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APPARENT 'GLUCOKINASE' ACTIVITY IN NON-HEPATIC TISSUES DUE TO *N*-ACETYL-D-GLUCOSAMINE KINASE

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Summary

1. Electrophoretic examination of tissue extracts from rat intestinal mucosa, kidney, lung, spleen, mammary gland, adipose tissue, heart muscle and placenta in agarose gels did not reveal the presence of any glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) activity corresponding to that present in rat liver.

2. All these tissues do contain an enzyme that possesses very high-K_m glucose-phosphorylating activity but which has a slightly lower electrophoretic mobility than glucokinase and can be separated from it by various means.

3. This phosphotransferase activity is due to *N*-acetyl-D-glucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59), which has been partially purified from intestinal mucosa tissue and shown to have similar kinetic properties to the same enzyme previously purified more extensively from liver and kidney.

4. It is suggested that many of the effects reported in the literature of 'glucokinase' activity in non-hepatic tissues are probably due to *N*-acetyl-D-glucosamine kinase.

Introduction

The discovery [1,2] that rat liver tissue contains a glucose-phosphorylating enzyme with a comparatively high K_m for glucose (glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2)) was followed by the demonstration that

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both glucokinase and hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activities are present in this tissue [3]. It was subsequently shown that the hexokinase activity can be resolved into three bands by ion-exchange chromatography on DEAE-cellulose [4]. These findings were corroborated by evidence from starch-gel electrophoresis [5,6].

Many other tissues have been examined for their content of glucose-phosphorylating enzymes usually by means of one or other of two methods. First, the rate of glucose 6-phosphate formation is measured spectrophotometrically, by coupling it with glucose-6-phosphate dehydrogenase to $NADP^+$ reduction. Two glucose concentrations, 100 and 0.5 mM, are used, it is assumed that the latter concentration gives a measure of hexokinase activity and that the glucokinase activity is given by the difference [7]. This is subject to several errors [6,8—10] and not all workers have corrected for the glucose dehydrogenase activity (at 100 mM glucose) in the exogenous glucose-6-phosphate dehydrogenase. The differential heat stability of the hexokinase isoenzymes has been incorporated into such procedures to obtain quantitative information [11]. Secondly, electrophoresis on starch gels has been employed under a range of conditions; several reporters have commented upon the variability of certain results depending, for example, upon the presence or absence of EDTA [12—14] and perhaps not fully appreciating the likely loss of glucokinase activity during the period (often overnight) of the electrophoresis.

Nevertheless, using such procedures a number of reports have suggested that small amounts of high-K_m glucokinase activity are present in tissues such as kidney [13,15], adipose tissue [11,16], intestinal mucosa [17—19], placenta [20], mammary gland [15,11], Islets of Langerhans [21,22], human brain [23], rabbit reticulocytes [24], pig erythrocytes [25] and leucocytes [11]. In some instances the activity was adaptive to physiological status [16,19].

In our recent studies on glucokinase development in neonatal rat liver [10, 26] we have stressed the need to confirm the authenticity of low glucokinase activities determined by the differential spectrophotometric assay by another technique and have used a rapid electrophoretic method in agarose gel [27] to detect glucokinase activity. This technique revealed the existence of more than one high-K_m glucose-phosphorylating activity [28]. We have now used this and other procedures to re-examine several tissues for the presence of glucokinase activity and demonstrate that many non-hepatic tissues contain an enzyme whose glucose-phosphorylating activity has a very much higher K_m for glucose than for glucokinase. This enzyme can be separated from true glucokinase and is in fact *N*-acetyl-D-glucosamine kinase. It is the 'GK_a' referred to in a preliminary note [28].

Methods

The sources of all the specialized chemicals have been given [29,27] and other chemicals were of analytical grade. Wistar rats from our own departmental colony of specific-pathogen-free animals were the source of tissues.

The agarose-gel electrophoresis procedure employs a discontinuous buffer system and takes only about 45 min to complete [27]. Gels were stained for glucose-phosphorylating activity by coupling glucose 6-phosphate formation to

the reduction of Nitro Blue Tetrazolium [27]. Variations of the conditions are as described. *N*-Acetyl-D-glucosamine kinase activity was detected by coupling ADP formation to the oxidation of NADH [27].

