Biochem. J. (1976) **153**, 363–373
Printed in Great Britain

The Purification in High Yield and Characterization of Rat Hepatic Glucokinase

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(*Received 25 July 1975*)

A new improved procedure for the purification of rat hepatic glucokinase (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2) is given. A key step is affinity chromatography on Sepharose–*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. A homogeneous enzyme, specific activity 150 units/mg of protein, is obtained in about 40% yield. The molecular weight of the pure enzyme was determined by several procedures. In particular, sedimentation-equilibrium studies under a variety of conditions indicate a molecular weight of 48000 and no evidence for dimerization; reports in the literature of other values are discussed in the light of this evidence on the pure enzyme. The amino acid composition suggests that hepatic glucokinase is closely related to rat brain hexokinase and also the wheat 'light' hexokinases.

The procedure devised in this laboratory (Parry & Walker, 1966; Walker & Parry, 1966) for the isolation of rat hepatic glucokinase (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2) did not yield a homogeneous preparation, but has formed the basis for other attempts to obtain the pure enzyme. Pilkis (1972) improved the procedure by modifying the last gel chromatography step and adding starch-gel electrophoresis. Further work (C. R. Lowe & D. G. Walker, unpublished work) indicated that the specific activity of the pure enzyme was probably several times that hitherto described, and encouraged us to seek new approaches to the purification problem, which many workers have found to be hampered by the low amount of the enzyme in liver and by its instability.

This paper describes a new procedure that differs from the earlier one in particular by (*a*) performing the initial step on DEAE-cellulose at a lower pH thereby achieving a greatly improved specific activity at an early stage and (*b*) the inclusion of an affinity-chromatography step described briefly by Chesher *et al.* (1973) and in detail in the preceding paper (Holroyde *et al.*, 1975). The experiences gained in ascertaining the conditions necessary for optimum use of this latter procedure are likely to be of wider value. During the course of this work Grossman *et al.* (1974) described an improved method that resulted in a homogeneous preparation of glucokinase. Our different method results in an apparently homogeneous enzyme of significantly higher specific activity and is obtained in very much greater yield. Certain characteristics of the pure enzyme will be described that permit conclusions to be drawn about its molecular nature.

Materials and Methods

Chemicals

Coenzymes, nucleotides and dithiothreitol were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sepharose 4B, Sephadex G-100, Sephadex G-200, Blue Dextran and DEAE-Sephadex A-50 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. CNBr was from R. Emanuel, Wembley, Middx., U.K. Coomassie Brilliant Blue R was obtained from George T. Gurr, High Wycombe, Bucks., U.K. DEAE-cellulose (DE 52) was supplied by Whatman Biochemicals, Maidstone, Kent, U.K., and guanidine hydrochloride by BDH Chemicals, Poole, Dorset, U.K. Dimethyl suberimidate was a gift from Mrs. H. R. Trayer. All other chemicals were AnalaR grade and used as supplied.

Yeast hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1; type C-302) (300 units/mg) and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ 1-oxidoreductase, EC 1.1.1.49, from yeast, type VII) (345 units/mg) were purchased from Sigma Chemical Co., who also supplied bovine serum albumin, chymotrypsinogen and cytochrome *c*. Pronase (B grade) was from Calbiochem, San Diego, Calif., U.S.A.

Preparation of rat hepatic glucokinase

All procedures described below were carried out at 4°C and all pH measurements were made at this temperature unless otherwise indicated. Although the following procedures describe the purification of glucokinase from livers of 50 rats, the method has been applied to 1 and 100 livers by adjusting the individual steps in proportion.

Step 1: *extraction and centrifugation.* Wistar rats weighing between 250 and 300g were fed *ad libitum* as described previously (Parry & Walker, 1966). The livers from 50 rats were homogenized in 2 vol. of 5mM-$NaHCO_3$, 1mM-EDTA, 1mM-$MgCl_2$, 0.15M-KCl and 1mM-dithiothreitol for 2min in an Ato-Mix blade-type blender. The crude homogenate was immediately centrifuged for 1h at 23500*g* and the resulting supernatant filtered through glass wool; it was not clear owing to the presence of the microsomal fraction. The filtrate was diluted with 0.5 vol. of the above solution without KCl to lower the KCl concentration to approx. 100mM and then adjusted to pH6.5 with 5% (v/v) orthophosphoric acid.

Step 2: *batchwise chromatography on DEAE-cellulose.* To this filtered supernatant (per 100ml) was added approx. 10g of DEAE-cellulose, previously equilibrated with 20mM-potassium phosphate buffer, pH6.5, containing 0.1M-KCl, 0.5mM-dithiothreitol, 1mM-EDTA, 1mM-$MgCl_2$ and 5% (v/v) glycerol, in a batchwise process until all the glucokinase activity was adsorbed. The DEAE-cellulose was collected on a Buchner funnel and washed with 2 litres of the 0.1M-KCl/phosphate buffer, pH6.5, under suction. The washed cellulose ion-exchanger was then packed into a column (approx. 27cm×4.5cm) and more buffer passed through until the E_{280} of the emerging eluate was less than 0.15. Glucokinase was step-eluted from this column by raising the KCl concentration in this buffer to 0.2M. All fractions containing glucokinase activity were pooled (approx. 250ml).

Step 3: *affinity chromatography on a Sepharose-N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose.* The pooled fractions were dialysed against three changes of 1.5 litres each of Buffer A [20mM-triethanolamine/HCl, pH7.0, containing 10mM-KCl, 4mM-EDTA, 7.5mM-$MgCl_2$, 1mM-dithiotheitol and 5% (v/v) glycerol] in a rocking dialyser for about 3h until the conductivity of the non-diffusible material was between 2 and 3mohm^{-1}. The fraction was then applied to the affinity column previously equilibrated with Buffer A. This column contained about 350g wet weight of Sepharose–glucosamine derivative which was necessary to handle this amount of enzyme; the *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was linked and then diluted to give a final concentration of ligand of 2μmol/g wet packed weight of Sepharose (see preceding paper, Holroyde *et al.*, 1975). After application of the enzyme the column (35cm×4.5cm) was washed with Buffer A at 50ml/h until the E_{280} of the emerging eluate was essentially zero. Elution of the enzyme was achieved by including 1M-glucose in Buffer A; the fractions containing activity were pooled.

