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Purification and some Kinetic Properties of Rat Liver Glucosamine Synthetase

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1. Glucosamine synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) was purified about 300-fold from rat liver by two techniques. One procedure utilized the protective action of fructose 6-phosphate and gave a relatively stable preparation, the other yielded an unstable enzyme (half-life of about 20h), free of contaminant activities, on which kinetic experiments were performed. Although the properties of the two preparations showed slight differences, the unstabilized form could be converted into the stabilized form. 2. During preparation the enzyme retained its sensitivity to the feedback inhibitor, UDP-*N*-acetylglucosamine. 3. The reversibility of the enzyme-catalysed reaction could not be demonstrated. There was no apparent requirement for a cofactor. 4. The pH optimum was at 7.5, at which pH the reaction obeyed a Ping Pong Bi Bi rate equation. At pH values outside the range 6.9–7.6 and at temperatures below 29°C the velocity was described by an ordered Bi Bi rate equation. 5. The molecular weight of the enzyme, determined by two procedures, was 360000–400000. 6. The aminotransferase was unable to utilize ammonia as a substrate.

Glucosamine synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) catalyses the reaction:

L-Glutamine + D-fructose 6-phosphate → D-glucosamine 6-phosphate + L-glutamate

and occurs as the first enzyme in the pathway of hexosamine biosynthesis (Phelps, Hardingham & Winterburn, 1970).

Previous work on this enzyme, initiated by Leloir & Cardini (1953) and extended by others (Gryder & Pogell, 1960; Ghosh, Blumenthal, Davidson & Roseman, 1960), revealed its widespread distribution in animal and bacterial sources. It was later shown by Kornfeld, Kornfeld, Neufeld & O'Brien (1964) and Kornfeld (1967) that glucosamine synthetase isolated from animal origins was susceptible to feedback regulation by the end product of the hexosamine pathway, UDP-*N*-acetylglucosamine, whereas the enzyme derived from the bacterial species studied was devoid of this regulatory ability.

Because of the instability of this enzyme, information on this reaction has been derived mainly from preparations of low purity from which contaminant activities were not excluded. In the present work the enzyme was isolated from rat liver, since previous work (Pogell & Gryder, 1957) had indicated that the highest activities were obtainable from this material. This source offered the additional advantage of an extensive documentation of metabolite concentrations and carbohydrate control mechanisms. The stabilizing influence of glucose 6-phosphate during purification (Gryder & Pogell, 1960; Kornfeld, 1967) was not used. The object of the purification reported in this paper was to explore systematically the steady-state kinetics of the enzyme. It was considered unwise to incorporate a factor whose mode of stabilizing was uncertain, as this might cast doubt on the subsequent kinetic patterns observed. Two preparative procedures are therefore described; in the first is generated an unstabilized preparation (preparation I) suitable for kinetic studies. Investigations into preparation I (Winterburn & Phelps, 1971*b*) revealed that the protective action of one of the substrates, fructose 6-phosphate, could be utilized for preparing a more stable and purer fraction (preparation II). During purification care was taken to ensure that no apparent desensitization of the enzyme to its feedback inhibitor occurred.

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MATERIALS AND METHODS

Materials. Pyridoxine, pyridoxamine phosphate, pyridoxal phosphate, tris, glutamine and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. ATP (sodium salt), UDP-*N*-acetylglucosamine, glucose 6-phosphate (sodium salt),

GSH and fructose 6-phosphate (barium salt) were products of Boehringer Corp. (London) Ltd., London W.5, U.K. The barium salt of fructose 6-phosphate was converted into the free acid on a column of Dowex 50 (X8; H^+ form, 50–100 mesh) and immediately neutralized to pH6.9 with NaOH. $(NH_4)_2SO_4$ was enzyme grade supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. Glucosamine 6-phosphate was prepared by the method of Jourdain & Roseman (1962). Mannose 6-phosphate was prepared by phosphorylation of D-mannose (BDH Chemicals Ltd.) by using ATP and hexokinase [Boehringer Corp., (London) Ltd.] essentially by the method of Slein (1957). Hydroxyapatite was prepared as described by Tiselius, Hjerten & Levin (1956) and stored as a suspension (100mg of solids/ml) in 5mM-KH_2PO_4 adjusted to pH6.5 with KOH. DEAE-cellulose was precycled according to the manufacturer's instructions. Rats were female Wistar strain weighing 150–300g.