Glucokinase and hexokinase activities were assayed as described previously [10] and any variations of the conditions are noted in the legends to the figures. *N*-Acetyl-D-glucosamine kinase activity was assayed at a concentration of 2 mM *N*-acetyl-D-glucosamine [27] unless otherwise stated. The preparation of tissue homogenates and supernatants and the basic protein-fractionation methods have been described [29]. Protein was determined by absorbance at 280 nm [30].

The antibody was a pooled sheep antiserum raised against an apparently homogeneous preparation of rat hepatic glucokinase prepared according to the method of Holyroyde et al. [29] to which a second affinity-chromatography step on Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was added [28]. This glucokinase preparation contained no band in the *N*-acetyl-glucosamine kinase region on agarose-gel electrophoresis (see above for method and Fig. 1). The antibody was diluted as necessary and a small excess of activity was incubated in the glucokinase-assay buffer at pH 7.5 with glucokinase for 30 min at 32°C and then at 0°C for 15 min; this procedure was adequate to precipitate out all glucokinase activity.

Results

Electrophoretograms in agarose gel of samples of supernatant fractions from homogenates of several rat tissues are shown in Fig. 1. The designation of the various bands indicated is based upon the rates of migration of purified [29, 31] or partially-purified [32] specimens of the various isoenzymes. Attention is drawn to the absence of glucokinase bands in the non-hepatic tissues and to the presence in all the tissues of an arrowed band labelled *N*-acetyl-D-glucosamine kinase. In order to show up the latter band adequately for photographic purposes the amount of sample applied was such as to overload all the gels, except (a), (b) and (k), with respect to sharp banding of the various hexokinases. Further, gels (b) to (j) contained 1 M glucose in the gel buffer to enhance the subsequent staining of the *N*-acetyl-D-glucosamine kinase bands. None of the bands stained in the absence of ATP. In other gels (not shown) in which glucose was omitted from the gel buffer and subsequent staining for enzymic activity was in the presence of 0.5 mM glucose, only the HK I, HK II and HK III bands appeared. Sample (b) is a partially-purified preparation from intestinal mucosa (see below for details) of a high-K_m glucose-phosphorylating enzyme of identical mobility to preparations of *N*-acetyl-D-glucosamine kinase from liver and kidney [27].

During the earlier studies [29] on the purification of glucokinase from rat liver clues had been noted suggesting that the enzyme responsible for the *N*-acetyl-D-glucosamine kinase band in liver might be different and separable from glucokinase; this information has been used to isolated it from intestinal mucosa, liver and kidney as now illustrated. Intestinal mucosa (Fig. 1c) contains no glucokinase but shows a comparatively strong *N*-acetyl-D-glucosamine kinase band; this activity will henceforth be designated *N*-acetyl-D-glucosamine kinase

