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Distribution, Purification and Properties of 1-Aspartamido-β-*N*-acetylglucosamine Amidohydrolase

BY J. CONCHIE AND I. STRACHAN
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

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1. The activity of the enzyme that splits 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose (1-aspartamido-β-*N*-acetylglucosamine) was measured in tissues from different mammalian species. 2. The enzyme from an aqueous extract of rat liver was purified 150-fold in 56% yield. 3. Optimum activity for the hydrolysis of 1-aspartamido-β-*N*-acetylglucosamine was at pH 7, and ammonia and *N*-acetylglucosamine were liberated in equimolar amounts. At pH 8·5, 1-amino-*N*-acetylglucosamine was the only sugar produced after short periods of incubation. On prolonged incubation there was spontaneous liberation of ammonia from this compound. 4. It is concluded that the enzyme is an amidase.

An important linkage between the carbohydrate and protein components of many glycoproteins is that which links *N*-acetylglucosamine and asparagine (Marks, Marshall & Neuberger, 1963). Preliminary reports indicated that enzymes that attacked 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose (1-aspartamido-β-*N*-acetylglucosamine) could be obtained from various sources, e.g. molluscs (Kaverzneva, 1965), ram testis (Roston, Caygill & Jevons, 1965), ram epididymis (Murakami & Eylar, 1965) and boar epididymis (Clamp, Dawson, Hough & Khan, 1966). The exact mode of action of the enzyme remained unknown, however, and it was generally considered to be an *N*-glycosidase until Makino, Kojima & Yamashina (1966) described an enzyme occurring in mammalian sera that split 1-aspartamido-β-*N*-acetylglucosamine with the production of 1-amino-*N*-acetylglucosamine and aspartic acid. The 1-amino-*N*-acetylglucosamine was then hydrolysed non-enzymatically at the acid pH of the assay to give *N*-acetylglucosamine and ammonia.

While the present work was in progress, a similar enzyme was found to be of widespread occurrence in rat tissues (Ohgushi & Yamashina, 1968), and the enzyme from pig plasma was found to attack both 1-aspartamido-β-*N*-acetylglucosamine and a glycopeptide from ovalbumin (Makino, Kojima, Ohgushi & Yamashina, 1968). More intensive studies on the purified enzyme from rat kidney have been made by Mahadevan & Tappel (1967), who confirmed that the enzyme was an amidase and showed it to be particle-bound and latent in sucrose homogenates. A similar enzyme has been described in hen oviduct by Tarentino & Maley (1969).

The present paper describes the occurrence of the enzyme in a variety of sources, together with the purification and properties of the rat liver enzyme, as a preliminary to its use for structural studies on glycopeptides that contain an aspartamido–*N*-acetylglucosamine linkage.

MATERIALS AND METHODS

We are indebted to Professor L. Hough, Department of Organic Chemistry, Queen Elizabeth College, London, for a generous supply of synthetic 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose, and for samples of analogues in which the aspartyl residue is replaced by glycyl, valyl or seryl residues, as well as of 1-amino-*N*-acetylglucosamine.

Enzyme assays

α-Mannosidase. The hydrolysis of 6mM-*p*-nitrophenyl α-D-mannoside in 0·125M-acetic acid–NaOH buffer, pH 5·0, at 38° in the presence of 0·01% bovine serum albumin was measured as described by Levvy & Conchie (1966).

β-N-Acetylglucosaminidase. The hydrolysis of 5mM-*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0·05M-citric acid–NaOH buffer, pH 4·4, at 38° was measured in the presence of 0·01M-NaCl and 0·01% albumin, as described by Levvy & Conchie (1966).