Assay of glucosamine synthetase activity. The activity was measured by using the following incubation system in a final volume of 0.5–1.0ml: 50mM-tris, 1mM-EDTA, 5mM-fructose 6-phosphate, 12mM-glutamine and enzyme adjusted to pH7.5 with HCl and to an ionic strength of 0.2 with KCl. These substrate concentrations were in excess of those required for saturation of the enzyme to compensate for substrate depletion by other activities present in the cruder extracts. Incubations, started by the addition of enzyme, were for 30 or 60min at 37°C. The reaction was terminated either by boiling for 1min or by the addition of $HClO_4$ to a final concentration of 6% (w/v) and subsequent neutralization. The $HClO_4$ procedure was preferable for incubation mixtures that contained more than 2mg of protein/ml. With this system the production of glucosamine 6-phosphate was linear with time up to 90min and was proportional to the enzyme concentration under all conditions tested.

Glucosamine 6-phosphate was assayed by the Morgan–Elson reaction as detailed by Good & Bessman (1964) except for the following modification. The extinction was measured at 545nm and, since the chromagen was unstable (about 14% linear decay/h), the extinctions were measured twice and extrapolated to a common zero time (the time when the samples were cooled after the 37°C incubation). Internal standards of glucosamine 6-phosphate were processed in control experiments to correct for interference by other compounds in the Morgan–Elson reaction.

During purification the sensitivity of the enzyme to UDP-*N*-acetylglucosamine was checked in the following manner. The maximal activity of a fraction was determined in the presence and absence of UDP-*N*-acetylglucosamine (final concn. 0.1mM), and the ratio of these two respective velocities was taken as indicative of the sensitivity of the enzyme to its feedback inhibitor. This ratio, referred to as the control ratio, varied slightly between preparations; however, all studies were performed on enzyme preparations exhibiting a control ratio less than 0.55.

Separation of reaction components. The sample was diluted with water to decrease the ionic strength below 0.01 and applied to a column (0.8cm×11cm) of Dowex 1 (X8; formate form; 200–400 mesh). The glutamine was eluted with 40ml of water. Glutamate and glucosamine 6-phosphate were eluted with a 200ml linear gradient of 0–0.08M-formic acid, the glucosamine 6-phosphate being eluted before the glutamate. Fructose 6-phosphate was eluted with a 200ml linear gradient of 0.08–5M-formic acid. Formic acid was removed by three extractions with an equal volume of diethyl ether and the aqeuous phase was freeze-dried.

Glutamate was determined by the method of Bernt & Bergmeyer (1963). Glutamine was hydrolysed in 2M-HCl at 100°C for 2h, neutralized with 10M-KOH and assayed for glutamate. Fructose 6-phosphate was determined by the method of Hohorst (1963).

Preparation of [U-^{14}C]*glucosamine 6-phosphate.* A mixture containing 30μmol of glucosamine hydrochloride, 30μmol of $MgCl_2$, 30μmol of ATP, 50μCi of [U-^{14}C]-glucosamine (170mCi/mmol; Volk Radiochemical Co., Skokie, Ill., U.S.A.) and 400μmol of tris in 20ml of water was adjusted to pH8.0 with 1M-HCl and incubated at 30°C for 2h with 4 units of hexokinase. After being boiled for 1min to stop the reaction, the mixture was diluted (40-fold) with water and applied to a column (1.3cm× 16cm) of Dowex 1 (X8; formate form; 200–400 mesh). The resin was washed with water and eluted with a 200ml linear gradient of 0–0.15M-formic acid; 5ml fractions were collected. Fractions that contained radioactivity, determined as described by Hardingham & Phelps (1968), were pooled and freeze-dried. The specific radioactivity of the [U-^{14}C]glucosamine 6-phosphate was 0.97mCi/mmol.

Other enzyme assays. Glucosamine 6-phosphate *N*-acetyltransferase (EC 2.3.1.4), glutaminase (EC 3.5.1.2), phosphoglucose isomerase (EC 5.3.1.9) and phosphomannose isomerase (EC 5.3.1.8) activities were assayed as described by Winterburn (1969); phosphofructokinase (EC 2.7.1.11) activity was assayed by the method of Underwood & Newsholme (1965) and glucosamine 6-phosphate isomerase (EC 5.3.1.10) activity as described by Comb & Roseman (1958).

Determination of protein. Protein measurements were subject to large errors caused by the presence of GSH and tris unless the following procedure was employed. The protein was precipitated with trichloroacetic acid (final concn. 6%, w/v) and collected by centrifugation. The precipitate was redissolved in a minimum of 0.7M-NaOH, and water was added to adjust the protein concentration to a suitable range for assay by either the biuret method of Gornall, Bardawill & David (1949) or the Folin–Ciocalteu method of Miller (1959). The procedure was standardized with dry bovine serum albumin.

RESULTS

Cell localization experiments showed that all of the glucosamine synthetase activity was present in the supernatant fraction, in agreement with Izumi (1965).