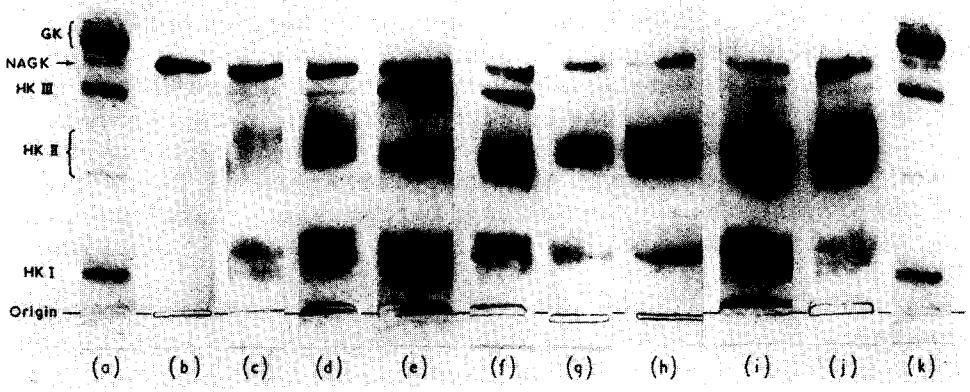

Fig. 1. Electrophoresis of tissue and enzyme preparations on agarose gels. This composite photograph contains gels run under two sets of conditions. Samples (a) and (k) were run under standard conditions [27] as described in the text, while 1 M glucose was included in the gel buffer for samples (b)—(j) in order to maximize the detection (at the time of staining) of the glucose-phosphorylating activity with low affinity for glucose. The rat tissue samples were supernatants from 30% (w/v) homogenates in 0.15 M KCl, 1 mM EDTA, 10 mM $NaHCO_3$ that had been centrifuged for 60 min at 100 000 × *g*. Tissue samples (a) and (c)—(j) and the enzyme preparation (b) were dialysed overnight at 4° C against 15 mM KH_2PO_4/1 mM EDTA/1 mM dithiothreitol, 20% (w/v) sucrose/50 mM glucose at pH 7.0. Samples (10 μl) were applied in each case as follows: (a) and (k), liver; (b), enzyme partially-purified from intestinal mucosa as described in text; (c), intestinal mucosa (original tissue preparation) (d), kidney; (e), lung; (f), spleen; (g), mammary gland; (h), adipose tissue; (i), heart muscle; (j), placenta. Gels were stained for enzymic activity as described in the text. HK I, HK II and HK III are the three low-K_m hexokinase isoenzymes; GK, glucokinase (or hexokinase IV); NAGK, *N*-acetyl-D-glucosamine kinase.

for reasons to be stated later. To isolate this enzyme, the glucose-phosphorylating activity in the supernatant fraction of a 30% (w/v) homogenate of rat intestinal mucosa was first obtained by means of batch DEAE-cellulose chromatography at pH 6.5 as previously described [29]. The *N*-acetyl-D-glucosamine kinase activity was separable on a DEAE-Sephadex column at pH 7.0 (Fig. 2) from the other glucose-phosphorylating activity eluting earlier, which is attributable to low-K_m hexokinase activity, but which does have a low activity towards *N*-acetyl-D-glucosamine as substrate.

In an earlier study [27] it was found that the *N*-acetyl-D-glucosamine kinase activity in liver can be separated from hexokinase and glucokinase. The high-K_m glucose-phosphorylating activity of a liver supernatant preparation was first obtained by batchwise chromatography with DEAE-cellulose at pH 6.5 [29] thereby eliminating some of the low K_m hexokinase activity [29]. This material was applied to a column of DEAE-Sephadex and then eluted (Fig. 3). The resulting *N*-acetyl-D-glucosamine kinase has been further purified and characterized [27,33]. As a third example, Fig. 4 shows the elution profile of *N*-acetyl-D-glucosamine kinase activity of rat kidney tissue which has been taken through the DEAE-cellulose batchwise step [29] and the applied to a DEAE-Sephadex column. All the hexokinase activity was eluted in earlier fractions (not shown) and the coincidence of *N*-acetyl-D-glucosamine kinase activity (measured with 10 mM substrate by the ADP assay) with high-K_m glucose-phosphorylating activity (measured with 1 M glucose by the glucose 6-phosphate formation assay) is apparent (Fig. 4).

