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PURIFICATION AND PROPERTIES OF GLUTAMINE SYNTHETASE FROM FLESHFLY FLIGHT MUSCLE

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Abstract—Glutamine synthetase (GS) of fleshfly flight muscle was unstable in crude extracts, but could be stabilised by the reagents glutathione and $MnCl_2$. The enzyme was purified to homogeneity and had a molecular weight of 324,000. Michaelis constants for the three substrates of the biosynthetic reaction were found to be 12.8 mM (glutamate), 130 μM (NH_4^+) and 240 μM ($MgATP^{2-}$). These results indicate an ammonium detoxifying role for this enzyme. Catalytic activity may be regulated *in vivo* by the divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+}. The high K_m for glutamate indicates that GS could not be solely effective in reducing the glutamate concentration in the synapse, unless present in great excess.

Key Word Index: Fleshfly, glutamine synthetase, glutamate, glutamine, neuromuscular junction, glutamatergic

INTRODUCTION

A number of investigations have indicated that glutamate acts as the insect neuromuscular transmitter. Electro-physiological studies (Kerkut *et al.*, 1965; Usherwood and Machili, 1966, 1968; Beránek and Miller, 1968; Faeder and O'Brien, 1970; Neal, 1975a, 1975b) have shown that the application of glutamate, at relatively low concentrations (as low as 240 μM), to insect muscle membranes elicits electrical depolarisations of these membranes. These depolarisations were similar to those elicited by the natural neuromuscular transmitter (Usherwood and Machili, 1968).

Biochemical studies of insect nerve-muscle tissue have indicated the presence of lipoprotein (Lunt, 1973; Fiszer de Plazas *et al.*, 1977) and membrane-bound (James *et al.*, 1977) glutamate receptors; the receptors showed both specificity (Lunt, 1973) and high affinity (James *et al.*, 1977) for glutamate. Investigations into the metabolism of glutamate in nerve-muscle tissue (Donnellan *et al.*, 1974; Langcake and Clements, 1974) have indicated the presence of a number of glutamate metabolising enzymes and their respective subcellular locations. However, the role of these enzymes in neurotransmitter metabolism was not made clear.

Investigations of *Lucilia sericata* larvae revealed that injected [^{3}H]glutamate caused reversible motor paralysis and that the injected [^{3}H]glutamate was mainly converted to glutamine (Irving *et al.*, 1979). The enzyme glutamine synthetase (GS, EC 6.3.1.2.) has been demonstrated in insect nerve-muscle tissue (Donnellan *et al.*, 1974). It is also present in mammalian brain (Ronzio *et al.*, 1969b) where there is strong evidence that it is involved in the metabolism of neurotransmitter glutamate (for reviews, see Hamberger *et al.*, 1978; Schousboe and Hertz, 1981; Schousboe, 1981). These findings suggest that the activity of glutamine synthetase in insect nerve-muscle tissue may play a central role in the metabolism of transmitter glutamate. Although the presence of GS in insect tissue has previously been reported (Kilby and Neville, 1957; Levenbook and Kuhn, 1962; Donnellan *et al.*, 1974), we believe that this study represents the first purification of this enzyme from insects to homogeneity.

The study of the insect neuromuscular junction is likely to uncover a unique biochemical region, in that other animals (except crustaceans) are only known to possess cholinergically mediated nerve-muscle transmission. For this reason, detailed investigations of insect nerve-muscle tissue may reveal unique characteristics at which insect-specific poisons can be directed.

MATERIALS AND METHODS

Insects

Parasarcophaga crassipalpis were reared in conditions of controlled temperature (27 ± 2°C) and fed sugar, water and liver. Sexually mature adults were used for all experiments.