1-Aspartamido-β-N-acetylglucosamine amidohydrolase. The incubation mixture contained 0·2M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0 (0·25ml.), 11·2mM-1-aspartamido-β-*N*-acetylglucosamine (0·25ml.), water (0·25ml.) and enzyme (0·25ml.). After incubation for 1hr. at 38°, the reaction was stopped by boiling for 3min. The mixture was centrifuged at 1500*g* for 10min. and 0·5ml. of the supernatant was removed for determination of *N*-acetylglucosamine by the method of Levvy & McAllan (1959). The substrate alone gave no colour with this procedure. Results were expressed as μmoles of *N*-acetylglucosamine liberated/hr./g. of tissue. Enzyme dilutions were adjusted to give less

than 10% hydrolysis of the substrate during the incubation period.

Asparaginase. Asparaginase activity was measured as described by Meister (1955), the ammonia liberated being determined as described below.

General methods

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

N-Acetylglucosamine determination. *N*-Acetylglucosamine was measured by the method of Levvy & McAllan (1959) except that the borate buffer was prepared by titration of boric acid to pH 9·1 with KOH. The borate solution was made 4% with respect to glycerol to prevent deposition of the salt (R. Begbie, personal communication). The samples of *p*-dimethylaminobenzaldehyde used (British Drug Houses Ltd., Poole, Dorset) were purified by fractionation from ethanol with water as described by Randle & Morgan (1955).

Ammonia determination. Ammonia was determined by a modification of the aeration method of Folin & Farmer (1912), followed by reaction with Nessler's reagent.

Thin-layer chromatography. Identification of sugars and amino acids was made by t.l.c., with cellulose as adsorbent. The solvents used for sugars were (A) ethyl acetate–pyridine–water (10:4:3, by vol.) and (B) ethyl acetate–propan-1-ol–water–aq. NH_3 (sp.gr. 0·88) (10:70:20:1, by vol.). For amino acids, butanol–acetic acid–water (4:1:4, by vol.) was used. For identification of sugars the chromatograms were sprayed with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) and for identification of amino acids, with ninhydrin.

EXPERIMENTAL AND RESULTS

Occurrence of the amidase

Enzyme preparations were made from a number of sources and assayed for activity. Tissues were homogenized in water with an Ultra-Turrax TP 18/2N disintegrator, and the resulting homogenates were made 0·1 M with respect to acetic acid–sodium hydroxide buffer, pH 5·2. After incubation for 1 hr. at 38°, the suspensions were centrifuged at 1500*g* for 15 min. and the supernatants assayed for amidase activity. A summary of total enzyme activities, measured at an arbitrary substrate concentration of 0·71 mM, is given in Table 1. For mammalian sera, incubations had to be done for 24 hr. to obtain satisfactory assay readings. In view of the fact that Makino *et al.* (1966) found that the pH optimum of the enzyme in pig serum was about 5·5, assays on pig and deer serum were repeated at pH 5, with results similar to those quoted in Table 1.

Of the enzyme sources examined, guinea-pig epididymis was the richest, although other guinea-pig tissues were not particularly rich in the enzyme. Boar epididymal preparations, after fractionation with ammonium sulphate between 20%- and 80%-saturation limits, were active when freshly prepared, but preparations that had been kept at −20° varied in activity with duration of storage and the number of times they had been thawed and refrozen. When boar epididymal preparations were further fractionated with acetone, as in the procedure for the purification of β-*N*-acetylglucosaminidase (Findlay & Levvy, 1960), the amidase activity was destroyed or removed.

After a study of some of the properties of the enzyme (see below) a substrate concentration of 2·8 mM was adopted for routine assay purposes. Table 2 shows the specific activities of a number of

Table 1. *Distribution of the enzyme*

Tissue extracts were prepared as described in the text. Sera were assayed directly. Assays were carried out as described in the Material and Methods section: the final substrate concentration was 0·71 mM. Results are expressed as μmoles of *N*-acetylglucosamine liberated/hr./g. of tissue (/ml. for sera).

