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Studies on the Control of Hexosamine Biosynthesis by Glucosamine Synthetase

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1. The nature of the feedback inhibition of hexosamine biosynthesis on rat liver glucosamine synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) by UDP-*N*-acetylglucosamine was investigated in detail. 2. Further modifiers of physiological importance are described. Glucose 6-phosphate and AMP potentiated the UDP-*N*-acetylglucosamine inhibition, and UTP behaved as an activator. These three compounds only exerted their action when the feedback inhibitor was bound to the enzyme. 3. ATP also inhibited the enzyme. 4. The actions of these various effectors are discussed in kinetic terms. 5. An interpretation of these findings with reference to the regulation of hexosamine biosynthesis is presented.

The purification and some properties of rat liver glucosamine synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) are described in the preceding paper (Winterburn & Phelps, 1971). The fractionation produced a relatively stable preparation that, while retaining the sensitivity to the feedback inhibitor UDP-*N*-acetylglucosamine, was free of contaminating activities. This regulation of the hexosamine pathway by UDP-*N*-acetyglucosamine, first described by Kornfeld, Kornfeld, Neufeld & O'Brien (1964), has been shown to operate *in vivo* (Bates, Adams & Handschumacher, 1966). The purpose of the present paper is to describe in detail the operation of the inhibition by UDP-*N*-acetylglucosamine and also the modulation of the inhibition by three hitherto undescribed modifiers. In addition, an attempt is made to relate the properties *in vitro* to those operating within the cell.

MATERIALS AND METHODS

Materials. The following chemicals and enzymes were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.: ADP (sodium salt), AMP (sodium salt), ATP (sodium salt), CoA, fructose 1,6-diphosphate (sodium salt), fructose 6-phosphate (barium salt), glucose 6-phosphate (sodium salt), GSH, GTP (lithium salt), hexokinase (EC 2.7.1.1), NAD^+, NADH (sodium salt), $NADP^+$ (sodium salt), NADPH (sodium salt), phosphoenolpyruvate (sodium salt), UDP (potassium salt), UDP-*N*-acetylglucosamine (sodium salt), UDP-glucose (sodium salt), UDP-glucuronic acid (sodium salt) and UTP (sodium salt). 3′:5′-Cyclic AMP, CTP (sodium salt), glutamine, IMP (sodium salt), ITP (sodium salt), UMP (sodium salt) and UDP-galactose (sodium salt) were from Sigma Chemical Co., St Louis, Mo., U.S.A. Glucose 1-phosphate (potassium salt) was from BDH Chemicals Ltd., Poole, Dorset, U.K. UDP-xylose was purchased from Calbiochem Ltd., London W.1, U.K. The barium salt of galactose 6-phosphate was a generous gift from Boehringer Corp. (London) Ltd. Mannose 6-phosphate was prepared by phosphorylation of D-mannose (Slein, 1957). 2-Deoxy-D-glucose 6-phosphate was prepared by phosphorylation of 2-deoxy-D-glucose by using ATP and hexokinase and isolated on a column of Dowex 1 (formate form) by the method of Winterburn (1969). Acetyl-CoA was prepared as described by Simon & Shemin (1953).

All barium salts of sugar phosphates were converted into the sodium form as described for fructose 6-phosphate by Winterburn & Phelps (1971).

Methods. Glucosamine synthetase was purified from rat liver as described by Winterburn & Phelps (1971). Enzyme from stage 4 of procedure II was used for the investigations reported in this paper.

Concentrations of substrate greater than $10K_m$ were assumed to saturate the enzyme. All kinetic data were evaluated by the matrix procedure by substrate concentrations (Winterburn & Phelps, 1971), glutamine concentration being varied in the range $K_m/4$ to $10K_m$ and that of fructose 6-phosphate between $K_m/2$ and $10K_m$. (Since fructose 6-phosphate was used to stabilize the enzyme preparation, it was technically difficult to obtain concentrations of this substrate below 0.05 mM.) Graphical representation of the velocities was according to the v versus $v/[S]$ plotting procedure and extrapolation of such graphs was used to obtain V'. The dependence of V' on the second substrate was referred to as a secondary plot. V'_{Gln} refers to V' with glutamine saturating and likewise $V'_{Fru\text{-}6\text{-}P}$ refers to V' with fructose 6-phosphate saturating.

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The control ratio was defined as the ratio of the activity in the presence of UDP-*N*-acetylglucosamine (0.1 mM) to the uninhibited rate as described by Winterburn & Phelps (1971). Since this index signified the extent of desensitization during purification, no preparation exhibiting a control ratio greater than 0.55 was used in these studies.

All the inhibitory and other effects that modified the activity of the enzyme were reversible. The production of glucosamine 6-phosphate was proportional to time for at least 60 min under all conditions tested and the presence of modifiers did not influence this linearity of product formation.

Details of the assay of glucosamine synthetase and all other procedures were as described by Winterburn & Phelps (1971).

RESULTS

*Inhibitory effect of UDP-*N*-acetylglucosamine.* The feedback inhibition, first reported by Kornfeld *et al.* (1964), was confirmed, although the potency was not as great as had been described: in the present work with both substrates saturating 0.1 mM-UDP-*N*-acetylglucosamine caused 45% inhibition in contrast with 80% reported by Kornfeld (1967) (see the Discussion section). The maximum attainable degree of inhibition (90–95%) was achieved at UDP-*N*-acetylglucosamine concentrations greater than 0.75 mM.

The inhibition was investigated in greater detail by varying the concentrations of the substrates (see the Materials and Methods section) at five concentrations of UDP-*N*-acetylglucosamine in the range 0–0.15 mM.

With glutamine as the variable substrate and the fructose 6-phosphate concentration held constant the inhibition caused by UDP-*N*-acetylglucosamine was uncompetitive (nomenclature of Cleland, 1963) on which was superimposed substrate inhibition by the glutamine. Fig. 1(*a*) shows one such series of experiments where fructose 6-phosphate was saturating (3 mM). This substrate inhibition by glutamine that was induced by UDP-*N*-acetylglucosamine had been reported previously for the rat liver enzyme (Bates & Handschumacher, 1969) and also in the retinal system (Mazlen, Muellenberg & O'Brien, 1969). The substrate inhibition was more pronounced at lower concentrations of fructose 6-phosphate. With fructose 6-phosphate at approximately K_m (0.15 mM) and UDP-*N*-acetylglucosamine concentrations greater than 0.1 mM the velocity was virtually independent of the glutamine concentration over the range 0.25–6.0 mM. By ignoring the substrate inhibition the uncompetitive inhibition by UDP-*N*-acetylglucosamine was linear for $1/V$ (Fig. 1*b*) and revealed a K_i of 7×10^{-5} M with respect to glutamine.