Step 4: *chromatography on DEAE-Sephadex.* Sufficient KCl was carefully added to this pool to give a final concentration of 0.1M. (Localized concentration of KCl caused denaturation of the enzyme.) The enzyme is not very stable in Buffer A and so was not stored at this stage above a few hours without the addition of the KCl. The material was then applied to a column (24cm×4cm) of DEAE-Sephadex previously equilibrated with 0.1M-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 1mM-$MgCl_2$, 0.5mM-dithiothreitol, 50mM-glucose and 5% (v/v) glycerol. After application of the enzyme fraction (in approx. 0.1M-KCl) the column was re-equilibrated with the above buffer and further developed by applying a linear gradient formed from 1 litre of the above buffer and 1 litre of this buffer containing 0.5M-potassium phosphate, pH7.0. The column was operated at 35–40ml/h, and eluted fractions were assayed for glucokinase activity. The active fractions, eluted in the region 0.28–0.30M-potassium phosphate, were pooled.

Step 5: *concentration of enzyme before gel filtration.* This fraction was slowly diluted, with stirring, with 2vol. of a solution containing 1mM-EDTA, 1mM-$MgCl_2$, 50mM-glucose and 0.5mM-dithiothreitol, adjusted to pH7.0, to lower the ionic strength. After dilution the enzyme pool was rapidly applied (70ml/h) to a small DEAE-cellulose column (10cm×0.8cm) equilibrated with 0.1M-KCl/50mM-glucose in Buffer A. The retained glucokinase was eluted from this column in a sharp peak by increasing the KCl concentration to 0.3M in the above buffer. The pool from Step 4 (approx. 220ml) was concentrated to about 10–15ml by this procedure.

Step 6: *gel filtration on Sephadex G-200.* The glucokinase fraction was now applied directly to a Sephadex G-200 column (150cm×3cm) operated in 0.3M-KCl/50mM-glucose in Buffer A at 15–20ml/h. The active fractions from this step were concentrated as described in Step 5 to a protein concentration of about 0.3mg/ml and stored at 4°C in 20mM-triethanolamine/HCl, pH7.0, containing 0.3M-KCl, 1mM-EDTA, 1mM-$MgCl_2$, 0.5mM-dithiothreitol, 50mM-glucose and 20% (v/v) glycerol.

Preparation and operation of Sepharose–N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose

The preparation and characterization of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose, its attachment to CNBr-activated Sepharose 4B (Axen *et al.*, 1967) and determination of the extent of coupling have already been described (Holroyde

et al., 1976). The concentration of ligand initially coupled to the gel was usually about 8 μmol/g wet packed weight of Sepharose 4B and this was then adjusted to the required concentration by the addition of unsubstituted Sepharose 4B. The washing procedures between affinity-column operations, as described in the preceding paper (Holroyde *et al.*, 1976), were strictly adhered to. The gel was stored between use in Buffer A containing 0.2% sodium azide.

Assay of glucokinase

Glucokinase activity was measured at 30°C in a total volume of 0.75ml by the coupled assay of Parry & Walker (1966) as described by Storer & Cornish-Bowden (1974). Low-K_m hexokinase (EC 2.7.1.1) activities were determined at a glucose concentration of 0.5mM where glucokinase activity was negligible. Glucose dehydrogenase activity, present at a low rate in the glucose 6-phosphate dehydrogenase, was measured in the glucokinase assay mixture by omitting ATP. These activities were subtracted from the total activity observed with 100mM-glucose as substrate to give the true glucokinase activity. In addition, the glucokinase activity found in the liver extracts was calculated by halving the total amount of $NADP^+$ reduced to account for an extra mol of $NADP^+$ reduced (per mol of glucose 6-phosphate produced) due to the presence of 6-phosphogluconate dehydrogenase activity. This latter correction was only necessary in liver extracts since this latter enzyme was quantitatively removed by Step 2 of the purification scheme. Similarly, glucose dehydrogenase and hexokinase activities were greatly diminished after this step and were totally absent from the preparations after affinity chromatography.

One unit of glucokinase activity is defined as that which catalyses the formation of 1 μmol of glucose 6-phosphate/min at 30°C.

Electrophoretic methods

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed, and the gels were stained with Coomassie Brilliant Blue as described by Weber & Osborn (1969); standard marker proteins were included on gels when appropriate. Electrophoresis in polyacrylamide gels, in the absence of sodium dodecyl sulphate, when the gels could be stained for enzymic activity as well as protein was essentially by the procedure of Grossman & Potter (1974). Starch-gel electrophoresis was performed as described by Smithies (1959).

Analytical methods

Conductivity measurements were made on a Radiometer conductivity meter, type CDM (Radiometer, Copenhagen, Denmark).

Protein concentrations for specific-activity determinations were made by the turbidimetric tannin micro method (Mejbaum-Katzenellenbogen & Drobry-szycka, 1959). Light-scattering was measured at 500nm in this method and bovine serum albumin was used as a standard.