Chemicals

All amino acids, glutathione, ADP, ATP, phosphoenol pyruvate, NADH, DL-methionine sulphoximine, L-glutamic acid monohydroxamate and bovine serum albumin were supplied by Sigma Chemical Co. Pyruvate kinase and lactate dehydrogenase (both crystalline from rabbit muscle) were supplied by Mannheim Boehringer. ADP-Sepharose 4B, Sephadex G-15, DEAE-Sepharose CL-6B and Sephacryl S-300 were supplied by Pharmacia.

Enzyme assays

All assays were performed at 30°C and absorbances measured with a Unicam SP1800 spectrophotometer. All enzyme activities [1 unit (U) = 1 μmol min^{-1}] were the means of duplicate assays and were corrected for blank rates (synthetase reaction, no glutamate; transferase reaction, no glutamine).

γ-Glutamyl transferase assay. The standard reaction mixture contained 60 mM $NH_2OH \cdot HCl$ (neutralised with 60 mM NaOH), 20 mM K-arsenate (pH 7.2), 100 mM glutamine, 0.4 mM ADP, 1 mM $MnSO_4$ and GS in a total

volume of 600 μl. Reactions were started by addition of enzyme. After 20 min incubation the reaction was stopped with 1.0 ml of a solution containing 0.03 M $FeCl_3$, 0.5 M HCl and 0.12 M trichloracetic acid. Reaction mixtures were centrifuged to remove precipitated protein and their absorbance at 540 nm determined. γ-Glutamyl hydroxamate was determined by comparison with a standard curve of authentic γ-glutamyl hydroxamate in the $FeCl_3$ reagent.

Biosynthetic, coupled enzyme assay. The method of Kornberg and Pricer (1951) was used (with modification) to follow the production of ADP. Assays contained 25 mM Tris–HCl (pH 8.4), 100 mM Na-glutamate, 12.5 mM $(NH_4)_2SO_4$, 2.8 mM ATP, 8 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 1 mM phosphoenol pyruvate, 0.20 mM NADH, 2.6 U of pyruvate kinase, 12 U of lactate dehydrogenase and 2–3 mU of GS in a total volume of 1.0 ml. Reactions were started by the addition of glutamine synthetase and the decrease in absorbance at 340 nm followed.

Biosynthetic, stopped time assay. Assays contained 25 mM Tris–HCl (pH 8.4), 100 mM Na-glutamate, 10 mM NH_4Cl, 2.8 mM ATP, 8 mM $MgCl_2$, 0.5 mM EDTA and 2–3 mU of GS in a total volume of 700 μl. The reaction was started by addition of GS and stopped, after 10 min incubation, with 100 μl of 1 M HCl. After 5 min at 0°C, 100 μl of 1 M NaOH was added to reneutralise the mixtures. ADP was then determined by adding 98 μl of a solution containing 25 mM Tris–HCl (pH 8.4), 102 mM KCl, 10.2 mM phosphoenol pyruvate and 2 mM NADH to each incubation, and the absorbance at 340 nm determined. Then 1.3 U of pyruvate kinase and 6 U of lactate dehydrogenase (2 μl) were added, and the decrease in absorbance at 340 nm measured.

Protein determination

Protein was determined according to Bradford (1976), using Coomassie brilliant blue G250. Bovine serum albumin was used as standard. Glutathione, $MnCl_2$ and ADP did not give significant colour with this reagent at the levels used.

Molecular weight determination

The molecular weight of GS was estimated by gel filtration chromatography with Sephacryl S-300 Superfine. The column (5.3 cm^2 × 58 cm) was equilibrated in 25 mM Tris–HCl (pH 7.2), 2.5 mM $MnCl_2$ and 5 mM β-mercaptoethanol. Thyroglobulin, ferritin, catalase and aldolase were used as standards.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was used in Tris–glycine buffer to check the relative purity of the enzyme (Davis, 1964). Protein samples were applied to the gel after 30 min pre-equilibration in Tris–glycine buffer containing 2.5 mM $MnCl_2$ and 10 mM glutathione.