With respect to fructose 6-phosphate the inhibition was of greater complexity. Fig. 2(*a*) shows

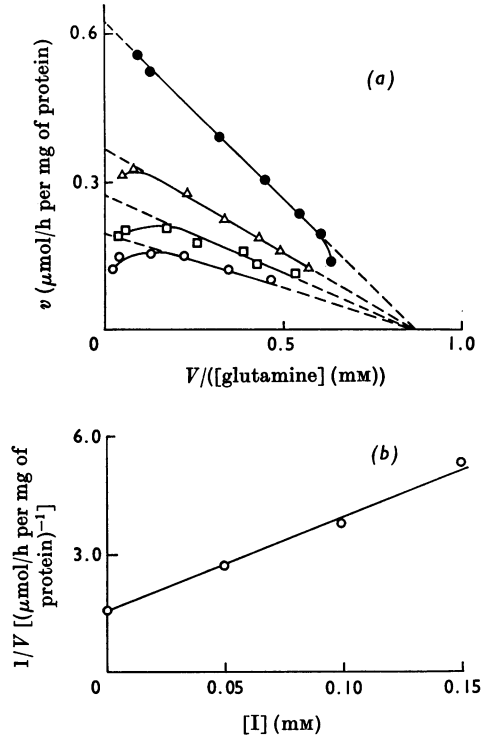

Fig. 1. (*a*) Inhibition of glucosamine synthetase by UDP-*N*-acetylglucosamine with fructose 6-phosphate saturating (3 mM) and glutamine as the variable substrate. Incubations were for 60 min in tris–HCl buffer, pH 7.5, at 37°C (for further details see the text). The UDP-*N*-acetylglucosamine concentrations were: ●, none; △, 0.05 mM; □, 0.10 mM; ○, 0.15 mM. (*b*) Dependence of the extrapolated maximum velocity, V, on the UDP-*N*-acetylglucosamine concentration, [I], from the data of (*a*).

that with glutamine saturating (8 mM) UDP-*N*-acetylglucosamine behaved as a non-competitive inhibitor, the extrapolated lines intersecting at a point to the left of the ordinate. The dependence of this inhibition on the UDP-*N*-acetylglucosamine concentration was hyperbolic for $1/V$ (Fig. 2*b*) and linear for K_m/V (Fig. 2*c*). At lower concentrations of glutamine the fructose 6-phosphate dependence of the velocity was non-linear. As shown in Fig. 3 the graphical pattern had two distinct regions linked by a transitional or step region. This effect was completely reproducible and always occurred at approx. 0.3 mM-fructose 6-phosphate. The region at fructose 6-phosphate concentration greater than 0.3 mM exhibited the non-competitive inhibition described above; however, as the concentration of the second substrate, glutamine, was lowered the point of intersection of the lines moved closer to the

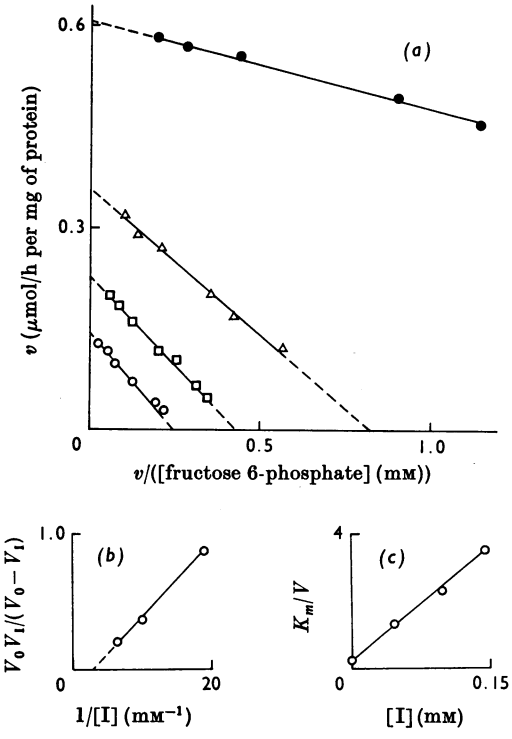

Fig. 2. (*a*) Inhibition of glucosamine synthetase by UDP-*N*-acetylglucosamine with glutamine saturating (8 mM) and fructose 6-phosphate as the variable substrate. Incubations were as described for Fig. 1. The UDP-*N*-acetylglucosamine concentrations were: ●, none; △, 0.05 mM; □, 0.10 mM; ○, 0.15 mM. The dependence of the extrapolated maximum velocity, V, and K_m/V on the UDP-*N*-acetylglucosamine concentration, [I], from the data of (*a*), is shown in (*b*) and (*c*) respectively. V_0 is V in the absence of inhibitor; V_1 is V in the presence of inhibitor.

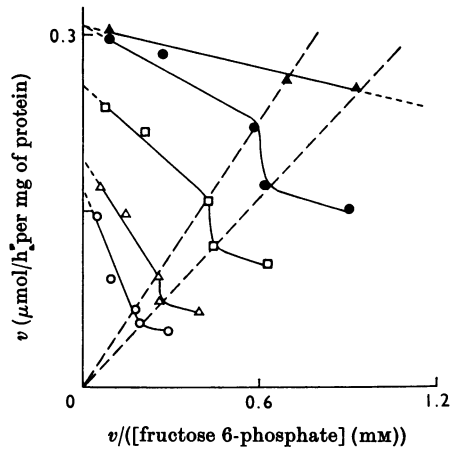

Fig. 3. Effect of UDP-*N*-acetylglucosamine on the velocity with fructose 6-phosphate as the variable substrate and glutamine constant at approximately K_m (0.67 mM). Incubations were as described for Fig. 1. The UDP-*N*-acetylglucosamine concentrations were: ▲, none; ●, 0.025 mM; □, 0.05 mM; △, 0.10 mM; ○, 0.15 mM.

ordinate until when the glutamine concentration was less than $K_m/2$ the mode of inhibition was competitive. Technical difficulties precluded the gathering of results at fructose 6-phosphate concentrations less than 0.1 mM.