Preparation I

Stages 1 *and* 2: *preparation of* 105000*g supernatant.* All operations were performed at 4°C unless stated otherwise, care being taken to avoid freezing the less pure fractions, since this resulted in complete

inactivation of the enzyme. Rat livers were rapidly removed from the exsanguinated corpse and chilled to 2°C in TEG buffer (50mM-tris–5mM-EDTA–5mM-GSH adjusted to pH7.8 with 2M-HCl)–100mM-KCl, pH7.8. The livers were homogenized in 3vol. of the above buffer in a Potter–Elvehjem homogenizer (stage 1). The homogenate was centrifuged at 18000*g* for 30min. The supernatant fluid was centrifuged at 105000*g* for 60min and the supernatant retained (stage 2).

Stage 3: *first DEAE-cellulose fractionation.* Ghosh *et al.* (1960) demonstrated that DEAE-cellulose was a useful fractionating system for this enzyme, and Kornfeld (1967) developed this into a column chromatography procedure. Because of the need for a rapid separation of this unstable enzyme, a column (5.5cm×5cm) of DEAE-cellulose equilibrated in TEG buffer–100mM-KCl, pH7.8, was used. By applying gentle suction the flow rate was maintained at 600–800ml/h. A column of these dimensions was used to fractionate up to 1g of 105000*g*-supernatant protein. After application of the supernatant, the column was washed with 3–4 bed volumes of the equilibrating buffer and the enzyme eluted with 150ml of TEG buffer–150mM-KCl, pH7.8.

Stage 4: *second DEAE-cellulose fractionation.* This step was designed to concentrate as well as to further purify the enzyme. The eluate from the first fractionation was diluted with 3vol. of 5mM-EDTA–5mM-GSH, pH7.0, to lower the ionic strength and adjusted to pH6.7 with 0.1M-cacodylic acid. Inactivation resulted if other dilutants were used and the substitution of imidazole for cacodylic acid inhibited the enzyme. This solution was applied to a column (2cm×7cm) of DEAE-cellulose equilibrated in TEG buffer–10mM-cacodylic acid, pH6.7, and adsorbed at a flow rate of 300ml/h. The enzyme was eluted with 2 bed volumes of TEG buffer–10mM-cacodylic acid–100mM-KCl, pH6.7. This fraction (about 15ml) was adjusted to pH7.5 by the careful addition of 0.1M-KOH and was used for the investigations.

Stage 5: *hydroxyapatite fractionation.* To the above fraction was added 10mg of hydroxyapatite/mg of protein. The solution was gently agitated for 10min before the hydroxyapatite was collected by brief low-speed centrifugation. The hydroxyapatite was washed for 10min with 10ml of 150mM-KH_2PO_4–5mM-EDTA–5mM-GSH adjusted to pH7.5 with 2M-KOH, recollected and the supernatant discarded. The enzyme was desorbed with 7ml of 220mM-KH_2PO_4–5mM-EDTA–5mM-GSH adjusted to pH7.5 with 2M-KOH for 10min.

The purification and yields at the various stages are shown in Table 1. The entire procedure was completed in 6h. If the 105000*g* supernatant was prepared the previous day and stored at 4°C

Table 1. *Purification of glucosamine synthetase from rat liver*

For experimental details and definition of control ratio see the text.

		Preparation I				Preparation II			
Stage	Description	Specific activity (μmol/h per mg of protein)	Purification	Yield (%)	Control ratio	Specific activity (μmol/h per mg of protein)	Purification	Yield (%)	Control ratio
1	1:3 (w/v) Homogenate	0.340	1	100	*	0.026	1	100	*
2	105000*g* Supernatant	0.071	2.1	86	0.33	0.058	2.2	91	0.33
3	First DEAE-cellulose column, pH7.8	0.53	16	34	0.34	0.57	22	67	0.38
4	Second DEAE-cellulose column, pH6.7	0.62	18	10	0.50	1.78	69	36	0.30
5	Hydroxyapatite	4.33	127	4	—	9.7	374	27	0.40
6	$(NH_4)_2SO_4$ fractionation	—	—	—	—	4.2	162	5	0.39

* UDP-*N*-acetylglucosamine rapidly degraded.

overnight (see below for stability) investigations were started after a 3h purification procedure.

Preparation II

A purer fraction of higher activity was prepared by a method developed from that described above. This utilized the stabilizing properties of fructose 6-phosphate (Winterburn & Phelps, 1971*b*); however, this preparation was unsuitable for some studies since no adequate method for the subsequent removal of the substrate was devised that did not result in a substantial loss of enzyme activity.

Stages 1–5. The 105000*g* supernatant was prepared as before except that all buffers contained 5mM-glucose 6-phosphate, since crude preparations contained a high phosphoglucose isomerase activity which generated sufficient fructose 6-phosphate. All subsequent steps required the incorporation of 1mM-fructose 6-phosphate. The hydroxyapatite fractionation followed the procedure of preparation I except that the phosphate concentrations in the buffers for washing and elution of the adsorber were 100mM and 200mM respectively.