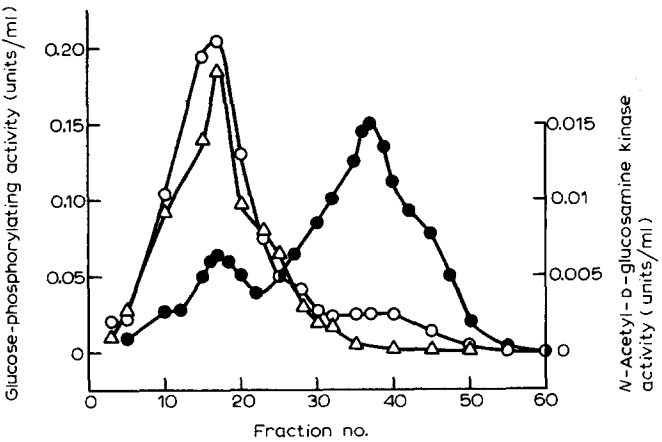

Fig. 2. Separation of *N*-acetyl-D-glucosamine kinase activity from other glucose-phosphorylating activities in a preparation from intestinal mucosa on a DEAE-Sephadex column. The tissue preparation had been taken through the DEAE-cellulose step as described in the text and 100 units of *N*-acetyl-D-glucosamine kinase activity was applied to the DEAE-Sephadex A50 column (4.5 × 7 cm) that had been equilibrated with 0.2 M KH_2PO_4/1 mM EDTA/50 mM glucose/5% (v/v) glycerol/0.5 mM dithiothreitol at pH 7.0. The column was eluted with a linear gradient formed from 400 ml of the equilibration buffer and 400 ml of the same buffer except with the concentration of KH_2PO_4 raised to 0.26 M. Fractions (10 ml) were collected. Hexokinase activity (△———△) was measured at 0.5 mM glucose, glucose-phosphorylating activity (○———○) at 100 mM glucose and *N*-acetyl-D-glucosamine kinase activity (●———●) at 2 mM *N*-acetyl-D-glucosamine; the latter was corrected for hexokinase activity due to the endogenous glucose.

The *N*-acetyl-D-glucosamine kinase activity from intestinal mucosa has been further purified as follows. Fractions 26—62 from the DEAE-Sephadex column (Fig. 2) containing *N*-acetyl-D-glucosamine kinase activity were pooled and applied to a column of phenyl-Sepharose (Fig. 5). Traces of hexokinase activity

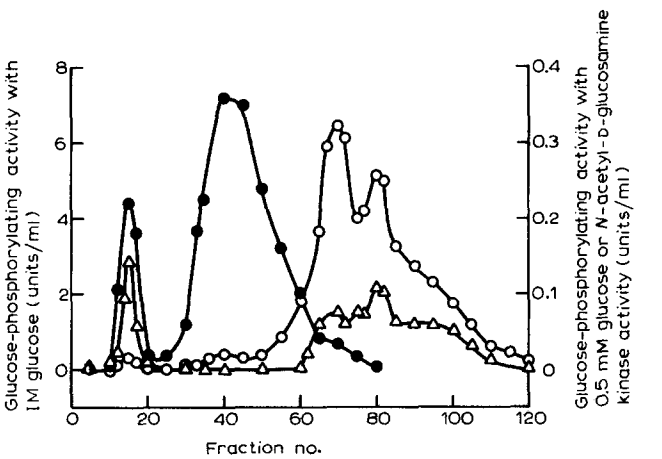

Fig. 3. Separation of *N*-acetyl-D-glucosamine kinase activity from other glucose-phosphorylating activities in a liver preparation on a DEAE-Sephadex column. The preparation starting with 350 g of rat liver had been through a DEAE-cellulose step as described in the text. Other details were as given in the legend to Fig. 2 except that the column size was 4 × 11 cm and the elution gradient consisted of 700 ml of each buffer. △———△, hexokinase activity measured at 0.5 mM glucose; ○———○, glucose-phosphorylating activity measured at 100 mM glucose; ●———●, *N*-acetyl-D-glucosamine kinase activity measured at 10 mM *N*-acetyl-D-glucosamine.

