamine kinase after step 4, i.e. before the second affinity chromatography stage; (c) liver *N*-acetyl-D-glucosamine kinase purified through to Step 5.

This is in marked contrast with the symmetrical elution profile (Fig. 7a) of muscle hexokinase type II, which is a monomeric enzyme of 96 000 mol.wt. (Holroyde & Trayer, 1976). The asymmetry of *N*-acetyl-D-glucosamine kinase activity has been found for less-pure preparations of the enzyme examined by gel chromatography on both Bio-Gel P-100 and Sephadex G-100 (not shown). Although interaction of the enzyme with the column material as the cause of the asymmetry cannot be positively excluded, the observations provide strong evidence that *N*-acetyl-D-glucosamine kinase is a dimer of mol.wt. 80 000, which is dissociated by sodium dodecyl sulphate into subunits of equal size to give the 39 000-dalton band seen in Fig. 6. The nature of the 90 000-mol.wt. protein is not known. It can be specifically eluted from the affinity column by either glucose or *N*-acetyl-D-glucosamine, and in these respects is so similar in

Fig. 6. Scans of electrophoresis of *N*-acetyl-D-glucosamine kinase activity isolated from (a) rat liver and (b) rat kidney

Samples of the *N*-acetyl-D-glucosamine kinase prepared from (a) rat liver and (b) rat kidney were subjected to electrophoresis in 7.5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate as described in the Experimental section. The gels were stained for protein with Coomassie Blue R250 and scanned at 542 nm in a Zeneih gel scanner. The origin is at the left and the anode to the right of these traces. The full scan illustrated represents a gel length of approx. 14 cm. The letters and arrows across the top of the Figure represent the distances run under identical conditions by standard marker proteins of the following mol.wts.: A, phosphorylase *a* (94 000); B, transferrin (77 000); C, bovine serum albumin (68 000); D, catalase (60 000); E, fumarase (49 000); F, ovalbumin (43 000).

properties to *N*-acetyl-D-glucosamine kinase that it is not separated on the basis of charge, size or hydrophobicity. It could be an *N*-acetyl-D-glucosamine kinase, but if so, it must have similar kinetic properties to the dimeric enzyme of mol.wt. 80 000, because the kinetic data presented in the following paper (Allen & Walker, 1980) give no indication of there being more than one active enzyme in our preparation.

The enzyme preparations from neither liver nor kidney contained detectable activities of the following enzymes: glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ATPase.

Stability of *N*-acetyl-D-glucosamine kinase preparations

The purified enzymes dissolved in the affinity-column elution buffer have been stored at -10°C for

Fig. 7. Gel filtration on Bio-Gel P-100 of (a) hexokinase and (b) *N*-acetyl-D-glucosamine kinase from liver. The enzyme samples were dialysed previously against the gel buffer which contained: 200 mM-KCl, 20 mM-triethanolamine buffer, pH 7.2, 1 mM-EDTA, 100 mM-glucose, 5% (v/v) glycerol and 1 mM-dithiothreitol. The column size was 32 cm × 2.5 cm, the flow rate was 20 ml/h and 1.5 ml fractions were collected. (a) Hexokinase activity (Δ); (b) *N*-acetyl-D-glucosamine kinase activity (O). The arrows marked A, B and C represent the positions of the elution peaks for Dextran Blue, haemoglobin and glucokinase respectively on the same column under identical conditions.

several months without any loss of activity. The preparations are also stable for several weeks in the absence of glucose or *N*-acetyl-D-glucosamine.

Sugar specificity of N-acetyl-D-glucosamine preparations

The specificity of the enzyme preparations with respect to the sugars that can be phosphorylated is shown in Table 3. Both enzymes showed some activity with D-glucosamine as substrate, but, apart from *N*-acetyl-D-glucosamine itself, the only other analogue phosphorylated was *N*-acetyl-D-mannosamine. It will be shown in the following paper (Allen & Walker, 1980) that the K_m values towards the two acetylated amino sugars differ by an order of magnitude in favour of the glucose derivative. That the 90 000-dalton component described above was not a specific *N*-acetyl-D-mannosamine kinase was established by gel filtration on Bio-Gel P-100, followed by assays for the two activities (Fig. 8). The activity ratios of *N*-acetyl-D-glucosamine kinase/*N*-acetyl-D-mannosamine kinase, which was within the range 1.0–1.1 across the whole peak, were consistent with both activities being catalysed by the same enzyme.