$V'_{Fru\text{-}6\text{-}P}$ was a linear function of glutamine concentration at all of the UDP-*N*-acetylglucosamine concentrations tested and did not reveal substrate inhibition. The step region was revealed also in the fructose 6-phosphate-dependence of V'_{Gln}.

In the presence of UDP-*N*-acetylglucosamine the Hill coefficient for the interaction of the glutamine sites was 1.0–1.4, the value varying inversely with fructose 6-phosphate concentration. No such information was available for the fructose 6-phosphate-binding sites because of the nature of the kinetic data. The Hill coefficient for the interaction of UDP-*N*-acetylglucosamine-binding sites was 1.1. This lack of co-operativity between the various binding sites had been noted by Kornfeld *et al.* (1964); however, the present work revealed that this situation held over a wide range of concentrations of the allosteric modifier and substrates.

Effect of other UDP-sugars. Kornfeld *et al.* (1964) had noted that, in addition to UDP-*N*-acetylglucosamine, other UDP-sugars were also inhibitory, although to a smaller degree. With both substrates saturating the concentrations of UDP-glucose, UDP-galactose, UDP-xylose and UDP-glucuronate required to give 20% inhibition were 1.5, 2.8, 2.6 and 3.4 mM respectively. For comparison under identical conditions, this extent of inhibition was achieved by 0.025 mM-UDP-*N*-acetylglucosamine. Kinetic analysis showed that UDP-glucose, UDP-xylose and UDP-glucuronate inhibited glucosamine synthetase in a similar manner to UDP-*N*-acetylglucosamine.

From these results it was suspected that all the UDP-sugars were bound at the same site. To investigate whether the nucleotide sugars could compete for this common site, UDP-*N*-acetylglucosamine (0–0.15 mM) and UDP-glucose (0–5 mM) were used at non-limiting substrate concentrations. At fixed concentrations of the hexosamine derivative an increase in that of UDP-glucose gave rise to activation (Fig. 4). UDP-glucuronate generated an effect on the inhibition due to UDP-*N*-acetylglucosamine similar to that observed for UDP-glucose, suggesting that all the UDP-sugars behaved in this manner. Such an activation effect

caused by two inhibitors acting in concert can be interpreted as a competition for a common site.

Effect of UTP. UTP by itself only slightly modified the kinetic parameters of the enzyme. The step phenomenon induced by UDP-*N*-acetylglucosamine was also produced by UTP at the same fructose 6-phosphate concentration (0.3mM).

Analysis with both substrates saturating revealed that the addition of UTP to a system inhibited with UDP-*N*-acetylglucosamine caused an activation (Fig. 5). The nature of this effect was investigated in greater detail. With glutamine as the variable substrate and the concentrations of UDP-*N*-acetylglucosamine (0.025mM) and fructose 6-phosphate held constant UTP (1.2mM) raised V, lowered the apparent K_m for glutamine and removed the substrate inhibition. This is typically shown in Fig. 6(*a*) where the fructose 6-phosphate concentration is approximately K_m. When the variable substrate was fructose 6-phosphate UTP raised V without influencing the apparent K_m (Fig. 6*b*).

Effect of AMP. In the absence of UDP-*N*-acetylglucosamine the influence of AMP, in common with UTP, was slight: 1mM-AMP had no effect on V or the K_m for either substrate.

The addition of AMP to a system inhibited by UDP-*N*-acetylglucosamine modified the action of the feedback inhibitor. The fructose 6-phosphate-dependence of the velocity at the several fixed glutamine concentrations in the presence of 0.025mM-UDP-*N*-acetylglucosamine revealed that 1mM-AMP converted the non-competitive inhibition of UDP-*N*-acetylglucosamine into a competitive mode. This effect generated activation at the higher concentrations of fructose 6-phosphate and potentiated the inhibition at the lower concentrations of this substrate. This is shown representatively in Fig. 7(*a*) where the glutamine concentration was 0.67mM. The step in the plots was apparent and its position unaltered (about 0.3mM-fructose 6-phosphate). The apparent K_i of the inhibitory combination of UDP-*N*-acetylglucosamine (0.025mM)+AMP (1mM) was 8×10^{-6}M.

At subsaturating concentrations of fructose 6-phosphate when glutamine was the variable substrate the addition of 1mM-AMP enhanced the substrate inhibition created by UDP-*N*-acetylglucosamine and slightly lowered the apparent K_m for glutamine. The overall uncompetitive nature of the inhibition was unaffected. This is shown in Fig. 7(*b*). When fructose 6-phosphate was saturating an activation by the AMP was observed, as would be expected by the change in mode of inhibition with respect to fructose 6-phosphate.

The fructose 6-phosphate-dependence of V'_{Gln} further demonstrated the competitive nature of the inhibition created by the UDP-*N*-acetylglucosamine and AMP acting in concert (Fig. 7*c*).

Of the other nucleotide 5′-monophosphates tested IMP and UMP displayed a potentiation of the action of UDP-*N*-acetylglucosamine similar to, although not as potent as, that shown by AMP. The magnitudes of the action of 1mM-AMP, 1mM-IMP and 1mM-UMP expressed as the effect on the control ratio (0.44) were 0.29 0.36 and 0.36 respectively.

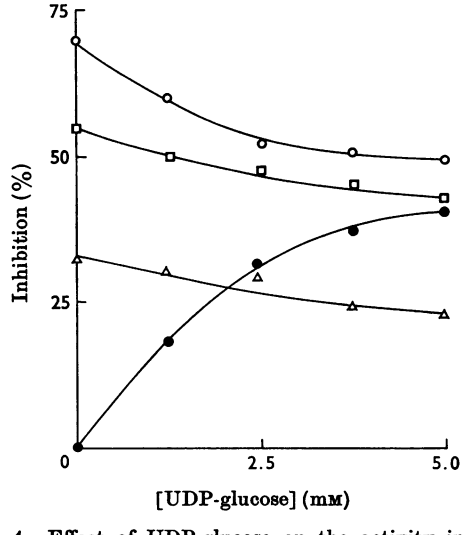

Fig. 4. Effect of UDP-glucose on the activity in the absence and presence of UDP-*N*-acetylglucosamine with both substrates saturating. The UDP-*N*-acetylglucosamine concentrations were: ●, none; △, 0.05mM; □, 0.10mM; ○, 0.15mM.