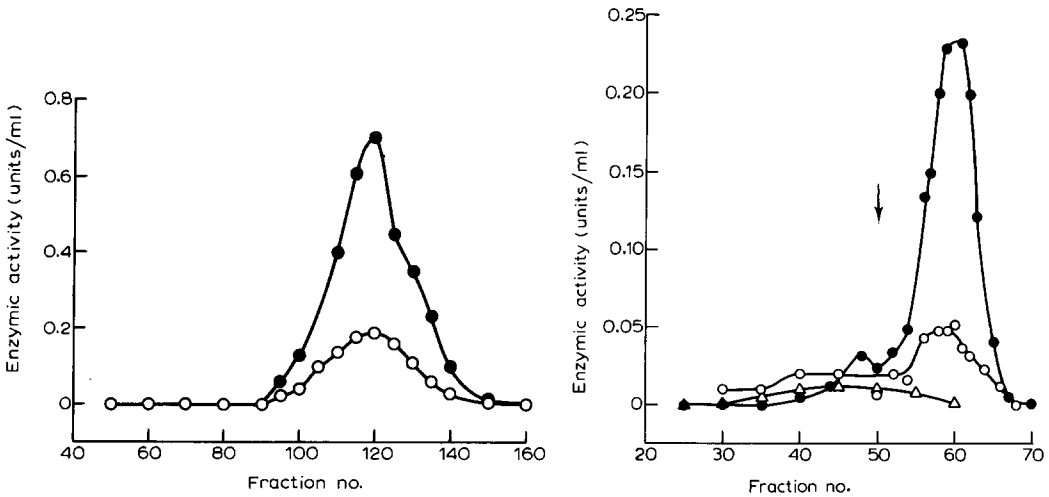

Fig. 4. Coincidence of *N*-acetyl-D-glucosamine kinase and high-K_m glucose-phosphorylating activity in a preparation from kidney tissue on elution from a DEAE-Sephadex column. The tissue preparation had been through the DEAE-cellulose step as described in the text. Other details are as given in the legend to Fig. 2 except that the column size was 4.5 X 10 cm and the elution gradient consisted of 700 ml of each buffer. ●———●, *N*-acetyl-D-glucosamine kinase activity measured at 10 mM *N*-acetyl-D-glucosamine; ○———○, glucose-phosphorylating activity measured at 1 M glucose.

Fig. 5. Hydrophobic chromatography of *N*-acetyl-D-glucosamine kinase from intestinal mucosa on phenyl-Sepharose. Fractions 26—62 inclusive, from the DEAE-Sephadex column illustrated in Fig. 2 were pooled and applied to a column of phenyl-Sepharose, 3 X 4.5 cm, previously equilibrated with 0.22 M KCl/0.02 M KH_2PO_4/1 mM EDTA/50 mM glucose/5% (v/v) glycerol/0.5 mM dithiothreitol, pH 6.5. The column was then washed with approx. 2 bed-volumes of the same buffer. At the point marked by an arrow the buffer was changed to 0.02 M triethanolamine-HCl/0.03 M KCl/1 mM EDTA/5% (v/v) glycerol/0.5 mM dithiothreitol, pH 7.2. ●———●, *N*-acetyl-D-glucosamine kinase activity with 2 mM *N*-acetyl-D-glucosamine; ○———○, glucose-phosphorylating activity at 100 mM glucose; △———△, glucose-phosphorylating activity at 0.5 mM glucose.

were removed by washing the column at the initial high salt concentration (0.22 M KCl) and the *N*-acetyl-D-glucosamine kinase was eluted as a sharp peak when the salt concentration was lowered to 0.03 M KCl. The fractions containing high activity were pooled and concentrated by ultrafiltration. The specific *N*-acetyl-D-glucosamine kinase activity was approx. 2 units/mg of protein. This preparation showed only one band of glucose-phosphorylating activity after agarose-gel electrophoresis (Fig. 1b), which ran at the same rate as samples of *N*-acetyl-D-glucosamine kinase purified more substantially from rat kidney and liver [27]. On other gels (not illustrated) this single band also stained with the ADP-formation procedure in the presence of 2 mM *N*-acetyl-D-glucosamine. Suitably-stained control gels were negative.

The substrate specificity of this partially-purified *N*-acetyl-D-glucosamine kinase activity from intestinal mucosa was examined using the ADP-formation assay at 10 mM concentration with respect to each substrate. The relative rates compared to that with *N*-acetyl-D-glucosamine as 100, were: *N*-acetyl-D-mannosamine, 95; *N*-acetyl-D-galactosamine, 2.6; D-glucosamine, 6.2; D-mannosamine, 3.1; D-galactosamine, 4.4; D-glucose, 4.4; D-mannose, 3.9; D-galactose, 2.6; D-fructose, 3.1; D-xylose, 2.3. Thus *N*-acetyl-D-mannosamine is the