Source	Activity
Rat liver	2·4
kidney	2·6
spleen	3·0
testis	0·71
epididymis	1·9
brain	0·24
preputial gland (male)	1·5
preputial gland (female)	0·94
Mouse liver	0·91
Guinea pig spleen	0·87
liver	0·37
kidney	0·71
epididymis	4·1
testis	1·7
Pig liver	Nil
epididymis	1·3
serum	0·04*
Sheep liver	0·3
serum	Nil*
Cattle liver	0·90
Human liver	0·1*
tumour (stomach)	0·57
tumour (lung)	0·56
serum	0·02*
foetal liver	1·1†
Reindeer liver	0·70†
Deer serum	0·03*
Jack bean meal	Nil
Pronase	Nil
Papain	Nil
Limpet visceral hump	1·5

* 24 hr. assay.
† with 2·8 mM substrate.

Table 2. *Specific activities of the enzyme from various sources*

Tissue extracts were prepared as described in the text. Enzyme activities were measured as described in the Materials and Methods section: the final substrate concentration was 2·8mM. Protein determinations were made by the method of Lowry *et al.* (1951). Results are expressed as μmoles of *N*-acetylglucosamine liberated/hr./mg. of protein.

Source	Specific activity
Rat liver	0·052
kidney	0·098
spleen	0·063
epididymis	0·217
testis	0·040
Mouse liver	0·009
Boar epididymis	0·070
Limpet visceral hump	0·021
Guinea pig epididymis	0·122
testis	0·112

Table 3. *Purification procedure*

For method of enzyme assay see the Materials and Methods section. A description of each stage is given in the Experimental section.

Stage	Description	Specific activity (μmoles of *N*-acetylglucosamine/hr./mg. of protein)	Total activity (μmoles of *N*-acetylglucosamine/hr.)
1	Whole extract	0·053	792
2(*a*)	0–40%-satd. $(NH_4)_2SO_4$	0·019	63
2(*b*)	40–65%-satd. $(NH_4)_2SO_4$	0·149	865
3	Dialysis	0·233	836
4(*a*)	0–35% acetone	0·074	69
4(*b*)	35–60% acetone	1·16	642
5(*a*)	Sephadex fractions 28–31, 41–45	1·75	141
5(*b*)	Sephadex fractions 32–40	7·84	443

preparations assayed at this substrate concentration. As before, assays were done on the buffer extract of a homogenate. The high specific activity of rat epididymal preparations reflects the low protein content of this tissue. However, the relative abundance of rat liver made it the preferred source for further studies on the enzyme.

Purification of the enzyme from rat liver

General comments. It was found in preliminary experiments that all the amidase activity in a buffer extract of a rat liver homogenate could be precipitated with ammonium sulphate between 40%- and 65%-saturation limits. In the procedure described below, volume changes were corrected for by adding water after mixing the reagents. All precipitates were left at 0° for 30min. before centrifuging. Except at the first stage, all precipitates were removed by centrifuging at 10000*g* for 10min. at 0°.

A summary of the results obtained at the various stages of purification is given in Table 3.

Stage 1 (*buffer extract*). Livers (260g.) from 20 rats (hooded Lister) were homogenized in ice-cold water with an Ultra-Turrax TP18/2N disintegrator and the suspension was made 0·1M with respect to acetic acid–NaOH buffer, pH5·2. The suspension (1300ml.) was incubated for 1hr. at 38° and stored overnight at −20°. After being thawed, the homogenate was centrifuged at 1500*g* for 15min. at room temperature and the supernatant kept for the next stage of purification.

Stage 2 (*ammonium sulphate fractionation*). (*a*) Solid ammonium sulphate was stirred into the supernatant to give a final saturation of 40%, and the precipitate formed was separated by centrifuging. (*b*) The supernatant was brought to 65% saturation with solid ammonium sulphate and the resulting precipitate was dissolved in water and used for the next purification stage. No further amidase activity was obtained by increasing the ammonium sulphate concentration.

Stage 3 (*dialysis*). The enzyme solution from stage 2(*b*) was dialysed against distilled water at 4° for 3–4 days with frequent changes of water. The inactive deposit that formed in the sac during dialysis was removed by centrifugation.

Stage 4 (*acetone fractionation*). To the dialysed solution, cooled to 0°, ice-cold acetone was added, with stirring, to give concentrations of (*a*) 35% and (*b*) 60% (v/v) acetone. The precipitates were removed and dissolved in water. The final supernatant had no amidase activity.