Stabilisation of GS

Flight muscle (from 100 thoraces) was dissected and homogenised in 20 ml of 50 mM Tris–HCl buffer. The crude homogenate was then dialysed against 1000 vol of test buffer and stored at 2–4°C. GS activity was measured at intervals using the transferase assay.

Purification of GS

All operations were performed at 2–4°C.

Crude extract. Thoracic flight muscle was dissected from 300 to 400 frozen (−25°C) fleshflies essentially according to Donnellan *et al.* (1974), except that the muscle tissue was not squeezed or scraped from the exoskeleton before homogenisation. Flight muscle was homogenised, using a mortar and pestle, in double-strength buffer (50 mM Tris–HCl, 20 mM glutathione, 5 mM $MnCl_2$; pH 7.2) at a concentration of 5 thoraces per ml. The homogenate was filtered through 3 layers of muslin.

Triton X-100 was added to the filtrate to a final concentration of 0.5% (w/v). After stirring for 30 min, the solution was centrifuged at 20,000 *g* and the pellet discarded.

Precipitation with ammonium sulphate. A solution of saturated ammonium sulphate was added to the supernatant to bring the saturation to 40%. This solution was stirred for 30 min, centrifuged at 20,000 *g* and the pellet discarded. Saturated ammonium sulphate was then added to the supernatant to bring the saturation to 55%. After stirring for 30 min, the solution was centrifuged at 20,000 *g* and the supernatant discarded. The pellet was dissolved in double-strength buffer (1–2 ml).

Removal of salt. The redissolved pellet was applied to a column filled with Sephadex G-15 (4.5 cm^2 × 9 cm) pre-equilibrated in single-strength buffer (25 mM Tris–HCl, 10 mM glutathione, 2.5 mM $MnCl_2$; pH 7.2). The void volume eluate (containing proteins) was collected and its conductivity checked (single-strength buffer conductivity = 1–2 mS).

DEAE chromatography. The void volume eluate was applied to a column filled with DEAE cellulose or DEAE-Sepharose CL-6B (0.6 cm^2 × 15 cm), the column washed to remove unbound protein and a salt gradient of 0–0.35 M KCl (in 300 ml single-strength buffer) applied to the gel. In both cases, the GS activity eluted at a conductivity of 7.5–9.5 mS.

ADP-Sepharose 4B affinity chromatography. The pooled, active fractions were applied directly to a column containing ADP-Sepharose 4B gel (2 ml of gel bed, pre-equilibrated in single-strength buffer). The column was washed with 10 ml of buffer to remove unbound protein and bound protein eluted by the addition of 5 mM ADP in single-strength buffer.

RESULTS

Stability

GS activity was unstable when flight muscle was homogenised in Tris–HCl buffer (10 mM, pH 7.2), losing up to 70% of its activity within 24 hr. In separate treatments, the addition of 5 mM β-mercaptoethanol (stabilising sheep brain GS, Ronzio *et al.*, 1969b), 2.5 mM $MnCl_2$ (stabilising *E. coli* GS, Woolfolk *et al.*, 1966; and pea leaf GS, O'Neal and Joy, 1973) and 10 mM Na-pyruvate (stabilising lupin nodule GS, Chen and Kennedy, personal communication) each had little stabilising effect. The addition of 10 mM glutathione and 2.5 mM $MnCl_2$ together retained approx. 100% activity over the course of 3 days. The buffer subsequently used to isolate GS was 10 mM Tris–HCl, 10 mM glutathione and 2.5 mM $MnCl_2$ (pH 7.2).

Purification

A typical purification is summarised in Table 1. The purified enzyme was much more stable than the crude, retaining close to 100% of its activity for 4–5 weeks at 4°C (after which the enzyme began to precipitate). PAGE indicated that the enzyme eluted from the ADP-Sepharose 4B column contained a single protein (Fig. 1). However, if Mn^{2+} and glutathione were not present during electrophoresis, 2–3 bands of protein were observed. Attempts to employ glutamate-agarose (Sigma) and ATP-agarose (Sigma) affinity chromatography under various conditions were unsuccessful.