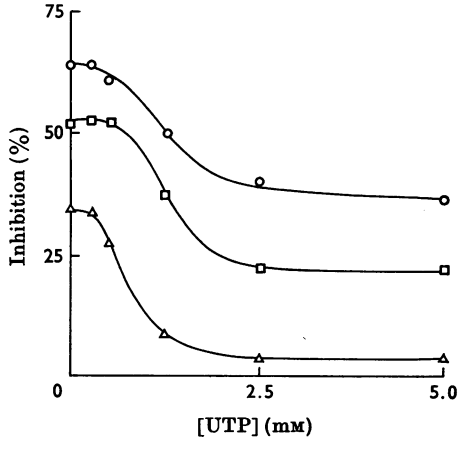

Fig. 5. Effect of UTP on the inhibition by UDP-*N*-acetylglucosamine, with both substrates saturating. The UDP-*N*-acetylglucosamine concentrations were: △, 0.05mM; □, 0.10mM; ○, 0.15mM.

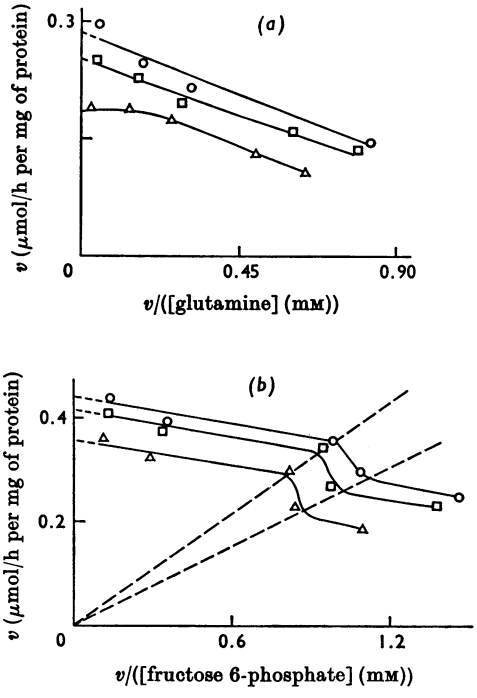

Fig. 6. (*a*) Effect of UTP on the inhibition by UDP-*N*-acetylglucosamine with glutamine as the variable substrate and fructose 6-phosphate concentration constant at approximately K_m (0.12mM). To the incubation mixtures, which were as described in Fig. 1, were added: ○, 1.2mM-UTP; △, 0.025mM-UDP-*N*-acetylglucosamine; □, 1.2mM-UTP+0.025mM-UDP-*N*-acetyglucosamine. (*b*) As for (*a*) except that fructose 6-phosphate was the variable substrate and the glutamine concentration was constant (1.33mM).

Effect of glucose 6-phosphate. In the presence of UDP-*N*-acetylglucosamine glucose 6-phosphate (2mM) exhibited only small effects on the kinetic behaviour of the enzyme: V and K_m values were unaltered and the step was induced in the fructose 6-phosphate-dependence at the same concentration as noted above (about 0.3mM).

Glucose 6-phosphate modified the inhibition due to UDP-*N*-acetylglucosamine in a manner similar to that described for AMP. The inhibition with respect to fructose 6-phosphate was converted into the competitive type whereas for glutamine the inhibition remained uncompetitive with superimposed substrate inhibition. The apparent K_i with respect to fructose 6-phosphate for the combination UDP-*N*-acetylglucosamine (0.025mM) +glucose 6-phosphate (0.5mM) was 3×10^{-6}M, whereas with respect to glutamine it was 1.5×10^{-4}M.

From the data it was concluded that the actions of glucose 6-phosphate and AMP on glucosamine synthetase were such as to modulate the effect of the feedback inhibitor in a similar manner.

In marked contrast with the modification of the inhibition by UDP-*N*-acetylglucosamine glucose 6-phosphate (1mM) did not exert any influence on the inhibition by two other UDP-sugars, namely UDP-glucose and UDP-glucuronate. This suggested that although glucose 6-phosphate affected the binding of hexosamine derivative it did not have a similar effect on other UDP-sugars.

Investigation into the specificity of the glucose 6-phosphate-binding site revealed that several other sugar phosphates, although not inhibitory themselves, enhanced the inhibition due to UDP-*N*-acetylglucosamine (Table 1). The nature of action of one of these sugar phosphates, mannose 6-phosphate, showed that it operated kinetically in the same way as glucose 6-phosphate.

Effect of ATP. Glucosamine synthetase was inhibited by ATP:7.0mM caused 50% inhibition with both substrates saturating; however, the addition of 0.1mM-UDP-*N*-acetylglucosamine decreased this inhibition to 10%. The graphical representation of the inhibition (Figs. 8*a* and 8*b*) revealed that ATP was a non-competitive inhibitor with respect to both substrates.

The addition of 1mM-magnesium chloride increased the inhibition by ATP (7.0mM) from 50% to 73%, suggesting that the $MgATP^{2-}$ complex may be more active than ATP^{4-}.

The examination of other nucleotide triphosphates and related compounds disclosed that, although neither ITP nor GTP was effective, CTP and phosphoenolpyruvate behaved in a similar manner to ATP. The 50% inhibitory concentrations of CTP and phosphoenolpyruvate, when assayed with both substrates saturating, were 6 and 8mM respectively.

Effect of other nucleotides and coenzymes. The following compounds did not exert any effect on glucosamine synthetase activity in the presence or absence of 0.1mM-UDP-*N*-acetylglucosamine at the concentrations quoted: 3′:5′-cyclic AMP (0.15mM), CoA (0.12mM), acetyl-CoA (0.10mM), $NADP^+$ (0.20mM), NADPH (0.20mM), NAD^+ (2.0mM) and NADH (0.1mM). These values for the concentrations were chosen after a review of the literature as being potentially at the upper physiological limit for rat liver.