Stage 5 (*Sephadex fractionation*). The 35–60% acetone fraction (stage 4*b*) was passed through a column of Sephadex G-100 at 4°, elution being carried out with 0·02M-Na_2HPO_4–KH_2PO_4 buffer, pH7·3 (Fig. 1). The fractions with the highest specific activities (32–40) were pooled (stage 5*b*), and a second, less active, preparation was obtained by pooling fractions 28–31 and 41–45 (stage 5*a*). The enzyme in fractions 34 and 36 had specific activities of 9·8 and 11·8 μmoles of *N*-acetylglucosamine liberated/hr./mg. of protein respectively. The latter activity represents a 213-fold purification

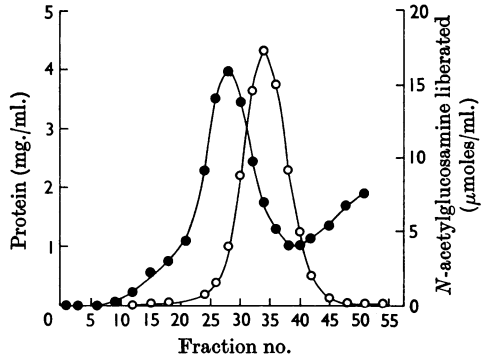


Fig. 1. Elution at 4° of the amidase (stage 4*b*) from a column of Sephadex G-100 (120cm.×3cm.) with 0·02M-Na_2HPO_4–KH_2PO_4 buffer, pH7·3. After 75ml. of eluate had been run to waste, 5ml. fractions were collected. Amidase activity (○) and protein content (●) were measured as described in the text.

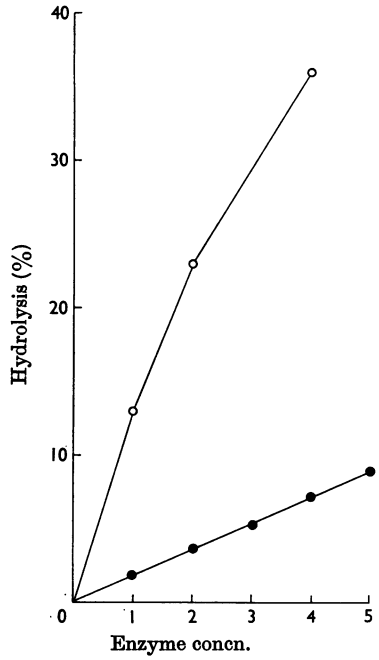


Fig. 2. Percentage hydrolysis of: ○, 0·71mM- and ●, 2·8mM-l-aspartamido-β-*N*-acetylglucosamine after incubation for 1hr. with various amounts of enzyme. Enzyme concentrations are in arbitrary units.

of the buffer extract. The more active of the combined fractions (stage 5*b*) showed a 148-fold purification, and contained about 56% of the original total

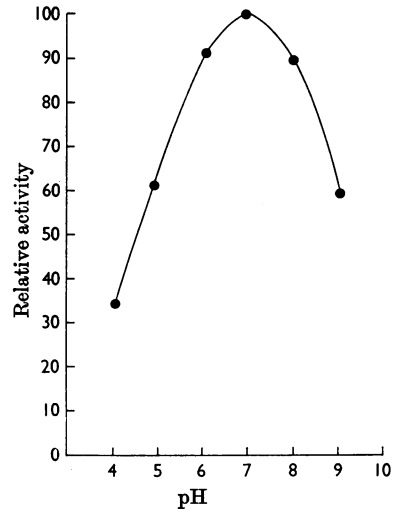


Fig. 3. Hydrolysis at various pH values of 2·8mM-l-aspartamido-β-*N*-acetylglucosamine in 0·05M-acetic acid–NaOH buffer (pH4–5) and 0·05M-Na_2HPO_4–KH_2PO_4 buffer (pH6–9) by purified rat liver enzyme. For other assay conditions see the Materials and Methods section. An activity of 100 is equivalent to 0·232μmole of *N*-acetylglucosamine liberated/hr.

activity. This product could be concentrated to 3–4ml. by freeze-drying, and the resulting solutions could be stored for many months at −20° without loss in activity.