Properties

For all studies into the nature of GS, the biosynthetic reaction was assayed.

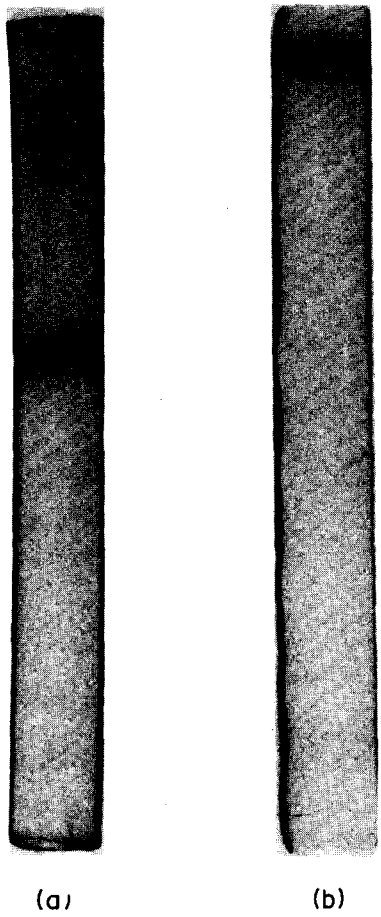

Fig. 1. PAGE of 5 μg of ADP-Sepharose 4B eluate in (a) the absence and (b) the presence of 2.5 mM $MnCl_2$ and 10 mM glutathione.

Table 1. Purification of fleshfly flight muscle GS

	Step	Units	Protein (mg)	Specific activity*	P.R.†	Yield (%)
I	Crude extract	25.6	558	0.046	1	100
II	$(NH_4)_2SO_4$	14.9	104	0.143	3.1	58
III	DEAE-Sepharose	11.9	13.1	0.912	20	47
IV	ADP-Sepharose	8.0	0.395	20.3	440	31

Activity was measured using the transferase assay. *Specific activity defined as U mg protein^{-1}. †P.R. is purification ratio.

Effect of EDTA. When the biosynthetic, coupled enzyme assay was initially used, very low activities were observed. Subsequently, it was found that Mn^{2+}, which was added with the GS to the assay, strongly inhibited the reaction. Since EDTA has a stability constant for Mn^{2+} 200,000-fold greater than that for Mg^{2+} (Sillen, 1971), it could be added to the assay to complex Mn^{2+} leaving excess Mg^{2+} free. EDTA was used at a concentration of 0.5 mM ($\cong 100 \times$ concentration of Mn^{2+} present) for all GS characterisation studies. Glutathione, which was also added to the assay with GS, had no effect on reaction rate.

pH optimum. Enzyme activity was assayed over the pH range of 7 to 9, using HEPES (25 mM, pH 7–8) and Tris–HCl buffers (25 mM, pH 8–9). A single peak of activity was observed at a pH of 8.4.

Mg^{2+} optimisation. According to Morrison (1979), it is important to optimise the concentration of free metal ion present when assaying reactions involving metal-complexed substrates (such as $MgATP^{2-}$), as excess free metal ion is often inhibitory. It was found that a free Mg^{2+} concentration of 5 mM was necessary for optimum activity (Fig. 2) and that higher levels were only slightly inhibitory.

Kinetic parameters. Kinetic parameters for all three substrates were calculated on an Apple IIe microcomputer using a BASIC non-linear regression programme designed by Duggleby (1981). Curves shown in Figs 3–5 were plotted by the microcomputer from the regression analyses, assuming the Michaelis equation $V = V_{max}[S]/(K_m + [S])$.

Plots of initial velocity versus substrate concentration for all three substrates (glutamate, NH_4^+, $MgATP^{2-}$) indicated hyperbolic kinetics (Figs 3–5). Non-linear regression analyses indicated a K_m for glutamate of 12.8 mM; for NH_4^+ of 130 μM; and for $MgATP^{2-}$ of 240 μM. The V_{max} was indicated as 12.3 μmol min^{-1} mg^{-1} protein.