ADP (2mM) potentiated the inhibition due to UDP-*N*-acetylglucosamine, as evidenced by a slight depression (8%) of the control ratio. This may have been caused by an observed AMP contamination of the ADP preparation.

UDP (2mM) caused a small elevation (11%) in the control ratio.

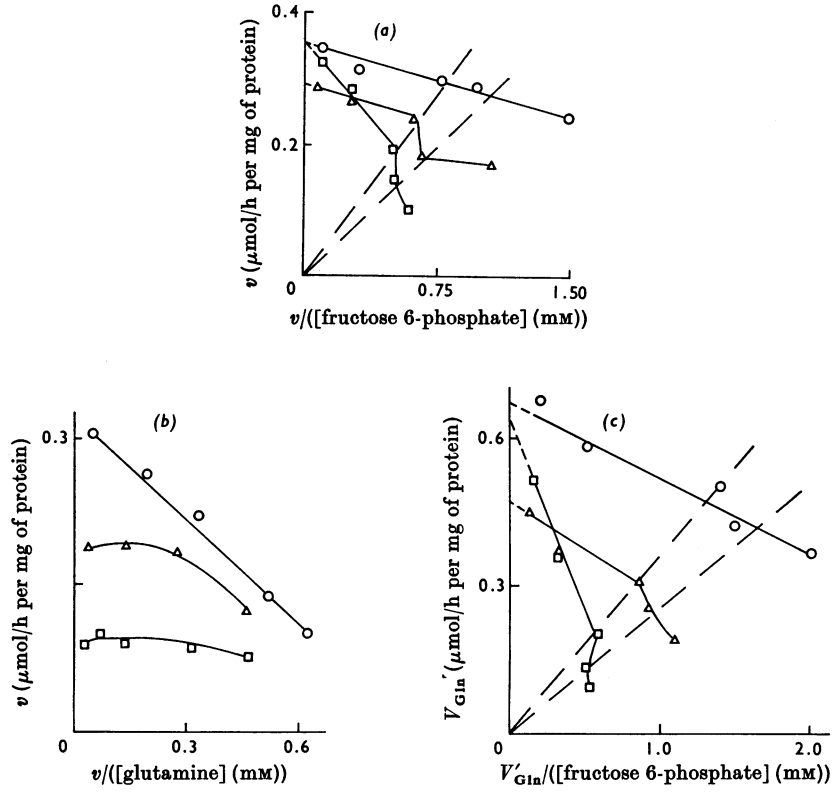

Fig. 7. (*a*) Effect of AMP on the inhibition by UDP-*N*-acetylglucosamine with fructose 6-phosphate as the variable substrate and glutamine concentration constant at approximately K_m (0.67 mM). To the incubation mixtures, which were as described in Fig. 1, were added: ○, 1 mM-AMP; △, 0.025 mM-UDP-*N*-acetylglucosamine; □, 1 mM-AMP + 0.025 mM-UDP-*N*-acetylglucosamine. (*b*) As for (*a*) except that glutamine was the variable substrate and the fructose 6-phosphate concentration was constant (0.17 mM). (*c*) Dependence of the velocity with glutamine saturating, V'_{Gln}, as derived by extrapolation of graphs such as (*b*), on the fructose 6-phosphate concentration in the presence of AMP and UDP-*N*-acetylglucosamine. The additions were as for (*a*).

Table 1. *Effects of various sugar phosphates on the UDP-*N*-acetylglucosamine inhibition of glucosamine synthetase by UDP-*N*-acetylglucosamine*

The glucosamine synthetase activity was determined with both substrates saturating in the absence and presence of UDP-*N*-acetylglucosamine (0.1 mM). The ratio of these activities is termed the control ratio. Hexose phosphates (1.5 mM final concn.) were added to the incubation mixtures and the control ratios calculated. See the Materials and Methods section for further details.

Hexose phosphate	Control ratio
None	0.47
Glucose 6-phosphate	0.16
Mannose 6-phosphate	0.30
2-Deoxyglucose 6-phosphate	0.27
Glucose 1-phosphate	0.41
Fructose 1,6-diphosphate	0.47
Galactose 6-phosphate	0.47

DISCUSSION

The previously reported inhibition by UDP-*N*-acetylglucosamine of glucosamine synthetase (Kornfeld *et al.* 1964; Kornfeld, 1967) has been confirmed and the nature of its action further elucidated. Although UDP-*N*-acetylglucosamine is a non-competitive inhibitor with respect to fructose 6-phosphate, the effect on the K_m term in the rate equation is greater than on the V term: the lines of the graph therefore converge at a point to the left of the ordinate. In the presence of glucose 6-phosphate or AMP this apparent competition between UDP-*N*-acetylglucosamine and fructose 6-phosphate is enhanced, as shown by the plots of v versus $v/[S]$, where the point of intersection of the lines becomes coincident with the ordinate. This may offer an explanation for the results reported

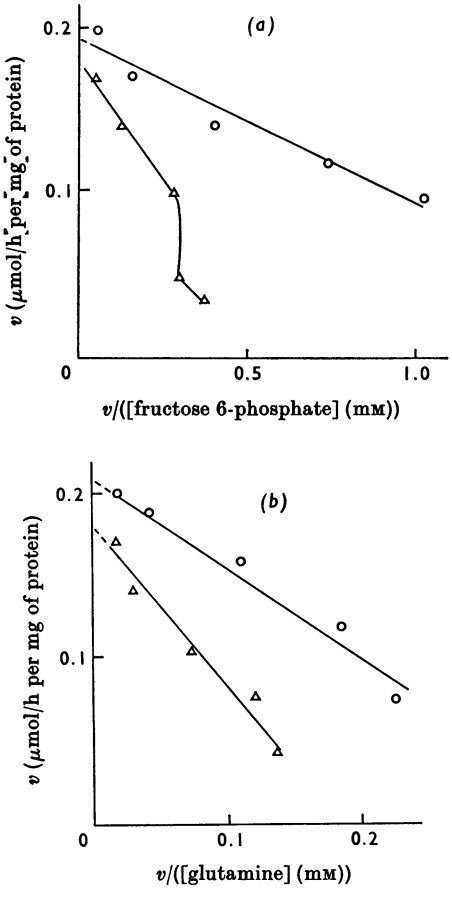

Fig. 8. (*a*) Inhibition by ATP with fructose 6-phosphate as the variable substrate and glutamine saturating. Incubations were as described for Fig. 1. The additions were: ○, none; △, 5 mM-ATP. (*b*) As for (*a*) except that glutamine was the variable substrate and fructose 6-phosphate was saturating.