Both final products (stages 5*a* and 5*b*) were free from L-asparaginase, β-*N*-acetylglucosaminidase and α-mannosidase activities.

Properties of the enzyme

Effect of varying the enzyme concentration. Since only a limited quantity of substrate was available, it could not be employed in the enzyme assay at its optimum concentration. At the usual activities employed for assay, it was found (Fig. 2) that the use of 0·71mM substrate did not give a linear relationship to enzyme concentration when there was more than 10% hydrolysis. This difficulty was avoided by the use of 2·8mM substrate. With possible exceptions in Table 1, in all measurements the degree of hydrolysis of substrate was less than 10%.

Variation of activity with pH. The pH optimum for the purified preparations from rat liver was 7·0 (Fig. 3). Preparations from limpet (*Patella vulgata*), rat liver and rat epididymis, after fractionation with ammonium sulphate within 20%- and 80%-saturation limits, had pH optima of 7·0, 7·0 and 8·0 respectively.

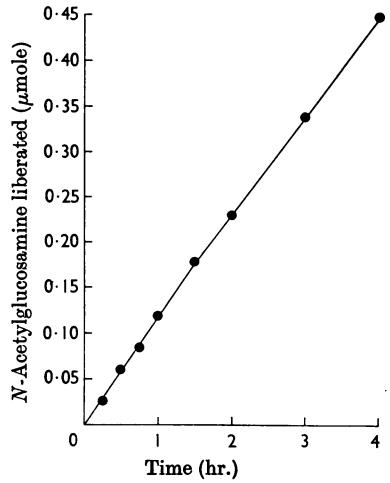


Fig. 4. Effect of increasing periods of incubation on the hydrolysis of 2·8 mM-l-aspartamido-β-*N*-acetylglucosamine by the rat liver enzyme. Assays were done, after the period shown, as described in the text.

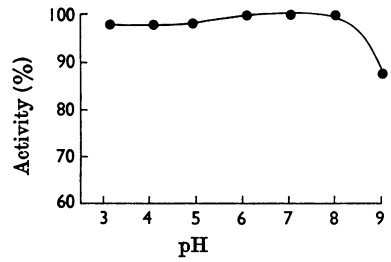


Fig. 5. Effect of preliminary incubation for 1 hr. at various pH values on the rat liver enzyme. Assays were subsequently done, as described in the text, at pH 7 and results are expressed as percentages of values for an untreated control. The control liberated 0·208 μmole of *N*-acetylglucosamine.

Effect of duration of hydrolysis. Measurement of the rate of hydrolysis of the substrate by the enzyme over various periods of time (Fig. 4) showed that the rate was unchanged for periods of incubation up to 1·5 hr., after which there was a slight decrease.

Variation in stability with pH. Samples of the purified enzyme were incubated for 1 hr. at 38° after adjustment with hydrochloric acid or sodium hydroxide to the required pH value. The solutions were then cooled and readjusted to the original pH (7·3) before assay in the usual way. Activities were compared with those of an untreated control that had been kept at 0°. Fig. 5 shows that the enzyme was almost completely stable over the pH range 3–8.

Effect of varying the substrate concentration. These experiments were limited to some extent by the need to keep the hydrolysis of substrate below 10% while still obtaining measurable activity. Substrate concentrations over a range 0·4–5·7 mM were employed (Fig. 6). Linear plots were obtained from which K_m was calculated by the method of Lineweaver & Burk (1934). The mean value obtained for a partially purified rat epididymal preparation (precipitated at 20–80%-ammonium sulphate saturation) was 0·65 mM and for a purified rat liver preparation, 0·58 mM. A substrate concentration of 2·8 mM was chosen for routine purposes, since it gave suitable assay readings with reasonable economy of substrate.