Sedimentation equilibrium

These studies were performed in a Spinco model E ultracentrifuge equipped with Rayleigh interference optics. The high-speed method of Yphantis (1964) was used, although occasionally column heights longer than 3mm were used (up to 5mm). Relatively high rotor speeds and low protein concentrations (0.2–0.4mg/ml) were used to ensure that the meniscus concentration was effectively zero. Experiments in 6M-guanidine hydrochloride (±0.1M-2-mercaptoethanol) were performed at 20°C whereas those in dilute aqueous salt solutions were conducted at 4°C. All runs were performed in 12mm double-sector cells with sapphire cell windows. Fluorocarbon FC-43 (Beckman Instruments, Palo Alto, Calif., U.S.A.) was used as a base fluid to give a transparent cell bottom. The Rayleigh patterns observed were recorded on Kodak (Kirkby, Lancs., U.K.) type II-G spectroscopic plates. Equilibrium was established by ensuring that no further increase in fringe displacement occurred with time. No correction to the observed fringe displacements for cell-window distortion was found to be necessary. Molecular-weight determinations in 6M-guanidine hydrochloride in the absence and presence of reducing agent (0.1M-2-mercaptoethanol) were performed after dialysis of the glucokinase preparation for at least 48h against these solvents. For molecular-weight estimations of the native enzyme, the glucokinase was first absorbed on DEAE-cellulose as in Step 5 of the purification scheme and eluted by 0.3M-KCl containing the appropriate additions (see Table 2). The eluted enzyme was then dialysed against the appropriate elution buffer for 2–3h before centrifugation. This procedure, especially the short dialysis times, ensured that no loss of enzymic activity occurred. If the glucokinase was dialysed overnight against the required buffer then some loss of activity did occur.

Interference patterns were read on a Nikon profile projector, model 6CT, fitted with photoelectric micrometers (supplied by Rank Precision Industries, Leicester, Leics., U.K.). The natural logarithm of the fringe displacements was plotted against the distance from the centre of rotation squared (r^2) and the slope of the line estimated by the method of least squares. The weight-average molecular weight was determined by the method of Yphantis (1964).

The partial specific volume calculated from the amino acid composition of glucokinase (Cohn & Edsall, 1943) is 0.730g/ml. The density of the 6M-

guanidine hydrochloride (1.1418g/ml) was found from tables after measuring its refractive index.

Analytical gel filtration

Estimation of the molecular weight of glucokinase by gel filtration on Sephadex G-100 was carried out as described by Fish *et al.* (1969) except that a series of dilute aqueous salt solutions were used as solvents (see Table 2). Blue Dextran was used to measure the column void volume, V_0, and Dnp-glycine was used as a marker to determine the total volume accessible to the solvent V_1. For the calculation of the molecular weight of glucokinase, K_d, as defined by Fish *et al.* (1969), was plotted against the logarithm of the molecular weight of the standard proteins used [molecular weights given by Fish *et al.* (1969)].

Amino acid composition

Amino acid analyses were performed with a Beckman model 120B amino acid analyser as described by Wilkinson *et al.* (1972). Duplicate samples were hydrolysed in 6M-HCl under vacuum for 24 and 72h.

Cross-linking experiments with dimethyl suberimidate

To 1ml of 20mM-triethanolamine/HCl, pH8.0, containing 0.3M-KCl, 4mM-EDTA, 7.5mM-$MgCl_2$, 0.2M-glucose and 10mM-ATP at 4°C was added glucokinase (0.3mg) followed by a solid dimethyl suberimidate (6mg) (Davies & Stark, 1970). The reaction was maintained at pH8.0 by the addition of 2M-KOH, and 0.2ml portions were withdrawn at hourly intervals and quenched by the addition of 5μl of 2M-NH_4Cl. To these samples was added 5mg of sodium dodecyl sulphate and 20μl of 2-mercaptoethanol and they were then heated at 100°C for 5min. The resulting solutions were dialysed against 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol before electrophoresis.

Results

Purification of rat hepatic glucokinase

Table 1 summarizes the purification procedure outlined in the Materials and Methods section for a typical preparation of glucokinase from rat liver. Final yields of the homogeneous enzyme of over 50% have been recorded but a 40% yield was more frequently obtained. The entire purification from 50 rat livers can be completed in 4–5 days. Several aspects of the procedure at each step are noteworthy.

Step 1. A lower speed of centrifugation was used here since it enabled larger quantities of material to be processed more rapidly than with the high-speed centrifugation used formerly (Parry & Walker, 1966; Grossman *et al.*, 1974).

Step 2. This batchwise ion-exchange step was performed at pH6.5 rather than pH7 or above because it resulted in a higher degree of purification. Far less protein was bound to the exchanger at this pH and, as a consequence, far less unwanted protein was eluted by the stepwise addition of KCl. Glucokinase activity was quantitatively recovered from this procedure. Slightly higher purifications of the enzyme could be obtained if the DEAE-cellulose was operated throughout this step in a column procedure and the glucokinase eluted by the application of a KCl gradient, but the additional time involved in processing large quantities of liver extracts in this manner was not warranted.

Step 3. No loss of glucokinase activity was found if only a short period of dialysis (see the Materials and Methods section) was used to lower the ionic strength of the enzyme solution before the affinity-chromatography step (Fig. 1). Glucokinase activity remained stable for about 48h in this buffer. Use of the conditions specified were critical.

Step 4. The turn-around between steps 3 and 4 was very rapid since the affinity-column eluate could be applied directly to the DEAE-Sephadex column. In agreement with Grossman *et al.* (1974), we have also found that elution of the enzyme from this ion-ex-

Table 1. *Purification scheme for rat hepatic glucokinase*

The results presented below are taken from a typical preparation starting with the livers of 50 rats. The procedure used to obtain each fraction is described in the Materials and Methods section.