Identification of reaction product

The reaction product was made as described in the Experimental section. The original reaction mix-

Table 3. Substrate specificity of the kinases isolated from rat liver and kidney

Kinase activity of the purified preparations was measured as the rate of ADP formation as described in the text. The substrate concentration was 10 mM in all cases and activities are expressed as a percentage of that given with *N*-acetyl-D-glucosamine as substrate.

	Relative activity	
	Liver	Kidney
<i>N</i> -Acetyl-D-glucosamine	100	100
<i>N</i> -Acetyl-D-mannosamine	78	98
<i>N</i> -Acetyl-D-galactosamine	7.6	3.3
D-Glucosamine	11.2	7.4
D-Mannosamine	2.7	1.6
D-Galactosamine	2.1	3.9
D-Glucose	4.5	1.4
D-Mannose	4.5	2.0
D-Galactose	0	0
D-Fructose	0	0
D-Xylose	2.1	1.5

Fig. 8. Distribution of *N*-acetyl-D-glucosamine kinase and *N*-acetyl-D-mannosamine kinase activity in fractions separated by gel filtration

A sample containing 5 units of the liver *N*-acetyl-D-glucosamine kinase was chromatographed through a Bio-Gel P-100 column under the same conditions as described in Fig. 7. Fractions (0.9 ml) were collected and the activities determined using either *N*-acetyl-D-glucosamine (O) or *N*-acetyl-D-mannosamine (Δ) as substrate.

ture at the conclusion of the 5 h incubation period was shown to contain some unchanged *N*-acetyl-D-glucosamine. The latter appeared as a spot on a chromatogram, developed with the butan-1-ol/acetic acid/water solvent and sprayed with the AgNO₃ reagent, having a R_{glucose} of 1.48 (Crumpton, 1959).

Another AgNO_3 -positive spot, with $R_{\text{glucose}} 0.22$, was present as a result of the incubation and stained positively for organic phosphate. Other chromatograms showed that the unchanged *N*-acetyl-D-glucosamine was completely removed by the treatment with the anion-exchange resin (see the Experimental section). A portion of the phosphate-containing material was incubated with alkaline phosphatase as described. As a result the organic phosphate-containing material disappeared, but the spot with $R_{\text{glucose}} 1.48$ reappeared, as did another spot corresponding to P_i . The product of the reaction must therefore have been a phosphate ester of *N*-acetyl-D-glucosamine.

A chromatogram spotted with a commercial preparation of *N*-acetyl-D-glucosamine 1-phosphate and subsequently developed did not stain appropriately with the AgNO_3 reagent, as might be expected because the latter is primarily a test for reducing sugars. The same compound did not react with the Reissig colour reagent, which is reported to only give a positive reaction if the C-1, C-3 and C-4 positions of a hexose are free (Reissig *et al.*, 1955). Our reaction product with $R_{\text{glucose}} 0.22$ was positive to both AgNO_3 and the Reissig reagent. This is strong evidence that the product was *N*-acetyl-D-glucosamine 6-phosphate.

Discussion

A combination of ion-exchange chromatography and hydrophobic chromatography together with affinity chromatography in the presence of a substrate analogue as ligand has permitted the greatly improved purification of *N*-acetyl-D-glucosamine kinase from kidney and liver tissue of the rat. The recoveries of purified enzyme were extremely good, but, because of the very low amount of activity in the starting material, the actual amounts isolated were small, and therefore limited the characterization studies that could be performed. Although direct comparisons are difficult to make, owing to several experimental differences and lack of certain information in earlier reports, our preparations are probably several-hundredfold more pure than those described previously for the enzyme from human gastric mucosa (Gindzienski *et al.*, 1974) and rat liver (Saeki *et al.*, 1971).