Effect of N-*acetylglucosaminono-*(1→5)-*lactone.* When assayed at pH 5 and pH 7 with 2-acetamido-2-deoxyglucono-(1→5)-lactone [*N*-acetylglucosaminono-(1→5)-lactone] (Conchie, Hay, Strachan & Levvy, 1967) at 50 mM concentration in the incubation mixture, no inhibition of rat liver or rat epididymal amidase was obtained. The amidase activity of a boar epididymal preparation was not inhibited at pH 5 or pH 7 by 100 mM-*N*-acetylglucosaminono-(1→5)-lactone in the assay mixture. Both epididymal preparations had been precipitated at 20–80%-ammonium sulphate saturation and the rat liver enzyme was a stage 5(*b*) preparation (Table 3).

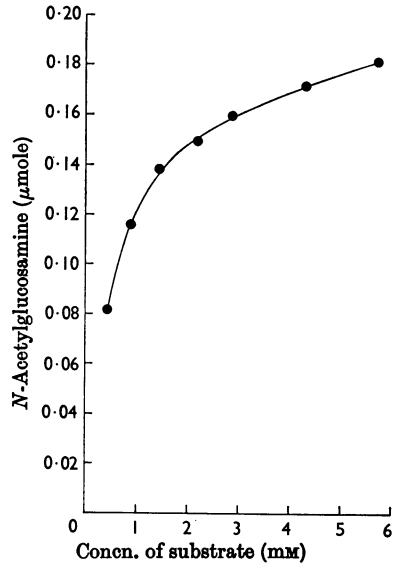


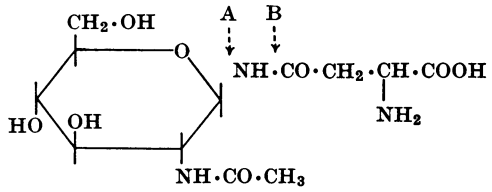
Fig. 6. Effect of varying the substrate concentration on the rate of hydrolysis of l-aspartamido-β-*N*-acetylglucosamine in 0·05 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7, by the purified rat liver preparation. For other conditions of assay see the Materials and Methods section.

Mechanism of the enzyme reaction

Investigations were made to determine whether the synthetic substrate (compound I) was hydrolysed by the rat liver enzyme at the *N*-glycoside (A) or at the amide (B) link between the sugar and the amino acid. In the first case the products of the reaction would be *N*-acetylglucosamine and asparagine, and in the second case 1-amino-*N*-acetylglucosamine and aspartic acid.

Table 4 shows liberation of ammonia and *N*-acetylglucosamine at two pH values. At pH 7, equivalent amounts of ammonia and *N*-acetylglucosamine were obtained and at pH values of about 8·5 there was less ammonia than *N*-acetylglucosamine in the incubation mixture, the actual ratio depending to some extent on the nature of the buffer.

The nature of the sugar component liberated was examined by t.l.c. with cellulose as adsorbent and solvents A and B (see the Materials and Methods section). *N*-Acetylglucosamine and 1-amino-*N*-acetylglucosamine were used as standards. At pH 7, the only sugar observed in the reaction mixture was *N*-acetylglucosamine. At pH 8·5, after incubation for 3 hr. in 0·05 M-barbital–NaOH buffer, the sole product was a compound with essentially the same R_X values (X = *N*-acetylglucosamine) in solvents A and B (0·41 and 0·66 respectively) as 1-amino-*N*-acetylglucosamine (0·42 and 0·67 respectively). After incubation for 27 hr., this compound was still the major component, but there was also a small quantity of sugar corresponding to *N*-acetylglucosamine. On examination of the products of incubation at either pH value for amino acids by t.l.c., only aspartic acid was detected.