Step	Stage	Volume (ml)	Protein concentration (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Fold purification
1	Extraction and centrifugation	2800	60.6	0.33	940	100	0.0055	1
2	Batchwise DEAE-cellulose	340	2.4	2.90	986	105	1.2	220
3	Affinity chromatography	130	0.24	6.04	785	85	21	3860
4	DEAE-Sephadex	230	0.025	2.24	514	55	91	16600
5	Concentration	20	0.27	24.20	483	51	91	16600
6	Sephadex G-200	160	0.016	2.45	393	42	150	27500
7	Concentration	10	0.25	37.6	376	40	150	27500

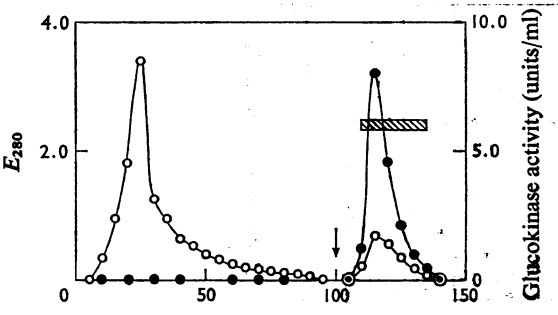


Fig. 1. *Affinity chromatography of glucokinase on Sepharose–N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose (Step 3)*

Enzyme from the DEAE-cellulose column (340ml containing 2.9 units of activity/ml) was applied to a column (18cm×3.8cm) of the Sepharose–glucosamine ligand, equilibrated with Buffer A. The concentration of glucosamine ligand attached to the Sepharose was 2μmol/g wet packed weight. After being washed, the enzyme was eluted by including 1M-glucose in the developing buffer (at the arrow). The column was operated at a flow rate of 35ml/h, and 6ml fractions were collected. The shaded area indicates the fractions pooled for subsequent operations. ○, E_{280}; ●, glucokinase activity.

change column with phosphate buffers gave sharper separations than when KCl was used (Fig. 2*a*). At this stage of the preparation, the glucokinase contained only one other major component. Purification to homogeneity could be achieved by either recycling through the affinity-chromatography step or by gel filtration. If the preparation is to be performed in the order of steps given here (see below) then we prefer to use the gel-filtration step since this does not involve decreasing the K^+ concentration of the buffer with a concomitant loss of activity.

Steps 3 and 4 were readily interchangeable provided the appropriate ionic conditions were established. When carried out as indicated above the preparation was more rapid. The advantage of performing the DEAE-Sephadex step first was that less non-specific protein was applied to the affinity column, thus greatly extending its operative life (see below).

Step 5. Concentration of the enzyme by using a small DEAE-cellulose column was preferred over other methods since less enzyme activity was lost. When using concentration methods which involved exposure of the enzymes at this stage of purity to a membrane surface (ultrafiltration, vacuum dialysis or any method in which the enzyme was contained in a dialysis bag) then far greater losses of activity were found.

Step 6. Fig. 2(*b*) shows a typical elution pattern for the glucokinase from the Sephadex G-200 column. Only one peak of material absorbing at 280nm was seen which was eluted coincidentally with the enzymic activity. At this stage of purity it was necessary to maintain both glycerol and glucose in the buffers in order for this step to be performed in high yield.

The subsequent characterization of the enzyme was performed on material purified in this manner. More recently, we have been able to adapt the affinity-chromatography step such that it can be operated after the DEAE-Sephadex column without the necessity of an intermediate desalting step. Fig. 3(*a*) shows the behaviour of the protein after step 2 applied directly to DEAE-Sephadex operated as described for step 4 above. The key to the subsequent affinity-chromatography step (Fig. 3*b*) was to operate the column at a higher ligand concentration than before, allowing the enzyme to be applied at a higher ionic strength. Less unwanted protein was therefore bound to the column and the subsequent elution with 0.2M-glucose provided highly purified glucokinase. When operated in this manner, the working life of these affinity columns was greatly prolonged and they have been used repeatedly over 12 months without any apparent loss in their effectiveness. The material eluted in Fig. 3(*b*) still required a gel-filtration step (Step 6) to complete the preparation.

This modified purification scheme has been used to achieve homogeneous preparations of pig liver glucokinase and rat skeletal-muscle hexokinase (type II) (M. J. Holroyde, A. S. Warsy & I. P. Trayer, unpublished work).

Purity of glucokinase preparations

The various stages of the purification scheme were monitored as a routine by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Plate 1). The enzyme became apparent as a discrete band after passage through the affinity-chromatography column. Two major components were present in the pooled fractions from the DEAE-Sephadex column, but gel filtration enabled the heavier impurity to be removed leaving an apparently homogeneous preparation of glucokinase (Plate 1). A variety of other electrophoretic methods, operated under conditions where separation depended on protein charge, also demonstrated the apparent homogeneity of the final preparation. Plate 1 also shows the results of two polyacrylamide-gel-electrophoresis runs, one stained for protein and one for activity. A similar result was obtained when a starch-gel support matrix was used.

Careful scrutiny of gel (*e*) in Plate 1, in which a relatively large amount of protein was applied (40μg), reveals a very minor band (less than 1% of the total

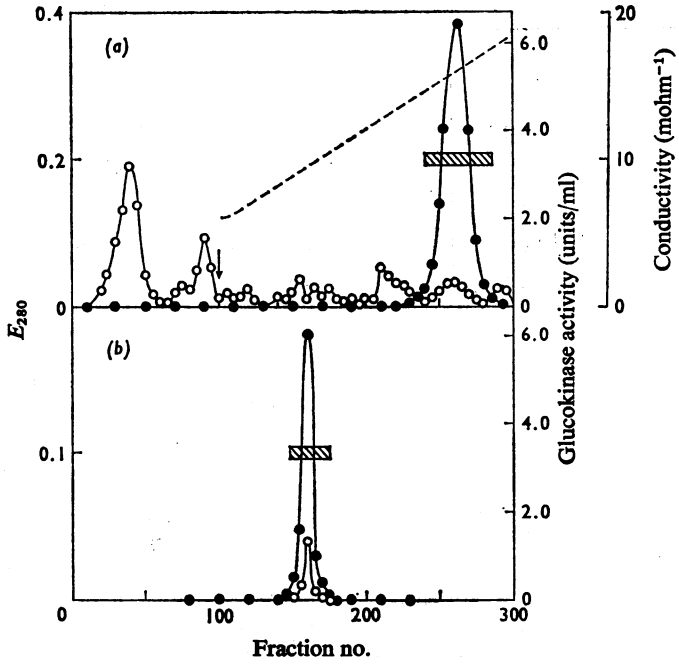


Fig. 2. *Chromatography of glucokinase on (a) DEAE-Sephadex (Step 4) and (b) Sephadex G-200 (Step 6)*