by other workers. Thus Kornfeld (1967) quoted the inhibition to be competitive; however, her enzyme preparation was stabilized with glucose 6-phosphate and therefore reflected the modified action of UDP-*N*-acetylglucosamine rather than that of the inhibitor as such. When the data of Mazlen *et al.* (1969) are replotted by using the more sensitive plot of v versus $v/[S]$ their results are more readily interpretable in terms of the inhibitory pattern reported in the present paper.

The explanation of the discontinuities induced by UDP-*N*-acetylglucosamine in the plots where fructose 6-phosphate was the variable substrate remains obscure. Such transitions might result either from heterogeneity of the enzyme or from impurity in the fructose 6-phosphate preparation. The former is discounted since gel filtration, ultracentrifugation and ion-exchange chromatography do not suggest the presence of a second enzyme, isoenzyme or association–dissociation phenomena (Winterburn & Phelps, 1971). Further, the reaction rate is proportional to enzyme concentration across a wide range. With regard to substrate impurity, the only contaminant in the fructose 6-phosphate preparation was glucose 6-phosphate, which amounted to less than 3% of the total material. The transition therefore is apparently not an artifact but is a property of the enzyme. Such a transition could result from strong positive homotropic interactions between fructose 6-phosphate-binding sites, as has been suggested for glutamate dehydrogenase (Engel & Dalziel, 1969). The remarkable property of this transition is that it always occurs across the same range of fructose 6-phosphate concentrations (approx. 0.3 mM) regardless of the concentration of the other ligands.

UDP-*N*-acetylglucosamine not only behaves as an uncompetitive inhibitor for the other substrate, glutamine, but it also induces substrate inhibition that is not otherwise evinced. This feature had been previously noted by Mazlen *et al.* (1969) and Bates & Handschumacher (1969).

Glucosamine synthetase, with a molecular weight of 360000–400000 (Winterburn & Phelps, 1971) and not only susceptible to feedback inhibition but also capable of binding further ligands, might be expected to exhibit properties in common with other classical allosteric enzymes (Monod, Wyman & Changeux, 1965; Kirtley & Koshland, 1967). However, under all conditions no sigmoid dependence of velocity on glutamine or modifier concentration is measurable, and the value of the Hill exponent does not exceed 1.4. Thus it appears that with the possible exception of the behaviour towards fructose 6-phosphate any homotropic interactions that occur in this enzyme are of a minor nature.

Kinetically glucose 6-phosphate and AMP have two effects on the enzyme: (*a*) the inhibition by UDP-*N*-acetylglucosamine is converted from a non-competitive into a competitive mode and (*b*) the apparent K_i for the feedback inhibitor is decreased, this stronger binding of UDP-*N*-acetylglucosamine being reflected as an increase in substrate inhibition of the enzyme by glutamine. It is noteworthy that glucose 6-phosphate and AMP have no significant kinetic action on the enzyme in the absence of UDP-*N*-acetylglucosamine. Apparently their action is merely to enhance the binding of the feedback inhibitor.

Like glucose 6-phosphate and AMP, UTP only demonstrates its effects in the presence of the feedback inhibitor. UTP relieves the inhibition created by UDP-*N*-acetylglucosamine as shown by an increase in the apparent K_i and by the removal

of substrate inhibition by glutamine. By virtue of this action UTP behaves as an activator for the glucosamine synthetase.

By contrast ATP inhibited the enzyme in the absence of UDP-*N*-acetylglucosamine (non-competitive with respect to both substrates), but this action was diminished on the addition of the feedback inhibitor. The potentiation of the ATP effect on addition of Mg^{2+} indicates that $MgATP^{2-}$ may be the reactive species and that this influence on the activity may be of metabolic importance.

The other UDP-sugars tested were all inhibitory but at concentrations two orders of magnitude higher than that of the *N*-acetylglucosamine derivative. Since the inhibition by UDP-glucose or UDP-glucuronate is not additive to that of UDP-*N*-acetylglucosamine and in fact a relief of inhibition is observed, the evidence is in agreement with there being a common binding site for all of the UDP-sugars. Data derived from Kornfeld (1967) indicate that UDP-*N*-acetylgalactosamine has an inhibitory potency intermediate between that of UDP-*N*-acetylglucosamine and the UDP-sugar analogues used in this work. This is indicative of the acetamido group being involved in inducing the inhibitory conformational change in the enzyme. Another significant difference concerns the action of glucose 6-phosphate on the inhibitions by UDP-sugars. Whereas glucose 6-phosphate causes a marked alteration in the action of the *N*-acetylglucosamine derivative it is without effect on the other UDP-sugars. Since the decrease in K_i for the UDP-sugar caused by glucose 6-phosphate is dependent on the presence of the acetamido group this further supports the postulate on the role of this grouping.

The glucose 6-phosphate-binding site exhibits a specificity for a phosphate group at the 6-position and an equatorial 4-hydroxyl group. These requirements are shown by the negligible effect of galactose 6-phosphate and glucose 1-phosphate. Since 2-deoxyglucose 6-phosphate and mannose 6-phosphate are both capable of potentiating the inhibition due to UDP-*N*-acetylglucosamine in a similar manner to that shown by glucose 6-phosphate, the specificity for the 2-hydroxyl group is apparently low.

The modulation of the glucosamine synthetase activity caused by glucose 6-phosphate, AMP and UTP is expressed only in the presence of UDP-*N*-acetylglucosamine. This led to the proposal that the regulation of this enzyme is best described in terms of a variation in the K_i for UDP-*N*-acetylglucosamine induced by these modifiers (Winterburn & Phelps, 1970).