Stage 6: *ammonium sulphate fractionation.* Although in the absence of a protector (glucose 6-phosphate or fructose 6-phosphate) the glucosamine synthetase was inactivated during fractionation with ammonium sulphate, the enzyme was stable when either of these agents was incorporated into the media. The buffered enzyme solution derived from stage 5 was fractionated by adding solid ammonium sulphate; the enzyme was precipitated between the limits of 1.5–2.3M. The precipitate was redissolved in TEG buffer–100mM-KCl–1mM-fructose 6-phosphate, pH7.5.

The purification and yields are shown in Table 1.

Properties

Purity. After the second DEAE-cellulose fractionation (stage 4), the material was shown to be free of the following contaminating enzymes: phosphomannose isomerase, phosphoglucose isomerase, phosphofructokinase, glucosamine 6-phosphate *N*-acetyltransferase, glutaminase and glucosamine 6-phosphate isomerase.

Sensitivity to the feedback inhibitor. Some enzymes capable of feedback regulation have been shown to exhibit desensitization to the modifier during purification. To guard against this possibility, the response of the enzyme to UDP-*N*-acetylglucosamine was tested at each stage of the purification. The control ratios are shown in Table 1. It was evident that for preparation II this index did not change significantly, although a slight desensitization occurred in preparation I.

Stability. The activity of the aminotransferase in the 105000*g* supernatant did not decline over the initial 100h. After this period the loss was rapid. The enzyme purified to stage 4 of the fractionation (preparation I) had a half-time of inactivation of 15–20h. Dialysis, gel filtration and freeze-drying all resulted in high losses of activity. The addition of 1mM-fructose 6-phosphate (preparation II) extended the 50% inactivation time to 8 days. This fraction was stable to dialysis if fructose 6-phosphate was also present in the dialysing medium. The fractions from hydroxyapatite and ammonium sulphate treatments had similar stabilities. Storage of fractions at −15°C did not improve the stability of any fraction.

Effect of metal ions. With preparation I, 1mM-Fe^{2+}, -Zn^{2+}, -Cd^{2+}, -Cu^{2+} and -Co^{2+} (as sulphates) inhibited the enzymes by 55%, 97%, 99%, 100% and 100% respectively.

Effect of ionic strength. The activity was found to increase with increasing ionic strength to a maximum in the region I 0.18–0.25 and then decline. The magnitude of this activation was dependent on the nature of the univalent ion employed. This effect was investigated by using a slight modification of the standard assay procedure. With tris–HCl buffer, pH7.5 (I 0.12), the salt concentration in the incubation mixture was increased to yield an ionic strength of 0.22 with the following additions: caesium chloride, potassium sulphate, potassium chloride, sodium chloride and lithium chloride; the activities expressed as percentages relative to the activity without added salt were 103%, 109%, 115%, 130% and 146% respectively. The similarity between the activations by potassium sulphate and potassium chloride suggest that the effect is not primarily an anionic one, and the gradation of the enhancement follows the increasing hydrated ion diameter of the cation.

Cofactor requirement. Unlike most glutamine-utilizing aminotransferases this hexosamine-forming enzyme has been found not to require the concomitant hydrolysis of a pyrophosphate bond (Ghosh *et al.* 1960). This has been confirmed in the present work in two ways. Addition of ATP to the reaction caused no enhancement of activity, and preincubation with potato apyrase (EC 3.6.1.5) to remove possible enzyme-bound ATP likewise did not affect the activity.

Many aminotransferases require pyridoxal phosphate as a cofactor. However, glucosamine synthetase was not inhibited or activated by 100μM-pyridoxal phosphate, -pyridoxamine phosphate or -pyridoxine, and also 1mM-isoniazid, a general pyridoxal inhibitor (Davison, 1956), had negligible effect. These results were consistent with the view that this enzyme, in common with other glutamine aminotransferases, does not require pyridoxal phosphate.

Reversibility and stoicheiometry. Ghosh *et al.* (1960) were unable to demonstrate the reversibility of this reaction in an attempted coupling of the production of fructose 6-phosphate via phosphoglucose isomerase and glucose 6-phosphate dehydrogenase to NADPH formation. In the present work the reversibility was investigated by using [U-^{14}C]glucosamine 6-phosphate. The incubation mixture consisted of the standard assay components except that fructose 6-phosphate and glutamine were replaced by 6mM-glutamate and 5mM-[U-^{14}C]glucosamine 6-phosphate (0.97mCi/mmol) in 0.6ml final volume. The reaction was terminated after 2h and 1.5μmol of unlabelled fructose 6-phosphate was added as carrier. The products of the reaction were separated on a Dowex 1 (formate form) column. No incorporation of radioactivity into the fructose 6-phosphate could be detected.