(*a*) Enzyme from the affinity-chromatography step (130ml containing 6.04 units of activity/ml) was applied to the DEAE-Sephadex column (18cm×3.8cm) equilibrated with 100mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 1mM-$MgCl_2$, 0.5mM-dithiothreitol, 50mM-glucose and 5% (v/v) glycerol. At the arrow, the column was developed with a linear gradient formed from 1 litre of this buffer and 1 litre of 0.5M-potassium phosphate buffer, pH7.0, containing the same additions as above. The column was operated at a flow rate of 40ml/h, and 5ml fractions were collected. (*b*) The fractions indicated by the shaded area in (*a*) were concentrated to 20ml and applied to a Sephadex G-200 column (150cm×3cm) operated in 0.3M-KCl/50mM-glucose/Buffer A at 15ml/h. The shaded area indicates the fractions pooled. ○, E_{280}; ●, glucokinase activity; ----, conductivity (mohm^{-1}).

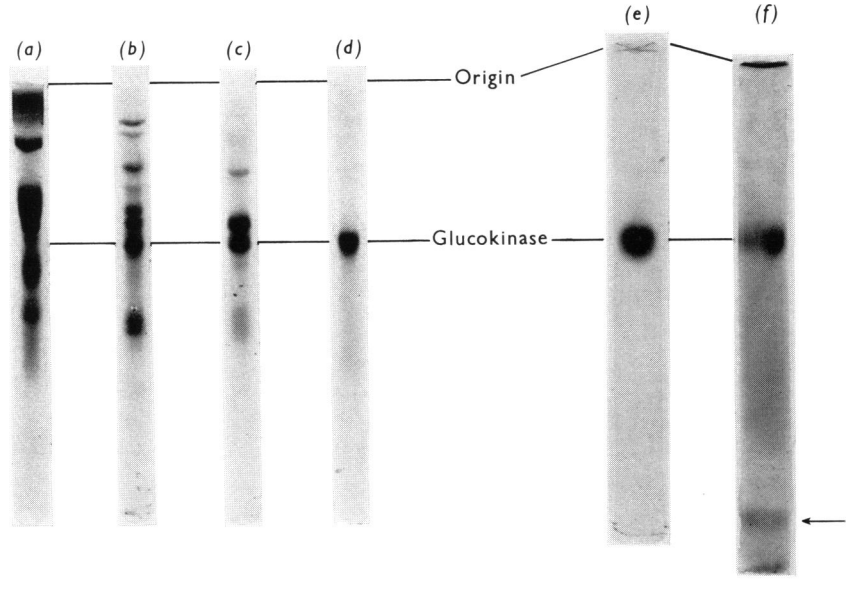
stained material) migrating more slowly than the glucokinase. This was ignored for the characterization purposes described below but can be removed (with some loss in the final recovery) to yield glucokinase that is suitable as an antigen (M. B. Allen & D. G. Walker, unpublished work).

Stability and storage of glucokinase

Enzyme obtained from the first two steps of the purification scheme was quite stable providing at least 100mM-K^+ ions and 0.5mM-dithiothreitol was present in the storage buffers. After the affinity-chromatography step, however, further additions are necessary to the buffers to maintain full activity and the enzyme is noticeably less stable. These observations differ from those of Grossman *et al.* (1974) but we can offer no reasonable explanation for this difference. The presence of glucose and EDTA certainly helped to preserve activity and these were included, wherever possible. If glycerol was added to the final preparations, the enzyme could be stored in solution at temperatures below zero. Further experimentation showed that glycerol helped stabilize the enzyme, over and above the effect of the additives described above, and it did not matter whether the enzyme was stored at 4°C or at −12°C. Thus glycerol was included in all buffers used in the preparation. If the pure glucokinase was stored at 4°C as described in the Materials and Methods section then less than 10% loss in activity was recorded after 6–8 weeks.

Molecular weight of glucokinase

The studies on sodium dodecyl sulphate-containing polyacrylamide gels indicated that the molecular weight of the glucokinase was about 52000. The same result was obtained whether the enzyme was reduced with 2-mercaptoethanol (as was the normal procedure) during treatment with sodium dodecyl sulphate before electrophoresis or not.



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of glucokinase preparations

Polyacrylamide gels were run either in the presence of sodium dodecyl sulphate [(*a–d*), Weber & Osborn, 1969] or as described by Grossman & Potter (1974) (*e*, *f*). (*a*) Material eluted from DEAE-cellulose (Step 2); (*b*) material after affinity chromatography (Step 3); (*c*) material after DEAE-Sephadex (Step 4); (*d*) final product after Sephadex G-200 (40 μg loading); (*e*) and (*f*) pure enzyme (40 μg) applied to polyacrylamide gels and stained for either protein (*e*) or glucokinase activity (*f*). The arrow indicates the position of the Bromophenol Blue dye in (*f*).

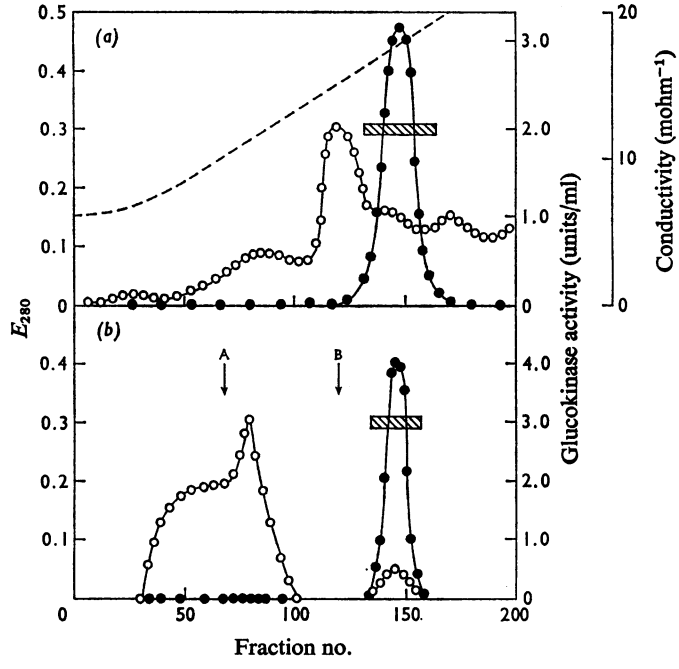


Fig. 3. *Chromatography of glucokinase (a) on DEAE-Sephadex after DEAE-cellulose (Step 2) and (b) on the Sepharose-N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose operated at higher KCl concentrations*

