

LOCALIZATION OF GLUTAMINE SYNTHETASE IN FLESHFLY FLIGHT MUSCLE

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(Received 13 November 1987; revised and accepted 13 May 1988)

Abstract—Antibodies to glutamine synthetase (GS; EC 6.3.1.2) from fleshfly flight muscle were raised after injection of biochemically pure GS into a rabbit. As crossed immunoelectrophoresis indicated that this antiserum was not monospecific, non-GS antibodies were adsorbed from the antiserum onto non-GS immunogens bound to Affi-Gel 10. After adsorption, the antiserum was judged monospecific by rocket immunoelectrophoresis, but not monospecific by crossed immunoelectrophoresis. The presence of reagents stabilizing GS (Mn^{2+} and 2-mercaptoethanol) improved the crossed immunoelectrophoretic pattern but did not prove monospecificity, suggesting that the immunoelectrophoretic patterns observed were complicated by the instability of the antigen during electrophoresis. When the adsorbed antiserum was subsequently used for immunohistological localization of immunogen in fleshfly flight muscle, immunogold label was found associated with the mitochondria, the myofibrils and the cytoplasm. These results were verified by subcellular fractionation studies in which fleshfly flight muscle was gently disrupted by incubation with collagenase and fractionated on Percoll density gradients. The physiological implications of these findings are discussed.

Key Word Index: Fleshfly, flight muscle, glutamine synthetase, glutamatergic, immunohistological, mitochondria, myofibril

INTRODUCTION

At mammalian glutamatergic synapses, neurotransmitter glutamate is inactivated primarily by uptake into glia and metabolism by glutamine synthetase (GS; EC 6.3.1.2) (Schousboe, 1981). At cockroach and locust neuromuscular junctions, autoradiographical evidence suggests that neurotransmitter glutamate may also be inactivated by uptake into glia (Faeder and Salpeter, 1970; Botham *et al.*, 1978). However, not all insect neuromuscular junctions are surrounded by glia (Shafiq, 1964; Osborne, 1967; Neal, 1975). The mechanism of inactivation of neurotransmitter glutamate at insect synapses that lack glia is unknown. Furthermore, the involvement of GS in the metabolism of neurotransmitter glutamate at either of these types of insect junctions (those with and those without glia) has not been previously investigated.

GS is present in adult fleshfly neuromuscular tissue (Donnellan *et al.*, 1974) that typically lack glia (Neal, 1975). This enzyme has been implicated in the metabolism of neurotransmitter glutamate at larval blowfly neuromuscular junctions (which also lack glia), as a paralysing dose of radiolabelled glutamate was largely converted to glutamine (Irving *et al.*, 1979). Subcellular localization studies suggested that GS is present in the cytosol (Donnellan *et al.*, 1974; Downton and Kennedy, 1985), although it is not known whether the enzyme is present in the nerve or muscle cell cytosol. In order to obtain a better understanding of the physiological role of GS at

insect neuromuscular junctions, the precise cellular and subcellular localization of this enzyme in fleshfly neuromuscular tissue was investigated.

MATERIALS AND METHODS

Insects

Parasarcophaga crassipalpis were reared as described by Downton and Kennedy (1985).

Chemicals

From Bio-Rad: Affi-Gel 10. From BDH: crystalline *p*-formaldehyde, polyethylene glycol 6000 and Triton X-100. From Calbiochem: Freund's complete adjuvant and PIPES. From Chemische Werke Lowi: Lowicryl K4M resin. From Hopkins and Williams: barbitone. From John Mathey: OsO_4 . From LKB: Silane. From Mannheim Boehringer: collagenase (*Clostridium histolyticum*), lactate dehydrogenase (rabbit muscle) and pyruvate kinase (rabbit muscle). From Pharmacia: agarose A, agarose B, DEAE-Sephacel CL-6B, Percoll, protein A (*Staphylococcus aureus*) and protein A-Sephacel CL-4B. From Polaron: glutaraldehyde and Spurr's resin. From Sigma: arginine, ADP, ATP, Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250, L-glutamic acid monohydroxamate, glutamate, glutamine, glutathione, DL- α -glycerophosphate, human serum albumin (fraction V), L-malic acid, 2-mercaptoethanol, NADH, β -phenylpyruvic acid, phosphoenol pyruvate, RIA grade bovine serum albumin and Trizma.

Production of antiserum to fleshfly flight muscle GS

Biochemically pure GS from fleshfly flight muscle (40 μ g protein in 1 ml) was prepared as described previously (Downton and Kennedy, 1985), dialysed extensively against distilled water, mixed with Freund's complete adjuvant (1:1 v/v) and injected intradermally into the thigh muscle of a 5 kg rabbit. A booster injection of similarly dialysed GS

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(20 µg protein in 1 ml) was given 2 weeks later. The rabbit was bled from the ear at fortnightly intervals and antiserum obtained from the blood collected. Control serum was obtained from the same rabbit prior to immunization. The immunoglobulin G fraction of both antiserum and control serum was prepared as described by Goding (1976) using protein A–Sephadex CL-4B.

Immuno-electrophoresis

Rocket, rocket-line and crossed immuno-electrophoresis were performed according to Svendsen *et al.* (1983) using Tris/barbital buffer. Antigen (fleshfly flight muscle extract containing 5–10 mU of GS transferase activity) was prepared in Tris/barbital buffer as described by Dowton and Kennedy (1985).