(I) 1-L-Aspartamido-β-*N*-acetylglucosamine

Table 4. *Relative amounts of* N-*acetylglucosamine and ammonia liberated from substrate by the amidase*

1-Aspartamido-β-*N*-acetylglucosamine (11·2 μmoles) and enzyme (0·05 ml., sp. activity 6·67 μmoles/hr./mg. of protein) were incubated in buffers in a total volume of 2 ml. for 22 hr. Ammonia and *N*-acetylglucosamine were determined as described in the Materials and Methods section.

pH of incubation	Buffer	Hydrolysis (%)	*N*-acetylglucosamine/ammonia ratio
7	0·05 M-Na_2HPO_4–KH_2PO_4	84	1:1
8·4	0·05 M-Na_2HPO_4–KH_2PO_4	91	1:0·6
8·5	0·05 M-barbital–NaOH	91	1:0·17

Action of the enzyme on other derivatives of 1-*amino*-N-*acetylglucosamine*

Both crude and purified rat liver preparations of the enzyme were incubated for 1 hr. at 37° with 1-*N*-glycyl-, 1-*N*-valyl- and 1-*N*-seryl-2-acetamido-2-deoxy-β-D-glucopyranosylamines at 2·8 mM concentration in 0·05 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. In no case was there any indication of enzyme activity, as measured by liberation of hexosamine.

DISCUSSION

The enzyme described in the present paper is widely distributed in mammalian tissues, and purified preparations from rat liver are in many respects similar in their properties to enzymes described by Makino *et al.* (1966) and Mahadevan & Tappel (1967). The pH optimum is higher with mammalian tissue preparations [7·0 and 8·0 for the preparations described here, 7·6 for those of Mahadevan & Tappel (1967)] than for the mammalian sera preparations of Makino *et al.* (1966) (5·5 for pig serum). All preparations studied so far, however, have much the same affinity for 1-aspartamido-*N*-acetylglucosamine, the K_m value for our rat liver preparation being 0·58 mM, that for rat kidney enzyme, 0·59 mM (Mahadevan & Tappel, 1967), and that for pig serum enzyme, 1 mM (Makino *et al.* 1966).

Makino *et al.* (1966) produced convincing evidence that the enzyme from pig serum acted on the amide linkage (B) of the substrate (compound I) with the production of 1-amino-*N*-acetylglucosamine and aspartic acid: at the pH optimum 1-amino-*N*-acetylglucosamine was very unstable and decomposed to give ammonia and *N*-acetylglucosamine. Chromatographic examination of the sugar component released during incubation of the substrate with our enzyme indicated that at pH 7 the sole component was *N*-acetylglucosamine, whereas at pH 8·5 the initial sugar released had R_X values (X = *N*-acetylglucosamine) identical to those for 1-amino-*N*-acetylglucosamine. Prolonged incubation at the latter pH resulted in the production of increasing amounts of *N*-acetylglucosamine, presumably due to slow decomposition of the 1-amino compound. Moreover, the differential release of

ammonia in incubation mixtures at pH 7 and pH 8·5 is in agreement with the known instability of 1-amino-*N*-acetylglucosamine at acid pH. Chromatographic examination for amino acids also indicated that aspartic acid and not asparagine was liberated from the substrate. The enzyme is thus an amide hydrolase, splitting the linkage at B (see formula I), and not an *N*-glycosidase.

An aqueous extract of the enzyme from homogenized rat liver could be purified 150-fold by the procedure described (Table 3). The purest fraction obtained with Sephadex G-100 had a specific activity of 11·8 μmoles of *N*-acetylhexosamine liberated/hr./mg. of protein when assayed at a substrate concentration of 2·8 mM. This compares favourably with the best fraction obtained from a purified rat liver preparation by Mahadevan & Tappel (1967), which had a specific activity of 9·8 μmoles of *N*-acetylglucosamine liberated/hr./mg. of protein when assayed at a substrate concentration of 5 mM.

The elimination of associated glycosidases and L-asparaginase makes the preparation particularly useful for enzymic studies on those glycopeptides that contain an aspartamido–*N*-acetylglucosamine linkage. It is noteworthy that Jenner & Pollitt (1967) found that 1-aspartamido-β-*N*-acetylglucosamine was excreted in large amounts (about 300 mg./day) in the urine of two mentally retarded siblings. This may be indicative of some defect in the metabolism of glycoproteins with this linkage.

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