(*a*) Enzyme from Step 2 (70ml) was diluted with an equal volume of 5% (v/v) glycerol containing 0.5mM-dithiothreitol and applied to DEAE-Sephadex, and the operation of the column was as described in the legend to Fig. 2(*a*). Only the portion after starting the gradient is shown. (*b*) The fractions indicated by the shaded area in (*a*) were pooled and diluted with 2vol. of 5% (v/v) glycerol, 1mM-$MgCl_2$, 1mM-EDTA, and 0.5mM-dithiothreitol, pH7.0. Glucose was removed without any loss of activity from this material by applying it to a DEAE-cellulose column as described in Step 5 of the Materials and Methods section and elution with 0.3M-KCl in Buffer A. After dilution with 2vol. of Buffer A (without KCl) this was applied directly to the affinity column (concentration of glucosamine ligand, 4μmol/g wet packed weight of gel). At A, the KCl concentration of the eluting buffer was increased to 0.12M and at B, 0.2M-glucose was also included. The shaded area indicates the fractions pooled.

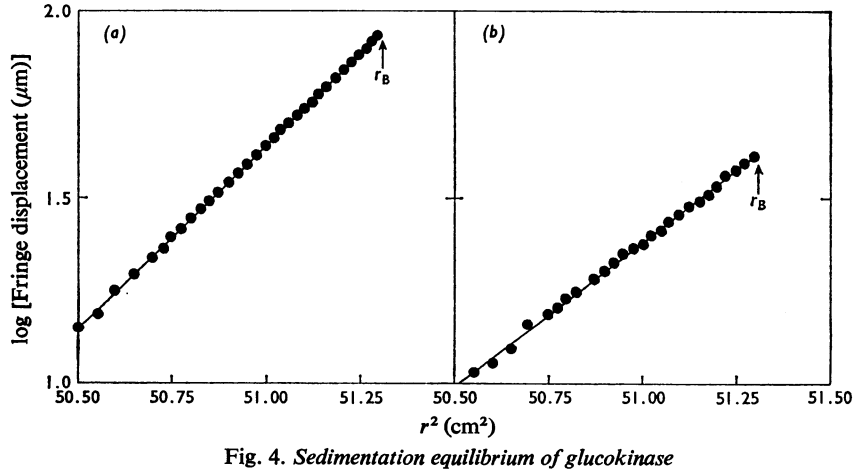


Fig. 4. *Sedimentation equilibrium of glucokinase*

(*a*) Glucokinase (0.25mg/ml) dissolved in 20mM-triethanolamine/HCl, pH7.0, containing 0.3M-KCl, 7.5mM-$MgCl_2$, 4mM-EDTA, 0.5mM-dithiothreitol, 5% (v/v) glycerol at 28132 rev./min. (*b*) Glucokinase (0.20mg/ml) dissolved in 6M-guanidine hydrochloride, pH5.0, at 29516rev./min. r_B represents position of cell bottom.

Table 2. *Molecular-weight determinations on rat hepatic glucokinase*

All methods used for making these measurements are given in the Materials and Methods section. Each value represents the average of at least two determinations on two different preparations of the enzyme.

Method	Solvent	Average molecular weight
Sedimentation equilibrium	20mM-Triethanolamine/HCl, pH7.0, containing 0.3M-KCl, 7.5mM-$MgCl_2$, 4mM-EDTA, 0.5mM-dithiothreitol, 5% (v/v) glycerol (Buffer B)	48600
	Buffer B+50mM-glucose	46800
	6M-Guanidine hydrochloride	47900
	6M-Guanidine hydrochloride, 0.1M-2-mercaptoethanol	47900
Gel filtration	Buffer B	49500
	Buffer B+50mM-glucose	48500
	Buffer B+5mM-$MgATP^{2-}$	48500
Sodium dodecyl sulphate/ polyacrylamide-gel electrophoresis	0.1% Sodium dodecyl sulphate, 0.01M-sodium phosphate buffer, pH7.0, ±1% (v/v) 2-mercaptoethanol	52000

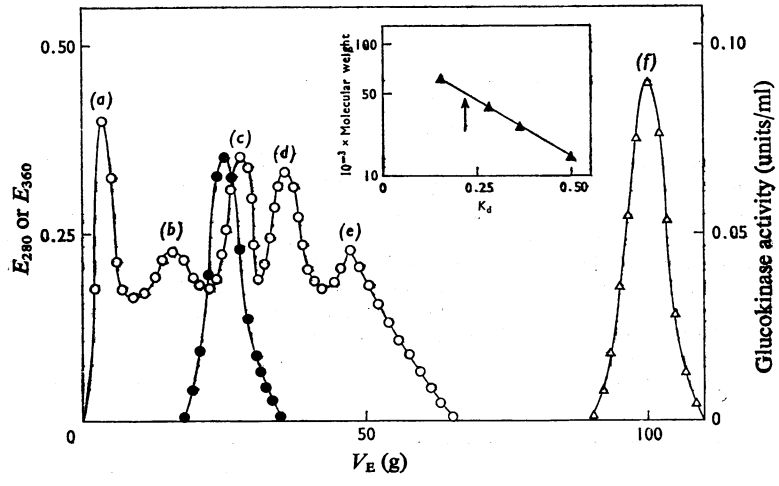


Fig. 5. *Molecular-weight estimation of glucokinase by gel filtration on Sephadex G-100*

A mixture containing 1.5mg each of bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome *c* and glucokinase (4 units) in a total volume of 0.3ml was applied to the Sephadex G-100 column (90cm × 1.5cm) operated in the buffer described in Fig. 4(*a*) above. The elution positions are indicated as follows: (*a*) Blue Dextran; (*b*) bovine serum albumin; (*c*) ovalbumin; (*d*) chymotrypsinogen A; (*e*) cytochrome *c*; (*f*) *N*-(2,4-dinitrophenyl)glycine. The inset describes a semi-logarithmic plot of molecular weight against K_d. ○, E_{280}; ●, glucokinase activity; △, E_{360}.