The main problem in its isolation from liver is that *N*-acetyl-D-glucosamine kinase behaves in many respects very similarly to glucokinase and at the same time represents only a small percentage of the total glucose-phosphorylating activity. This problem reveals itself if assays involving the detection of either the glucose 6-phosphate or ADP formed are used, and may not have been apparent to the other groups of workers who measured the disappearance of *N*-acetyl-D-glucosamine. Our estimate of the

amount of *N*-acetyl-D-glucosamine kinase activity in liver tissue compares well with the estimate by Leloir *et al.* (1958). As illustrated in Fig. 4(b), it is the lower affinity of *N*-acetyl-D-glucosamine kinase, compared with glucokinase, for glucose that permitted the separation of the two enzymes on an affinity column bearing the same ligand as had previously been employed successfully for the purification of glucokinase (Holroyde *et al.*, 1976a,b). This further illustrates the versatility of this and related ligands for the purification of sugar kinases (Wright *et al.*, 1978).

The specific activities of our two preparations were very similar. The enzymes from both liver and kidney showed only one band on agarose-gel electrophoretograms that stained for glucose-phosphorylating (Fig. 5) and *N*-acetyl-D-glucosamine kinase activity. Both preparations appeared to contain approximately equimolar amounts of a protein of mol.wt. 90 000 and one of 39 000 (Fig. 6) under dissociating conditions. Under non-dissociating conditions on Bio-Gel P-100 (Fig. 7) an asymmetric enzyme distribution was observed, with the main peak of activity appearing to have a mol.wt. of 80 000, but with a substantial proportion of the activity in the 40 000-dalton region. This may indicate either (a) that both proteins seen after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are enzymically active or (b) that *N*-acetyl-D-glucosamine kinase is a dimer of mol.wt. 80 000, the active monomers of which are in some form of equilibrium with the dimer, and that the 90 000-dalton protein is an impurity that has co-purified with *N*-acetyl-D-glucosamine kinase. Kinetic evidence in the following paper (Allen & Walker, 1980) favours the second alternative. The molecular weight of the enzyme from human gastric mucosa determined by gel filtration on Sephadex G-100 was 77 000 (Gindzienski *et al.*, 1974).

As well as their behaviour during the various purification procedures, the *N*-acetyl-D-glucosamine kinases from both kidney and liver showed similar preferences for substrates (Table 3). Whereas *N*-acetyl-D-mannosamine is also a good substrate for what appears to be the same enzyme (Fig. 8), neither *N*-acetyl-D-galactosamine nor the other amino sugars and monosaccharides tested at a concentration of 10 mM showed more than 11.2% of the activity towards *N*-acetyl-D-glucosamine. Kinetic evidence in the following paper (Allen & Walker, 1980) provides justification for referring to our enzymes as *N*-acetyl-D-glucosamine kinase rather than an *N*-acetyl-hexosamine kinase of broader specificity. From a physiological viewpoint the activity of the liver enzyme towards D-glucose must be noted, but so should the very high K_m value (Allen & Walker, 1980). Our results confirm the report by Leloir *et al.* (1958) that the product of the reaction with *N*-

acetyl-D-glucosamine is *N*-acetyl-D-glucosamine 6-phosphate; likewise, from the very nature of the assay employed, the product of glucose phosphorylation is glucose 6-phosphate.

During the purification of the kidney enzyme on DEAE-Sephadex (Fig. 1a), we removed another enzyme that phosphorylated *N*-acetyl-D-glucosamine (detected by ADP formation) and coincided with low- K_m hexokinase activity (measured with 0.5 mM-glucose). This activity showed up as hexokinase isoenzyme II activity on agarose-gel electrophoretograms. Earlier reports (e.g. Walker, 1966) indicated that liver hexokinase (i.e. a mixture of the unresolved isoenzymes) does not phosphorylate *N*-acetyl-D-glucosamine, although the latter is a competitive inhibitor of glucose phosphorylation. This matter warrants further investigation.