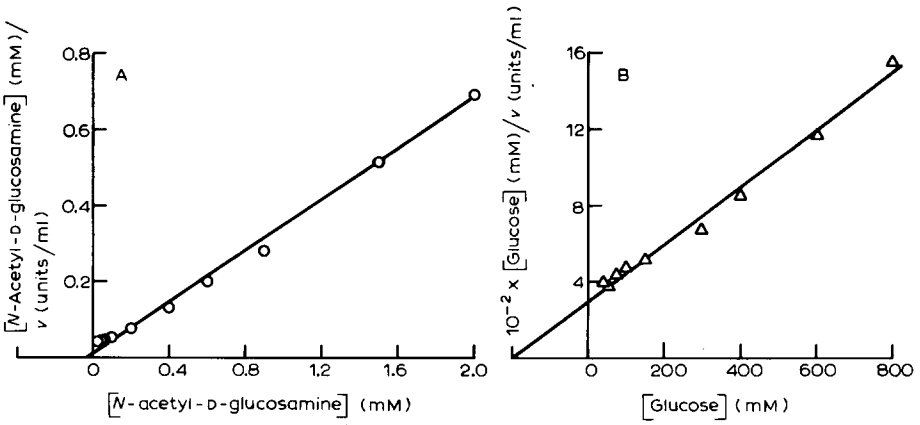

Fig. 6. Effect of concentrations of (A) *N*-acetyl-D-glucosamine and (B) glucose upon the enzymic activity prepared from intestinal mucosa. The enzyme preparation was a pool of fractions 58—67 from Fig. 5. Enzymic activity was determined by measuring in (A) ADP formation and in (B) glucose 6-phosphate formation as described in the text and the substrate concentrations were varied as indicated.

only other substrate to be phosphorylated by the enzyme at an appreciable rate. No increased rate of formation of ADP occurred in the presence of both 2 mM *N*-acetyl-D-glucosamine and 2 mM *N*-acetyl-D-mannosamine over that with only one of those substrates.

Fig. 6 presents [s]/*v* vs. [s] plots for both *N*-acetyl-D-glucosamine and D-glucose upon enzymic activity, which give K_m values of 0.05 mM and 210 mM, respectively, for the two substrates. Slight upward curvature of the plot at low *N*-acetyl-D-glucosamine concentration can be seen in Fig. 6A. The data was

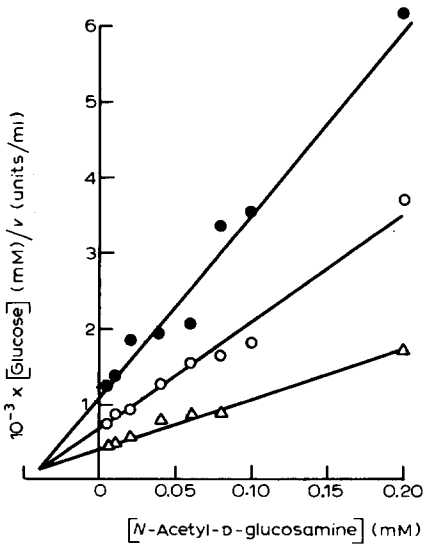

Fig. 7. Inhibition by *N*-acetyl-D-glucosamine of glucose phosphorylation by the enzyme preparation. This was as in Fig. 6. Enzymic activity was determined by measuring glucose 6-phosphate formation as described in the text. The *N*-acetyl-D-glucosamine concentration was varied as indicated. △———△, 100 mM glucose; ○———○, 300 mM glucose; ●———●, 600 mM glucose.

TABLE I

INHIBITION BY ANTI-GLUCOKINASE

Experimental details are given in the text. Treated samples were incubated with the anti-glucokinase preparation; the latter was replaced by control serum in the control incubation. *N*-Acetyl-D-glucosamine kinase activity was determined with 2 mM *N*-acetyl-D-glucosamine by the ADP formation assay. High-K_m glucose-phosphorylating activity was determined with 100 mM glucose by the glucose 6-phosphate formation assay.

Enzyme sample	*N*-Acetyl-D-glucosamine kinase activity (A) (units/ml) (×10)		High-K_m glucose-phosporylating activity (B) (units/ml) (×10)		Ratio A/B	
	control	treated	control	treated	control	treated
Partially-purified liver glucokinase [a]	0	0	2.5	0.03	—	—
Purified *N*-acetyl-D-glucosamine kinase from liver [b]	1.04	1.01	0.14	0.14	7.4	7.2
Purified *N*-acetyl-D-glucosamine kinase from kidney [c]	0.58	0.84	0.08	0.11	7.3	7.6
Partially-purified intestinal mucosal *N*-acetyl-D-glucosamine kinase [d]	0.84	0.90	0.11	0.12	7.6	7.5

[a] Taken through columns of DEAE-cellulose and phenyl-Sepharose, free of *N*-acetyl-D-glucosamine kinase activity, specific activity approx. 2 units/mg of protein.
[b] Prepared as in [27], specific activity 39 units/mg of protein, and diluted.
[c] Prepared as in [27], specific activity 42 units/mg of protein, and diluted.
[d] This paper, specific activity approx. 2 units/mg of protein, and diluted.

replotted (not shown) in the form log $(v/V - v)$ against log [s] when the straight line obtained gave a value for the Hill coefficient, h, of 1.58. The corresponding value for glucose (data of Fig. 6B replotted) was 1.0. The K_m for $MgATP^{2-}$ with either acceptor substrate (data not shown) was 0.25 mM.