Several other methods were used to examine the molecular weight of the native and denatured enzyme. Sedimentation-equilibrium measurements were made in the analytical ultracentrifuge under a variety of conditions (Table 2). All centrifuge measurements were made on two preparations of the enzyme and at concentrations ranging from 0.2 to 0.4mg/ml. At each concentration studied, data were collected at, at least, two speeds. A linear relationship was always found between the logarithm of the fringe displacement and the square of the distance from the centre of rotation from the cell bottom to less than 100 μm fringe displacement (Fig. 4), indicating a monodisperse system. A mol.wt. of 48000 for the glucokinase was calculated from these studies. The same value was obtained when the runs were performed under denaturing conditions (6M-guanidine hydrochloride ±0.1M-2-mercaptoethanol) and under conditions where the enzyme retained activity both in the absence and presence of glucose (Table 2). The runs using the native enzyme were performed at 4°C; no enzyme activity was lost during the centrifuging operation.

Table 3. *Amino acid composition of rat hepatic glucokinase compared with other hexokinase enzymes*

Details of sample preparation and acid hydrolyses are given in the Materials and Methods section. Except for amino acids estimated by extrapolation (see text), the results of duplicate hydrolyses at different times were consistent to ±4% or better. All values are calculated as residues per 48000g.

Amino acid	Rat hepatic glucokinase*	Rat brain hexokinase†	Wheat-germ hexokinase‡		Yeast hexokinase§	
			L_I	L_{II}	P_I	P_{II}
Aspartic acid	46	49	48	49	49	49
Threonine	24	25	22	20	26	26
Serine	31	24	28	26	22	21
Glutamic acid	59	44	55	55	45	48
Proline	14	18	19	26	21	24
Glycine	40	40	46	45	37	36
Alanine	30	24	43	34	30	30
Half-cysteine	9	9	4	3	4	3
Valine	32	32	36	36	26	21
Methionine	18	15	7	6	10	10
Isoleucine	17	25	22	21	28	32
Leucine	41	42	39	40	39	32
Tyrosine	8	4	13	11	15	14
Phenylalanine	18	18	16	16	16	21
Histidine	11	9	8	7	8	5
Lysine	25	30	31	32	32	32
Arginine	28	27	17	17	15	17

* From this study.
† From Chou & Wilson (1972).
‡ From Meunier *et al.* (1971).
§ From Gazith *et al.* (1968).

Conflicting results have been obtained from gel-filtration studies on this enzyme by Pilkis (1972). Our experiments conducted on Sephadex G-100 at 0.3M-KCl and 0.50mM-KCl (not shown here) and in the absence or presence of one or other of the substrates, resulted in a single peak of activity being eluted from the column under all conditions tested corresponding to an apparent mol.wt. of 49000 (Fig. 5, Table 2).

The values obtained by the several methods under different conditions (Table 2) are in excellent agreement with one another and indicate that the active enzyme is a monomer. To test further whether the reacting enzyme could form dimers, incubations were set up under assay conditions and the protein was 'frozen' by the additions of a bifunctional cross-linking reagent, dimethyl suberimidate (Davies & Stark, 1970). The cross-linked enzyme was then subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Under all conditions tested, a single protein band migrated on these gels corresponding to a mol.wt. of 52000 and no suggestion of any higher-molecular-weight cross-linked material was observed.

Amino acid composition of glucokinase

The amino acid composition of glucokinase is presented in Table 3. The calculated value for each amino acid except isoleucine, valine, threonine, serine and half-cysteine is the average of two times of hydrolysis (24 and 72h) each performed in duplicate. The values for serine and threonine were estimated by linear extrapolation to zero time of hydrolysis. Valine and isoleucine values were obtained by the average of the 72h hydrolyses only. Half-cysteine was estimated as cysteic acid after performic acid oxidation (Moore, 1963). In Table 3 the amino acid composition of rat liver glucokinase is compared with published values for rat brain hexokinase I (Chou & Wilson, 1972), the wheat-germ hexokinase (Meunier *et al.*, 1971) and the yeast hexokinases (Gazith *et al.*, 1968); all values are normalized to 48000g of protein. The similarity between the rat brain hexokinase and rat liver glucokinase is particularly striking.

Discussion

Glucokinase from rat liver can be purified some 30000-fold and in at least 40% yield by the procedures described here. The overall yield of enzyme has steadily increased as we have gained more experience with the techniques and introduced the various modifications mentioned in the text. All of the purification steps described can be operated at over 80% yield and most of our losses occur in the turn-round

stages between the steps. The procedures represent a considerable improvement over the available methods for purifying this enzyme (Gonzalez *et al.*, 1967; Pilkis, 1972; Grossman *et al.*, 1974) which has proved difficult because of its instability and the small amounts present in liver. The liver of a normal adult Wistar rat fed on a normal diet contains approx. 0.15mg of soluble glucokinase having a turnover number of 7200mol of D-glucose phosphorylated by ATP/min per mol of glucokinase at 30°C, assuming one catalytically active site, a mol.wt. of 48000 and a specific activity of 150 units/mg of pure enzyme. The yield of the enzyme obtained by our procedure nevertheless now permits us to perform structural and immunological studies on it to complement the kinetic studies.