Incubation of the standard assay mixture for 4h, isolation and quantitative determination of the reactants and products revealed that the substrates were stoicheiometrically converted into products, in confirmation of the reaction as indicated in the introduction.

Molecular weight. This was measured in two ways.

(*a*) Gel filtration. A column (1cm×56cm) of Sephadex G-200 was prepared as described by Andrews (1964) and equilibrated in TEG buffer–100mM-HCl, pH7.8, at a flow rate of 5ml/h. The column was calibrated with six proteins of known molecular weight. The glucosamine synthetase activity was eluted in a single peak; the elution volume corresponded to a molecular weight of 360000 with the assumption that the shape of the aminotransferase was approximately spherical.

(*b*) Sedimentation. The sedimentation coefficient of the enzyme was determined in an ultracentrifuge (Spinco model E) by using the moving-partition cell of Yphantis & Waugh (1956) as described by Schachman (1957). A series of runs was performed of different durations such that the ratio of activity remaining centripetal to the platform to that at the beginning of the run varied in the range 0.2–0.7. These results gave a mean sedimentation coefficient of 13.6 (S.D.±0.3)S, which corresponded to a molecular weight of about 400000 for a spherical protein particle.

Effect of pH *on the enzyme activity.* To explore the pH-dependence, a series of buffers covering the pH range 6.2–8.8 was constructed by mixing tris and cacodylic acid such that the pH and buffer concentration generated the requisite pH on addition of the enzyme and maintained a constant ionic strength of 0.2 with respect to tris and of 0.1 with respect to cacodylic acid. Neither of these buffer salts showed any effect on the enzyme activity. The concentration of fructose 6-phosphate was maintained at 2mM with respect to the predominant

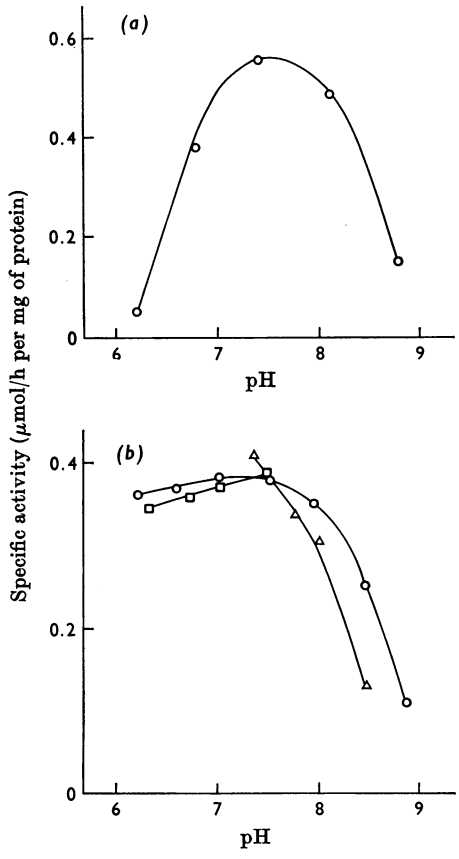

Fig. 1. Effect of pH on the activity of glucosamine synthetase in the presence of saturating concentrations of both substrates. (*a*) Preparation I with tris–cacodylic acid buffers; (*b*) preparation II with the following buffers: ○, tris–cacodylic acid; □, potassium phosphate; △, barbitone. The buffer systems and corrections for substrate pK values are detailed in the text.

(dianionic) species at 7.5 assuming pK_2 6.1(Kumler & Eiler, 1943). Similarly the glutamine concentration was maintained at 4mM with respect to the predominant (zwitterionic) species at pH7.5 assuming pK_2 9.1 (Meister, 1965*a*). Fig. 1(*a*) shows the pH-dependence of the velocity of the enzyme-catalysed reaction with material from preparation I. The pH-dependence for preparation II differed, as shown in Fig. 1(*b*): in contrast with the bell-shaped pH–activity profile obtained with preparation I, the decline in activity on the acid side of the pH optimum was not so marked.

Effect of substrates at the optimum pH. A 10×10 matrix of substrate concentrations was used to investigate the kinetic parameters of preparation I at pH7.5: the rate was measured at each of ten

concentrations of fructose 6-phosphate in the range 0.09–2.9mM and at each of ten concentrations of glutamine between 0.16 and 6mM. The measured velocities were treated graphically by the v against v/[A] linear transform of the Michaelis–Menten equation as recommended by Dowd & Riggs (1965) and the apparent V' and K_m values determined for each of the fixed concentrations of the second substrate, B. The true values of V, K_m and K_i were obtained by secondary plots of the apparent V' against V'/[B].