The effects of various concentrations of *N*-acetyl-D-glucosamine on glucose phosphorylation was examined by following the formation of glucose 6-phosphate (Fig. 7). This plot of $[s]/v$ against inhibitor concentration [34] suggests mixed inhibition between *N*-acetyl-D-glucosamine and D-glucose.

Further evidence that this kinase, present in several tissues, is not due to true glucokinase was obtained by use of an antibody raised against a preparation of pure glucokinase [28] which was known not to contain *N*-acetyl-D-glucosamine kinase activity. Table I shows that this antiserum which, under identical conditions, completely removed the glucokinase activity present in a partially-purified hepatic glucokinase preparation had no effect upon the *N*-acetyl-D-glucosamine kinase activity of preparations from liver, kidney and intestinal mucosa.

Discussion

The electrophoretic evidence illustrated in Fig. 1 suggests that many of the tissues mentioned in the Introduction do not contain glucose-phosphorylating activity with the same electrophoretic mobility as true hepatic glucokinase but that they do contain an enzyme having a slightly lower mobility which has a

high K_m for glucose. This enzyme has now been partially purified from intestinal mucosa tissue such that it apparently possesses only one glucose-phosphorylating activity (Fig. 1b) and has the same electrophoretic mobility as the enzymes purified more substantially from rat liver and kidney tissue [27]. The activity of this enzyme with respect to the substrates it phosphorylates, its relative affinities towards *N*-acetyl-D-glucosamine and glucose (Fig. 6), the cooperative nature of its interaction with *N*-acetyl-D-glucosamine but not with glucose and the nature of the competition between these two substrates (Fig. 7) are all very similar to those of the enzymes isolated from liver and kidney and designated *N*-acetyl-D-glucosamine kinase [27,33].

The distinct nature of this enzyme from true hepatic glucokinase is also apparent from the separations effected both by ion-exchange on DEAE-Sephadex (Fig. 2—4) and by hydrophobic chromatography on phenyl-Sepharose (Fig. 5). Fig. 5 also illustrates that this *N*-acetyl-D-glucosamine kinase is, like glucokinase, a rather hydrophobic enzyme. The two enzymes are separable by affinity chromatography on Sepharose *N*-(6-aminohexanoyl)-2-deoxy-D-glucopyranose [27].

In our hands none of the tissues, other than liver, that are both illustrated in Fig. 1 and listed in the Introduction, i.e., intestinal mucosa, kidney, mammary gland, adipose tissue and placenta, contain any glucokinase as measured by the difference between the glucose-phosphorylating activity at 100 mM and 0.5 mM glucose after appropriate control corrections [10]. This spectrophotometric information is supported by the electrophoretic evidence and confirms the observations of others [6,35]. While it has not been our intention to re-examine all the reports of non-hepatic glucokinase mentioned by Weinhouse [9] and in the Introduction, we conclude that in many instances the activities were due not to true hepatic glucokinase but rather to *N*-acetyl-D-glucosamine kinase. Davagnino and Ureta [36] have suggested the same possibility in a brief report. It is possible that the 'slow' glucokinase sometimes observed on starch gel electrophoretograms may be due to this enzyme; it is the 'GK_a' referred to in our preliminary report [28]. At present we have no evidence that true glucokinase (EC 2.7.1.2) is other than an exclusively hepatic enzyme. Certainly, our results support the negative findings of glucokinase in rat intestinal mucosa [36] and make is doubtful whether the adaptive behaviour of so-called 'glucokinase' activity in that tissue [19] should be interpreted in that way. The appearance of glucose-phosphorylating activity with a higher K_m for glucose during the aging of human [37] and rabbit [38] erythrocytes, when these cells are no longer synthesizing protein, has been interpreted as the age-dependent post-translational modification of a primary low-K_m hexokinase; this is another possibility to be kept in mind when considering other tissues. The procedures used here and elsewhere [27] should provide the means of re-examining other tissues such as the various tumours that have been reported to contain glucokinase [9].

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