Molecular weight. Using gel filtration chromatography, the molecular weight of GS was estimated as 324,000 ± 10,000.

Inactivation by methionine sulphoximine. The conditions involved in inactivation of GS by methionine sulphoximine were investigated (Table 2). For significant inactivation to occur, the enzyme had to be pre-incubated with methionine sulphoximine and $MgATP^{2-}$. Little inactivation was seen in the absence of $MgATP^{2-}$. If glutamate was present during pre-incubation, much less inactivation was observed. NH_4^+ had no effect. Furthermore, inactivation was dependent upon both the concentration of methionine sulphoximine used (Fig. 6) and the time of pre-incubation (Fig. 7).

Effect of divalent cations. Both Mn^{2+} and Ca^{2+} were found to be strong inhibitors of the biosynthetic GS activity. Using the coupled enzyme assay [Na-glutamate, 100 mM; $(NH_4)_2SO_4$, 12.5 mM; $MgCl_2$, 8 mM; ATP, 2.8 mM; EDTA, 0.5 mM], I_{50}s of 20 μM (Mn^{2+}) and 160 μM (Ca^{2+}) were found.

Effect of L-amino acids, amino compounds and phosphate. A number of compounds were investigated for inhibition/activation of GS activity in both glutamate limiting (10 mM) and glutamate saturating (50 mM) conditions (all other substrates and assay as for the coupled enzyme assay referred to in the Materials and Methods section). Alanine, arginine, asparagine, aspartic acid, GABA, glutamine, glycine, histidine, lysine, *p*-aminobenzoic acid and serine (all at 10 mM) had no significant effect on activity at either glutamate level.

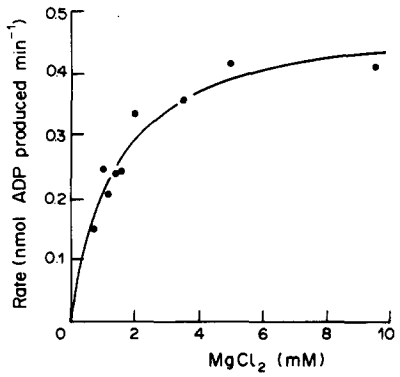

Fig. 2. Optimisation of free Mg^{2+} concentration for GS activity. Na-glutamate, 100 mM; $(NH_4)_2SO_4$, 12.5 mM; ATP, 1 mM; EDTA, 0.5 mM. Assays used the coupled enzyme assay referred to in the Materials and Methods section.

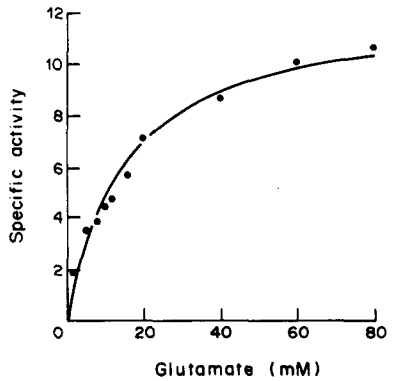

Fig. 3. Effect of glutamate concentration on GS activity. ATP, 2.8 mM; $(NH_4)_2SO_4$, 12.5 mM; $MgCl_2$, 8 mM; EDTA, 0.5 mM. Assays used the coupled enzyme assay.