One role suggested for soluble *N*-acetyl-D-glucosamine kinase in a cell (e.g. Saeki *et al.*, 1971; Gindzienski *et al.*, 1974) is that it acts to reutilize free *N*-acetyl-D-glucosamine released as a result of lysosomal degradation of glycoproteins (Aronson & de Duve, 1968). The cytosolic enzyme that we have isolated from kidney and liver is very hydrophobic in character, as indicated by the behaviour on phenyl-Sepharose. The property will facilitate interaction of the enzyme with membranes and this could be of relevance to glycoprotein synthesis. There may well be other related kinases associated with cellular organelles. This paper offers a basis for procedures that should be of value in the isolation and purification of such kinases that may be present in tissues in very small absolute quantities.

This work was supported in part by a grant from the Medical Research Council to D. G. W., Dr. I. P. Trayer and Dr. A. Cornish-Bowden.

References

- Allen, M. B. & Walker, D. G. (1976) *Biochem. Soc. Trans.* **4**, 1057
 Allen, M. B. & Walker, D. G. (1980) *Biochem. J.* **185**, 577–582
 Alvares, F. L. & Nordlie, R. C. (1977) *J. Biol. Chem.* **252**, 8404–8414
 Andrews, P. (1965) *Biochem. J.* **96**, 595–606
 Aronson, M. M. & de Duve, C. (1968) *J. Biol. Chem.* **243**, 4564–4573
 Bandurski, R. S. & Axelrod, B. (1951) *J. Biol. Chem.* **193**, 405–410
 Cardini, C. E. & Leloir, L. F. (1957) *J. Biol. Chem.* **225**, 317–324
 Crumpton, M. J. (1959) *Biochem. J.* **72**, 479–486
 Datta, A. (1970) *Biochim. Biophys. Acta* **220**, 51–60
 Datta, A. (1971) *Arch. Biochem. Biophys.* **142**, 645–650
 Davagnino, J. & Ureta, T. (1978) *Arch. Biol. Med. Exp.* **11**, 12
 Ghosh, S. & Datta, A. (1967) *Trans. Bose Res. Inst. Calcutta* **30**, 167–180
 Gindzienski, A., Zwierz, K. & Rozanski, A. (1971) *Biochem. Med.* **5**, 188–194
 Gindzienski, A., Glowacka, D. & Zwierz, K. (1974) *Eur. J. Biochem.* **43**, 155–160
 Holroyde, M. J. & Trayer, I. P. (1976) *FEBS Lett.* **62**, 215–219
 Holroyde, M. J., Cheshier, J. M. E., Trayer, I. P. & Walker, D. G. (1976a) *Biochem. J.* **153**, 351–361
 Holroyde, M. J., Allen, M. B., Storer, A. C., Warsy, A. S., Cheshier, J. M. E., Trayer, I. P., Cornish-Bowden, A. & Walker, D. G. (1976b) *Biochem. J.* **153**, 363–373
 Layne, E. (1957) *Methods Enzymol.* **3**, 447–454
 Leloir, L. F., Cardini, C. E. & Olavarria, J. M. (1958) *Arch. Biochem. Biophys.* **74**, 84–91
 Niemeyer, H., Cardenas, M. L., Rabajille, E., Ureta, T., Clark-Turri, L. & Penaranda, J. (1975) *Enzyme* **20**, 321–333
 Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966
 Saeki, H., Suzuki, R. & Tsuiki, S. (1971) *Tohoku J. Exp. Med.* **105**, 87–98
 Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 7–14
 Susor, W. A. & Rutter, W. J. (1971) *Anal. Biochem.* **43**, 147–155
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
 Ureta, T. (1975) in *Isoenzymes* (Markert, C. L., ed.), vol. 3, pp. 575–601, Academic Press, New York
 Walker, D. G. (1966) *Essays Biochem.* **2**, 33–67
 Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
 Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50
 Wright, C. L., Warsy, A. S., Holroyde, M. J. & Trayer, I. P. (1978) *Biochem. J.* **175**, 125–135