Physiological considerations. Rat liver glucosamine synthetase is located in the cytosol, as are the remainder of the enzymes responsible for UDP-*N*-acetylglucosamine formation from fructose 6-phosphate (Izumi, 1965; Winterburn & Phelps, 1971). Also, in this context, the data of Hardingham & Phelps (1968) for neonatal rat skin were consistent with there being no compartmentation of intermediates in the hexosamine biosynthetic pathway. In consequence glucosamine synthetase is situated at a branch point in carbohydrate metabolism and will compete for fructose 6-phosphate with other pathways utilizing hexose monophosphates (glycolysis, glycogenesis, pentose phosphate pathway etc.). This enzyme has the lowest extractable activity of the enzymes in the hexosamine pathway (Izumi, 1965) and is also rate-limiting as demonstrated by flux measurements made with rat skin *in vivo* (Hardingham & Phelps, 1968). The maximal rate of glucosamine 6-phosphate synthesis in rat liver, obtained by extrapolation from a crude homogenate to the activity *in vivo* (see Table 1 in Winterburn & Phelps, 1971), is approx. 2 μmol/h per g wet wt. Although this is a calculation that must be regarded with circumspection, this activity is certainly great enough to account for the observed production of hexosamine *de novo* in this tissue: 0.1 μmol/h per g wet wt. as calculated from the data of Spiro (1959*a*). Although maximal glycolytic or gluconeogenic rates in rat liver are 25–50 μmcl of glucose/h per g wet wt., the normal liver will not exhibit such high rates and the flux is probably nearer to the value of 6 μmol/h per g wet wt. quoted for mouse liver (Reich *et al.* 1968). These rates represent a 0.5–2.0% commitment of fructose 6-phosphate to hexosamine synthesis. Although this is only a small proportion of total carbohydrate utilization, in other tissues the synthesis of hexosamines may account for the fate of a larger percentage of the incorporated glucose, e.g. 15–20% in rat skin (Hardingham & Phelps, 1968).

Kornfeld *et al.* (1964) observed that hexosamine biosynthesis was subject to feedback regulation by UDP-*N*-acetylglucosamine. This inhibition was on the initial enzyme of the pathway, catalysing the apparent irreversible formation of glucosamine 6-phosphate (Winterburn & Phelps, 1971). The intracellular concentration of UDP-*N*-acetylglucosamine is 0.55 mM, calculated from the data of Bates & Handschumacher (1969) and assuming a homogeneous distribution. Since the intracellular UDP-*N*-acetylglucosamine concentration is far greater than the K_i of the enzyme for this ligand, it has been predicted that in the cell glucosamine synthetase will be combined with its feedback inhibitor (Winterburn & Phelps, 1970). Experimental evidence in support of this postulate is that UDP-*N*-acetylglucosamine apparently protects the enzyme *in vivo* from irreversible alkylation by Duazomycin A (Bates & Handschumacher, 1969). Although at

this UDP-*N*-acetylglucosamine concentration the enzyme is heavily inhibited (90%), the rate is still in excess of the rate observed *in vivo*. The availability of this latent potential will permit the rapid synthesis of hexosamine when triggered by the appropriate metabolic control without biosynthesis of enzyme *de novo*. Although the artificial induction of a fivefold increase in the concentration of UDP-*N*-acetylhexosamine does not stimulate glycoprotein synthesis, a depletion of the pool size by inhibiting glucosamine synthetase with a glutamine analogue, Duazomycin A, does lead to a retardation in polymer formation (Bates *et al.* 1966). This implies that although the upper limit of the pool size of this UDP-sugar may not be important the lower limit is critical for glycosylation.

Although other UDP-sugars relieve the inhibition by UDP-*N*-acetylglucosamine the effects are not within a physiological range. The UDP-glucuronate and UDP-glucose concentrations in rat liver are about 0.5mM (Wong & Sourkes, 1967; Hornbrook, Burch & Lowry, 1965). This potential mode of activation does not apparently constitute a cross-relationship between the uronate and hexosamine pathways.

The intracellular glutamine concentration is approx. 10mM (Brosnan, Krebs, & Williamson, 1970) assuming that the intracellular water for rat liver is 50% of the wet weight. Since the K_m is 7.5×10^{-4}M (Winterburn & Phelps, 1971) glucosamine synthetase will be saturated with respect to this substrate.

The apparent K_m for fructose 6-phosphate is 1.1×10^{-4}M and this probably approximates to that of the native enzyme (Winterburn & Phelps, 1971). This value is within the fluctuating physiological range of this metabolite: 0.04–0.14mM as calculated from the data of Start & Newsholme (1968) and assuming no compartmentation of intermediates (Till *et al.* 1968). This variable concentration results from alterations in the balance of the fluxes of carbohydrates through the various pathways in response to nutritional, hormonal and other changes.

The observed effects of glucose 6-phosphate on the enzyme are within the range of the intracellular concentration of this substrate, 0.16–0.6mM (Start & Newsholme, 1968). The combined action of the substrate, fructose 6-phosphate, and the inhibitory modifier, glucose 6-phosphate, renders the enzyme sensitive to (*a*) the fructose 6-phosphate/glucose 6-phosphate concentration ratio and (*b*) the total concentration of these two metabolites of the hexose monophosphate pool. This can best be illustrated by examples: (*a*) if the concentration of fructose 6-phosphate remains constant but the fructose 6-phosphate/glucose 6-phosphate concentration ratio increases, i.e. glucose 6-phosphate is depleted, there is a relief of the inhibition and the hexosamine synthetic rate increases; (*b*) if the ratio remains constant while the hexose monophosphate pool size diminishes, i.e. phosphoglucose isomerase activity is not limiting, the declining velocity caused by substrate depletion is offset by the relief of inhibition as the glucose 6-phosphate concentration decreases. The result of this latter action enables hexosamine synthesis to proceed virtually independent of the fluctuations in the concentration of its precursor, fructose 6-phosphate.