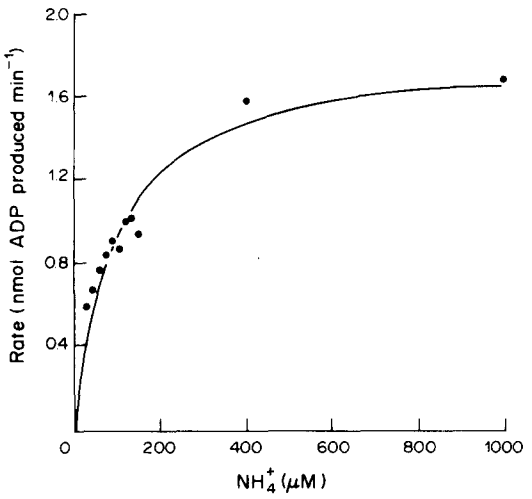

Fig. 4. Effect of NH_4^+ concentration on GS activity. Na-glutamate, 100 mM; ATP, 2.8 mM; $MgCl_2$, 8 mM; EDTA, 0.5 mM. Assays used the stopped time assay referred to in the Materials and Methods section.

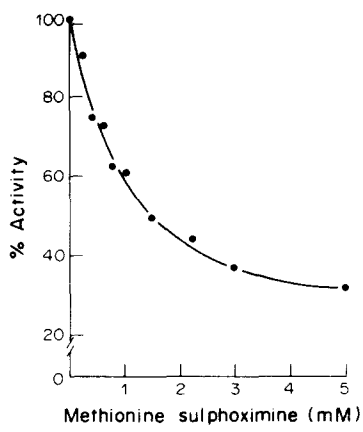

Fig. 6. Effect of DL-methionine sulphoximine concentration on GS activity. Tris–HCl (25 mM; pH 8.4), 8 mM $MgCl_2$, 2.8 mM ATP and 2–3 mU of GS were incubated, for 10 min at room temperature, in a total volume of 200 μl. Incubations were then immediately assayed for residual GS activity using the coupled enzyme assay.

Table 2. Inactivation of GS by methionine sulphoximine

Pre-incubation medium	% Inhibition
GS + MS	17
GS + MS + Glu	11
GS + MS + NH_4^+	21
GS + MS + Mg-ATP	62
GS + MS + Mg-ATP + NH_4^+	63
GS + MS + Mg-ATP + Glu	26
GS + MS + Mg-ATP + Glu + NH_4^+	30

Conditions of pre-incubation and residual GS activity as in Fig. 6, with 5 mM methionine sulphoximine (MS), 100 mM Na-glutamate (Glu), 10 mM NH_4Cl, 8 mM $MgCl_2$, 2.8 mM ATP and 0.5 mM EDTA.

Na-phosphate (10 mM) caused a slight inhibition (25% inhibition at 50 mM glutamate).

DISCUSSION

The aim of our investigation was to study an isolated protein involved in the metabolism of the putative neuromuscular transmitter glutamate. In doing so, we have had to overcome one of the common problems that insect biochemists face, that of instability of proteins in tissue extracts. For example, the membrane-bound glutamate receptor fraction isolated from locust muscle lost 50% of its glutamate-binding capacity within 2–3 days (James *et al.*, 1977); and the glutamate decarboxylase activity studied by Langcake and Clements (1974) lost up to 50% of its activity overnight. Furthermore, certain adult fly mono-oxygenase preparations have been found to be unstable (Jordan and Smith, 1970). Instability is thought to be associated with either the enzyme complex tyrosinase (Williamson and Schecter, 1970; Crankshaw *et al.*, 1977) or its end-products, *o*-quinones (Krieger and Wilkinson, 1971) which bind proteins together in the formation of cuticle (Mason, 1955). Upon tissue homogenisation, *o*-quinones may react generally with proteins, cross-linking and inactivating them. It should be possible to prevent cross-linking by addition of reagents that react with *o*-quinones. Glutathione is one such re-

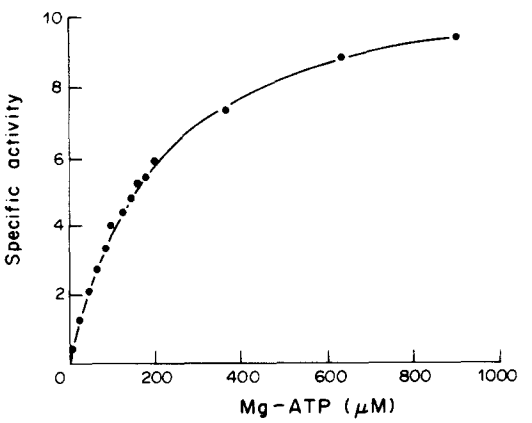

Fig. 5. Effect of $MgATP^{2-}$ concentration on GS activity. Na-glutamate, 100 mM; $(NH_4)_2SO_4$, 12.5 mM; $MgCl_2$, 8 mM; EDTA, 0.5 mM. Assays used the coupled enzyme assay.