Results, which were graphically linear, were fitted to either the ordered Bi Bi or the Ping Pong Bi Bi rate equations (Cleland, 1963), to obtain the kinetic parameters and their associated standard errors (S.E.M.) by using computer programs developed by Haarhoff (1969).

The results fitted a Ping Pong Bi Bi rate equation with the lines intersecting at a point coincident with the abscissa. The secondary plots are shown in Figs. 2(*a*) and 2(*b*). The computed values for the K_m were 2.4 ($\pm$0.1) $\times 10^{-4}$ M for fructose 6-phosphate and 6.9 ($\pm$0.2) $\times 10^{-4}$ M for glutamine. As a corollary to this treatment, the value of n in the Hill plot was unity within the range of substrate concentrations covered, signifying no homotropic co-operative effects between substrate-binding sites.

Glucosamine synthetase as present in the 105000g supernatant gave similar K_m values for both substrates: 2.4×10^{-4} M for fructose 6-phosphate and 5.1×10^{-4} M for glutamine, assuming that the equilibrium between fructose 6-phosphate and glucose 6-phosphate was attained rapidly by the high phosphoglucose isomerase activity present in this crude fraction.

Enzyme preparation II was similarly investigated and showed some differences. The graphs of v versus v/[glutamine] deviated from linearity at low glutamine concentrations although retaining the basic Ping Pong Bi Bi pattern of converging lines

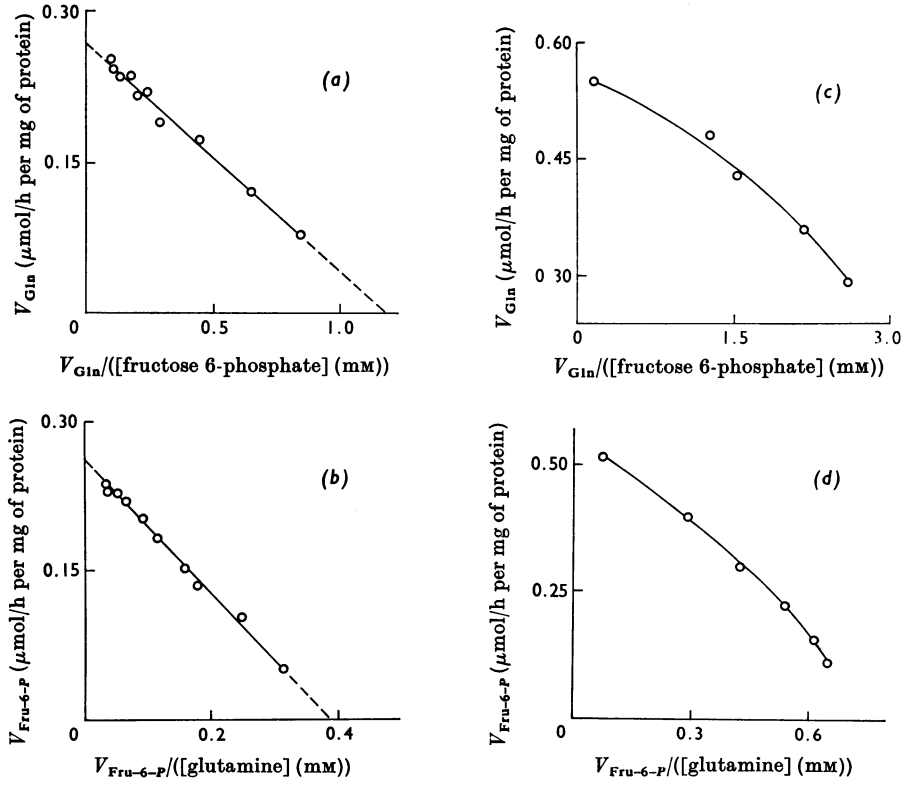

Fig. 2. Secondary plots showing the dependence of V on the concentration of the second substrate at pH 7.5 and 37°C. Each value of V was obtained by extrapolation of v versus v/[A] graph at a fixed concentration of the second substrate, B. In (*a*) and (*c*) A is glutamine and B is fructose 6-phosphate; in (*b*) and (*d*) A is fructose 6-phosphate and B is glutamine. (*a*) and (*b*) were obtained with stage 4 of preparation I and (*c*) and (*d*) were with stage 4 of preparation II.

noted with preparation I. The plots of V versus $V/[S]$ were non-linear with respect to both substrates (Figs. 2*c* and 2*d*), and a Hill coefficient of 1.2 was measured. The K_m for glutamine was 7.5×10^{-4}M, the same as obtained with preparation I, and the K_m for frucose 6-phosphate, which varied slightly between preparations, had a mean value of 1.1×10^{-4}M.

The kinetic characteristics of preparation I could be converted into those of preparation II by a 24h incubation at 4°C with 0.5mM-fructose 6-phosphate. This indicated that the differences in properties were caused by the presence of the protecting substrate rather than by the preparative procedure itself.