This sensitivity of glucosamine synthetase to glucose 6-phosphate may offer an explanation of the anomalous incorporations of glucose into carbohydrate-containing polymers in the diabetic animal. Spiro (1959*b*) noted that, whereas glucose incorporation into glycogen by rat liver was negligible in the diabetic state, the rate of synthesis of hexosamine-containing glycoproteins was only marginally affected. In the diabetic rat liver the fructose 6-phosphate and glucose 6-phosphate concentrations are decreased to approx. 30% of the normal values (Start & Newsholme, 1968). The present studies show that the apparent lack of dependence of hexosamine synthesis on fructose 6-phosphate concentration can be explained in terms of the action of glucose 6-phosphate rather than the suggestion of two hexose monophosphate pools originally invoked.

The liver AMP concentration, 0.25–0.6mM (Start & Newsholme, 1968; Brosnan *et al.* 1970), similarly places the effects on glucosamine synthetase activity within a physiological range. Regulation of enzymic activity by the state of the adenine nucleotides has been implicated in several pathways, including glycolysis and gluconeogenesis, either through the ATP/AMP concentration ratio (Gevers & Krebs, 1966) or by the total energy charge of the adenylate system (Atkinson, 1968). An increase in AMP will retard hexosamine production, so enabling a further channelling of carbohydrates through glycolysis. In liver, an inhibition of a pathway that consumes such a low percentage of available hexose monophosphate seems superfluous; however, in other tissues where the commitment is larger this regulation may be of greater importance. The cellular ATP concentration, 3.5–5.0mM (Start & Newsholme, 1968; Brosnan *et al.* 1970), is large enough to warrant speculation on the significance of the inhibition by this compound. The effect is not concerned with a control of activity by the UTP/ATP concentration ratio (P. J. Winterburn, unpublished work). An hypothesis based on ATP acting at the AMP site and control being exerted through the ATP/AMP concentration ratio cannot be overlooked without further experimentation.

The activation by UTP explains two hitherto

incompletely comprehended effects. The incorporation of orotate into the diet of rats causes a rise in the concentrations of uridine nucleotides (Euler, Rubin & Handschumacher, 1963) by an effect on the control of their synthesis (Windmueller & Bieri, 1965). The increased concentrations also included an elevated (fivefold) concentration of UDP-*N*-acetylhexosamine that was not caused by a decreased utilization (Bates *et al.* 1966). One suggestion proposed by these workers was that other uridine nucleotides modified the feedback inhibition. The present work demonstrates that UTP plays precisely this role. The administration of oestradiol-17β results in a twofold increase in the concentrations of UTP and UDP-*N*-acetylhexosamine in rat and rabbit uteri (Gorski & Mueller, 1963; Endo & Yosizawa, 1968). This effect can be explained in the same terms as that induced by orotate. In connexion with this oestrogen effect, not all UDP-*N*-acetylglucosamine is committed to glycoprotein biosynthesis. An alternative fate lies in the glycosylation of steroids (Collins, Jirku & Layne, 1968).

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REFERENCES

Atkinson, D. E. (1968). *Biochemistry, Easton*, **7**, 4030.

Bates, C. J., Adams, W. R. & Handschumacher, R. E. (1966). *J. biol. Chem.* **241**, 1705.

Bates, C. J. & Handschumacher, R. E. (1969). In *Advances in Enzyme Regulation*, vol. 7, p. 183. Ed. by Weber, G. Oxford: Pergamon Press Ltd.

Brosnan, J. T., Krebs, H. A. & Williamson, D. H. (1970). *Biochem. J.* **117**, 91.

Cleland, W. W. (1963). *Biochim. biophys. Acta*, **67**, 173.

Collins, D. C., Jirku, H. & Layne, D. S. (1968). *J. biol. Chem.* **243**, 2928.

Endo, M. & Yosizawa, Z. (1968). *Archs Biochem. Biophys.* **127**, 585

Engel, P. C. & Dalziel, K. (1969). *Biochem. J.* **115**, 621.

Euler, E. H., Rubin, R. J. & Handschumacher, R. E. (1963). *J. biol. Chem.* **238**, 2464.

Gevers, W. & Krebs, H. A. (1966). *Biochem. J.* **98**, 720.

Gorski, J. & Mueller, G. C. (1963). *Archs Biochem. Biophys.* **102**, 21.

Hardingham, T. E. & Phelps, C. F. (1968). *Biochem. J.* **108**, 9.

Hornbrook, K. R., Burch, H. B. & Lowry, O. H. (1965). *Biochem. biophys. Res. Commun.* **18**, 206.

Izumi, K. (1965). *J. Biochem., Tokyo*, **57**, 539.

Kirtley, M. E. & Koshland, D. E. (1967). *J. biol. Chem.* **242**, 4192.

Kornfeld, R. (1967). *J. biol. Chem.* **242**, 3135.

Kornfeld, S., Kornfeld, R., Neufeld, E. F. & O'Brien, P. J. (1964). *Proc. natn. Acad. Sci. U.S.A.* **53**, 371.

Mazlen, R. G., Muellenberg, C. G. & O'Brien, P. J. (1969). *Biochim. biophys. Acta*, **171**, 352.

Monod, J., Wyman, J. & Changeux, J. P. (1965). *J. molec. Biol.* **12**, 88.

Reich, J. G., Till, U., Gunther, J., Zahn, D., Tschisgale, M. & Frunder, H. (1968). *Eur. J. Biochem.* **6**, 384.

Simon, E. J. & Shemin, D. (1953). *J. Am. chem. Soc.* **75**, 2520.

Slein, M. W. (1957). In *Methods in Enzymology*, vol. 3, p. 154. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Spiro, R. G. (1959*a*). *J. biol. Chem.* **234**, 742.

Spiro, R. G. (1959*b*). *Ann. N.Y. Acad. Sci.* **82**, 366.

Start, C. & Newsholme, E. A. (1968). *Biochem. J.* **107**, 411.

Till, U., Blume, E., Gunther, J., Reich, J. G., Zahn, D., Klinger, R., Jaroszewicz, K. & Frunder, H. (1968). *Eur. J. Biochem.* **6**, 373.

Windmueller, H. G. & Bieri, J. G. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 669.

Winterburn, P. J. (1969). Ph.D. Thesis: University of Bristol.

Winterburn, P. J. & Phelps, C. F. (1970). *Nature, Lond.*, **228**, 1311.

Winterburn, P. J. & Phelps, C. F. (1971). *Biochem. J.* **121**, 701.

Wong, K. P. & Sourkes, T. L. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 835.