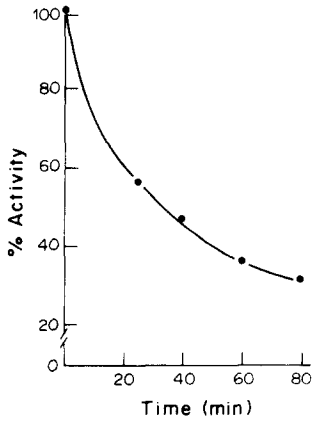

Fig. 7. Effect of time of pre-incubation of GS with methionine sulphoximine. Conditions of pre-incubation and assay of residual GS activity as in Fig. 6. Methionine sulphoximine concentration was 2 mM.

agent (Snell and Weissberger, 1939; Mason, 1955) forming reduced addition compounds that can no longer cross-link proteins. We suggest that glutathione may be generally useful in stabilising proteins of interest in other insect preparations.

The purification of GS to homogeneity was greatly facilitated by the successful use of an affinity chromatography step utilising the ADP-dependent transferase function of the enzyme. In the presence of Mn^{2+}, GS bound firmly to ADP-Sepharose 4B and was readily eluted with ADP. We suggest that all GS enzymes may be purified using ADP-Sepharose 4B chromatography, since all catalyse the transferase activity. Lupin nodule GS, a plant enzyme, has also been purified in this way (Chen and Kennedy, personal communication).

Kinetic studies of the biosynthetic reaction catalysed by GS indicate that the enzyme can act as an NH_4^+ scavenger, as it has a very low Michaelis constant for this substrate ($K_m = 130\,\mu M$). Its relatively high Michaelis constant for glutamate ($K_m = 12.8$ mM) indicates that the enzyme is unlikely to act as the immediate inactivator of neurotransmitter. Possibly, transport proteins such as the uptake glutamate receptor proposed by James *et al.* (1977) are primarily involved in the removal of glutamate from the synapse into a cellular compartment against a concentration gradient. Irving *et al.* (1979) have presented evidence of such an uptake of glutamate in *Lucilia* and *Locusta* larvae, while Faeder and Salpeter (1970) have indicated that the sheath cells in adult cockroach nerve-muscle tissue show highest glutamate uptake. GS would presumably be present in the uptake compartment. We propose to use biochemically-pure GS to raise monospecific antiserum to GS, and to use this antiserum to immunochemically localise this enzyme.

The activating and inhibiting effects that certain divalent cations have on GS activity may regulate this enzyme *in vivo*. Thus Mg^{2+} activates GS, while Mn^{2+} and Ca^{2+} strongly inhibit the Mg^{2+}-activated enzyme. This result is similar to that found with sheep brain GS (Elliott, 1951). Ca^{2+} and Mg^{2+} are known to flux rapidly in and around nerve-muscle tissue during both nerve impulses and muscle contractions.

Comparison of the properties of fleshfly flight muscle GS with sheep brain GS indicates that the two enzymes are fairly similar. Both are inhibited by Ca^{2+} and Mn^{2+}, have a similar Michaelis constant for NH_4^+ ($K_m = 180\,\mu M$, Rowe *et al.*, 1970) and are inhibited by methionine sulphoximine (Ronzio *et al.*, 1969a). These similarities preclude targetting insect GS with insect-specific poisons, although more detailed studies of insect GS may uncover other, unique properties of the enzyme.

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