The key step in the purification is the chromatography of the enzyme on Sepharose–*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. Glucokinase can be purified to about 95% purity, as judged by polyacrylamide-gel electrophoresis, by two passages down the affinity column after the DEAE-cellulose chromatography followed by the gel-filtration step. The overall yield is lower, however, since the pooling of fractions from the Sephadex G-200 column must be curtailed to exclude a protein having a mol.wt. of 26000 on polyacrylamide gels in the presence of sodium dodecyl sulphate. This impurity, which possibly exists as a dimer of 52000 in dilute aqueous salt solutions which would explain its elution volume on Sephadex G-200, is progressively eliminated by further passages down the affinity column. Grossman *et al.* (1974) have also noted the presence of this component in their purification scheme. We find it to be more efficient to eliminate it entirely by the combined action of the affinity column and chromatography on DEAE-Sephadex.

Of the reported purifications of this enzyme in the literature, only one (Grossman *et al.*, 1974) results in a homogeneous product. Their procedure, however, takes considerably longer and results in an approximately 20-fold smaller yield than that from the procedure reported here. The specific activity of their enzyme is 80 units/mg whereas our preparations exhibit a specific activity nearly twice this value (150 units/mg). Our enzymic measurements were made at 30°C (rather than 25°C) which would account for only part of this difference. Since the two preparations appear to be equivalent in terms of their purity with respect to protein, the inherent instability of the enzyme is more likely to account for this difference. Our procedures are more rapid and involve an affinity-chromatography step which can probably distinguish between active and inactive enzyme. Some of our later preparations of glucokinase of apparent similar purity to those described above have been found to have specific activities in the region of 200 units/mg of protein.

Subsequent structural and comparative work on the enzyme is made easier by a precise knowledge of its molecular weight and the reported values to date have varied widely (48000–68000) (Pilkis, 1972; Grossman *et al.*, 1974). Sedimentation-equilibrium measurements gave a mol.wt. of 48000 under a variety of conditions and there was no evidence of dimer formation. Gel filtration, again performed under a variety of conditions, always gave identical results and the values obtained by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, although slightly higher, were within the limits of experimental error for this procedure. Thus we feel confident in reporting that the enzyme is a monomer of mol.wt. 48000. Grossman *et al.* (1974) obtained values of 53000 by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and 57000 by gel filtration, which are not so different from our values that they cannot be reconciled with them. Pilkis (1972) found that the apparent molecular weight obtained by gel filtration on Sephadex G-100 varied from 48000 (in 0.15M-KCl) to 65000 (in the absence of salt) and reported values of 68000 by gel filtration on Bio-Gel P-100 and by sucrose-density centrifugation. The suggested cause for these discrepancies (Pilkis, 1972) was that glucokinase undergoes a conformational change during gel filtration in buffers of low ionic strength. These methods are known to give erroneous results when the protein under study is not globular, but our gel-filtration results were consistent with those obtained by sedimentation-equilibrium experiments. In addition, in a single experiment performed at 10mM-KCl we obtained the same molecular weight as those reported in Table 2. A more likely explanation for the results of Pilkis (1972) is that his glucokinase preparations contained impurities and the protein–protein interactions that would most likely occur at low ionic strength could affect the apparent filtration rate. Since our gel-filtration estimations (obtained by using standard proteins of accepted globular conformation) agree so well with those found by sedimentation equilibrium it seems highly likely that glucokinase is a globular protein.

It is interesting to compare the molecular weight of glucokinase with those found for hexokinases from other sources. The yeast enzyme exists as a dimer of two polypeptide chains, each with a mol.wt. of about 50000, whereas the mammalian hexokinases appear to be monomers of mol.wt. about 100000 [see review by Colowick (1973) and references therein]. In the wheat germ, on the other hand, four hexokinase isoenzymes are known to occur (Meunier *et al.*, 1971) of which two have mol.wts. of 50000 (Meunier *et al.*, 1974; Higgins & Easterby, 1974) whereas the two others have mol.wts. of 100000. There is reasonable evidence that the 'light' hexokinases exist as monomers and show no tendency to polymerize and the

'heavy' hexokinases, although by no means so well studied, also appear to be monomeric structures. Thus, on the basis of molecular-weight data alone, mammalian hepatic glucokinase appears to resemble the 'light' wheat hexokinases more closely than any of the others. We were unable to find any conditions under which the liver enzyme showed any tendency to form higher-molecular-weight structures. This was especially important to establish since the glucokinase exhibits a co-operative interaction with glucose (A. C. Storer & A. Cornish-Bowden, unpublished work) as does at least one of the 'light' wheat enzymes (Meunier *et al.*, 1974) and the microsomal rat liver glucokinases (Berthillier *et al.*, 1970; Berthillier & Got, 1972).

When the amino acid compositions of the enzymes shown in Table 3 are compared by the 'difference index of compositional relatedness' of Metzger *et al.* (1968) several notable features emerge. Rat liver glucokinase appears most closely related to the rat brain hexokinase (difference index, 4.5), followed by the wheat 'light' hexokinases ($L_{II} = 9.6$, $L_I = 9.8$) and the yeast hexokinases ($P_I = 11.2$, $P_{II} = 13.3$). These values may be significant, particularly the comparison between the mammalian enzymes, since many proteins with a high degree of sequence homology often show less compositional relatedness than this (Metzger *et al.*, 1968). Since the brain hexokinase is twice the size of the liver enzyme (Chou & Wilson, 1972), it may perhaps have arisen by gene duplication of some common ancestral gene. This sort of analysis is, however, prone to errors and any evolutionary relationships in this class of enzymes must await sequence analysis.

We thank the Wellcome Trust and then the Medical Research Council for subsequent grants in support of this work. M. J. H. holds a Science Research Council Training Award and A. C. S. is a University of Birmingham Research Student.

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