Effect of pH *on the kinetic parameters.* The values for K_m, K_i and V were calculated for preparation I at a series of pH values from 6.4 to 8.5. The substrate concentrations, employed in a 4×4 matrix, were corrected as necessary for the pK of an ionizing group on the substrate (see section above). The concentrations of fructose 6-phosphate and glutamine were varied in the respective ranges 0.09–1.8mM and 0.25–4.0mM. At the extremes of the pH range, the substrate ranges were extended to allow for variations in the Michaelis constants. The pH-dependence of log V is shown in Fig. 3(*a*). The method described by Dixon (1953) for estimating pK values revealed groups ionizing with pK values of 6.95 and 8.2 present in the rate-determining step of the reaction sequence (assuming that the same step was rate-determining throughout the pH range investigated).

Graphs of pK_m versus pH were virtually linear in the range investigated. This would be expected if there were only small differences between the ionization constants in the enzyme forms under investigation and those present in the rate-limiting step. Therefore the results were plotted as $\log(V/K_m)$ versus pH for each substrate to determine the apparent pK values of ionizing groups present in the enzyme form that bound the particular substrate. This is shown in Fig. 3(*b*). For fructose 6-phosphate pK values of 6.9 and 7.8 were obtained and for glutamine only one group at 7.0 was detected.

It is noteworthy that the rate equation obeyed by the data depended on pH. Within the pH range 6.9–7.6 the data fitted the Ping Pong Bi Bi equation, whereas outside this range they obeyed the ordered Bi Bi equation since the value of K_i for glutamine was significantly greater than zero.

Effect of temperature on the kinetic parameters. The values of V were determined for preparation I by using a 5×5 matrix of substrate concentrations at a series of temperatures in the range 25–38°C. The Arrhenius plot of log V versus $1/T$ revealed a slight biphasic response with a transition temperature of 29°C. The apparent enthalpy changes for the two

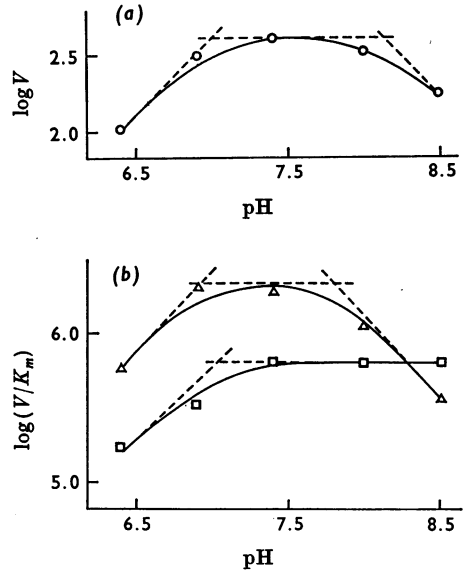

Fig. 3. Dependence of log V and log (V/K_m) on pH with preparation I. V and K_m were calculated by using a computer program that fitted the data either to the Ping Pong Bi Bi or to the ordered Bi Bi equations, the kinetic values used being those obtained from the application of the better fitted rate equation. (*a*) Effect of pH on log V; (*b*) effect of pH on log V/K_m for: △, fructose 6-phosphate; □, glutamine.

segments were 15 and 22kcal/mol for above and below the transition temperature respectively. Above the transition temperature the velocity was described by the Ping Pong Bi Bi rate equation, whereas below this point the velocity obeyed the ordered Bi Bi mechanism.

Utilization of ammonia as amino donor. Most known glutamine amide aminotransferases have been found to utilize ammonia as an alternative substrate, the K_m for the un-ionized form of ammonia being approx. the same as that for glutamine (Meister, 1965*b*). No production of amino sugar could be detected with 60mM-ammonium chloride in place of glutamine, even at pH 8.7 where the concentration of the un-ionized species would have been about 14mM. With glutamine as the substrate no inhibitory competition by ammonia for the glutamine-binding site could be demonstrated.

Product inhibition. Investigations into possible product inhibition by glutamate revealed that there was negligible effect. At concentrations above 25mM a slight inhibition was observed, but this could not be divorced from an ionic-strength effect.

Product inhibition studies with glucosamine 6-phosphate were not technically feasible.

DISCUSSION

This paper describes a rapid and reproducible method for extracting and purifying glucosamine synthetase from rat liver. This enzyme, which catalyses the initial reaction on the pathway of biosynthesis of hexosamines, has been little studied previously. One pertinent reason is its inherent instability, which has hindered the purification to a stage where meaningful kinetic work coud be undertaken. Some workers (Gryder & Pogell, 1960; Kornfeld, 1967) have used the protective action of glucose 6-phosphate to stabilize the enzyme. However, its use is not recommended because it modifies the inhibition by the feedback inhibitor UDP-*N*-acetylglucosamine (Winterburn & Phelps, 1971*a*). The development of the purification in the absence of a stabilizing agent (preparation I) has permitted the investigation of the mode of action of potential protectors. Subsequently the yields and the stability of the preparation were improved by adding a compound of known influence on the system, namely one of the substrates, fructose 6-phosphate (preparation II). The stabilizing potential of this substrate is aptly reflected in the comparison of the yields of the enzyme obtained by the two preparative procedures (Table 1). Moreover the ability to convert preparation I into preparation II by incubation with fructose 6-phosphate demonstrates that the two methods prepare essentially identical enzymes with only minor differences in properties. Credence is given to the postulate that this preparation does not differ significantly from that present *in vivo* by two observations: (*a*) the control ratio exercised by UDP-*N*-acetylglucosamine remains virtually constant throughout the purification procedure and (*b*) the Michaelis constants for both substrates are similar in crude and purer fractions. Inasmuch as this preparation has been shown to be free of any relevant contaminant activities it offers a better authenticated enzyme than previous workers have used, where the possibility of side reactions could not be excluded (e.g. phosphoglucose isomerase, glutaminase etc.).

The molecular weight of glucosamine synthetase from rat liver has been shown to be in the range 360000–400000 by two independent methods, and this agreed closely with a previously reported value of 340000 (Kornfeld, 1967) obtained by sucrose-density-gradient centrifugation. This should be contrasted with the value of 90000–107000 for the bacterial enzyme (Kornfeld, 1967), which suggests that the mammalian enzyme may comprise four subunits. Further, this smaller, possibly monomeric, bacterial enzyme is devoid of feedback regulation by UDP-*N*-acetylglucosamine.

In agreement with Ghosh *et al.* (1960) there is no definitive evidence supporting the existence of a cofactor in this reaction. No pyridoxal-dependent transfer of the amino group is indicated because: (*a*) addition of pyridoxal phosphate, pyridoxamine phosphate or pyridoxine does not alter the activity, (*b*) the velocity cannot be described by a Ping Pong Bi Bi rate equation under all conditions, (*c*) the specific pyridoxal phosphate inhibitor isoniazid is ineffective and (*d*) the absorption spectrum of the purest enzyme fractions shows no absorption in the region 350–400nm (Winterburn, 1969).

Considerable care was taken to assess the possible reversibility of this reaction. That no such phenomenon could be observed, a fact deduced from the absence of radioactivity incorporated from [U-^{14}C]-glucosamine 6-phosphate into fructose 6-phosphate, hints at a large negative ΔG accompanying this reaction. This irreversibility is apparently held in common with all other glutamine amide aminotransferases (Meister, 1965*b*). Yet in contrast with some other such enzymes this aminotransferase does not utilize un-ionized ammonia as a substrate, neither does it require the hydrolysis of a pyrophosphate bond. The sole driving force for the reaction is the scission of the amide linkage.

From the variation in the plot of $\log V$ versus pH, two pK values were derived that corresponded to ionizing groups present in the enzyme–substrate form involved in the rate-limiting step. The pK values of 6.95 and 8.2 are within 1 pH unit of the pK values of groups present in the free substrates and may therefore relate to substrate ionizations within the enzyme–substrate complex. However, the close similarities of the pK values of these groups to those observed in the measurements of the effect of pH on $V_{max.}/K_m$ suggest that the ionizations represent groups bound to the enzyme. The enzyme form that binds fructose 6-phosphate apparently involves groups with pK values of 6.9 and 7.8, whereas glutamine utilizes only the more acidic of these two (Fig. 3*b*). It is tempting to speculate from these data that the binding of fructose 6-phosphate involves the more basic of these two groups and that, since in this process the pK apparently increases from 7.8 to 8.2, it exists on the free enzyme in its acidic form. In a preferred pathway of ordered addition of substrate where fructose 6-phosphate is bound by the enzyme before glutamine (Winterburn & Phelps, 1971*b*), the involvement of the group with pK 7.8 in binding fructose 6-phosphate would account for its absence from the enzyme form that binds glutamine.

The fact that a two-substrate–two-product enzyme-catalysed reaction obeys a Ping Pong Bi Bi rate equation does not necessarily signify that the enzyme follows a Ping Pong mechanism. If the ordered addition of the first substrate in a preferred pathway is essentially irreversible, K_i tends

to zero and the equation becomes that of a Ping Pong mechanism, although not one involving a substituted enzyme intermediate. It is considered that the existence of Ping Pong-type kinetics under certain conditions of temperature and pH is solely an effect on the rate constants in a preferred pathway of ordered addition. This point is further discussed by Winterburn & Phelps (1971*b*).

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