Detection of antibodies specific to fleshfly flight muscle GS

Antibodies specific to fleshfly flight muscle GS were detected by their inhibition of the GS reaction. GS was partially purified by ammonium sulphate fractionation and DEAE–Sephadex CL-6B chromatography (Dowton and Kennedy, 1985) and preincubated with aliquots of the immunoglobulin G fraction of antiserum or control serum. After 10 min preincubation at room temperature, the residual GS activity was determined by addition of substrates for the biosynthetic, coupled enzyme assay of GS (Dowton and Kennedy, 1985).

The presence of antibodies to fleshfly flight muscle GS in the antiserum was also detected by enzyme staining of rocket immuno-electrophoresis immunoprecipitates. Rocket immuno-electrophoresis was performed essentially as described by Svendsen *et al.* (1983), except that after immuno-electrophoresis the agarose gels were pressed only once before GS transferase substrates (Dowton and Kennedy, 1985) were allowed to soak into the gel for 40 min at 37°C. GS reaction product was subsequently detected by applying the FeCl₃ colour developing reagent (0.03 M FeCl₃, 0.5 M HCl, 0.12 M trichloroacetic acid) to the gel surface.

Immuno-adsorption of contaminating antibodies

Fleshfly flight muscle was extracted and GS purified by ammonium sulphate fractionation and DEAE–Sephadex CL-6B chromatography as described previously (Dowton and Kennedy, 1985). Proteins from fraction 52 (0.3 mg total protein) of the DEAE–Sephadex CL-6B chromatography run contained immunogens (as detected by rocket immuno-electrophoresis) but no GS activity. This fraction was bound to Affi-Gel 10 according to the manufacturer's instructions, antiserum incubated with the ligated Affi-Gel at 4°C for 14 h with continual agitation using a Chiltern scientific orbital shaker, the mixture centrifuged at 1400 g for 5 min and the supernatant containing unbound material collected. The bound antibodies were subsequently obtained by the addition of 0.58% v/v acetic acid to the pelleted Affi-Gel 10, centrifugation at 1400 g for 5 min and collection of the supernatant. The supernatant was neutralized by the addition of 0.5 vol of 0.5 M NaHCO₃ buffer pH 9.5.

Preparation of fleshfly flight muscle for immunohistology

The six pairs of giant cells of the dorsal longitudinal flight muscles of adult fleshflies were dissected as described by Donnellan *et al.* (1974) into 8% *p*-formaldehyde (in 0.1 M sodium phosphate buffer, pH 7.2) and incubated in the fixative for 3–4 days at 4°C. The schedule for dehydration and embedding was adapted from that of Wells (1985). The tissue was dehydrated through a graded series of ethanol (30% v/v for 1 h at 4°C, 50% for 1 h at –20°C, 70% for 1 h at –35°C, 95% for 1 h at –35°C, and twice in 100% for 1 h at –35°C) and infiltrated with Lowicryl K4M resin (1:1 v/v resin:ethanol for 1 h at –35°C, 2:1 resin:ethanol for 1 h at –35°C, 3:1 resin:ethanol for 1 h at –35°C, 100% resin overnight at –35°C, and 100% resin for 8 h at

–35°C). The resin was then polymerized with indirect long wave u.v. light for 24 h at –40°C and 48 h at room temperature. Sections of flight muscle were cut using a glass knife with an LKB Bromma Ultratome and collected onto copper grids (200 mesh with a carbon-parlodion film).

Preparation of colloidal gold and the protein A–colloidal gold complex for immunolabelling

All glassware was thoroughly cleaned, siliconized (LKB–Silane) and rinsed with double distilled water. All reagents were prepared in double distilled, membrane-filtered water. Colloidal gold was prepared essentially as described by Frens (1973) and Roth (1983). HAuCl₄, 100 ml of 0.01% w/v, was reduced with 3 ml of 1% w/v trisodium citrate. Gold colloids of 20.6 nm dia were formed, as measured by electron microscopy. Staphylococcal protein A was adsorbed onto colloidal gold as described by Roth (1983) and stored at 4°C in 0.01 M sodium phosphate (pH 7.2) containing 0.15 M NaCl and 0.5 mg/ml of human serum albumin. This solution could be stored for periods of up to 6 months at –15°C when diluted 1:1 with glycerol.

Immunolabelling of fleshfly flight muscle sections

Tissue sections were labelled by the indirect method using a procedure modified from that of Robertson *et al.* (1984). Grids were placed section side down onto 50-µl drops of Tris-buffered saline (TBS; 10 mM Tris–HCl pH 7.4, 0.15 M NaCl, 0.02% w/v Na₂S₂O₃, 0.05% w/v polyethylene glycol 6000 and 0.2% w/v BSA) containing 2% w/v BSA (1 h at 37°C), transferred onto drops of antiserum diluted 1:25 with TBS (1 h at 37°C), washed for 15 min in TBS (five changes at room temperature), transferred onto drops of protein A–colloidal gold diluted 1:10 with TBS (1 h at 37°C), washed at room temperature in TBS for 9 min (three changes), in TBS containing 0.1% w/v Triton X-100 for 6 min (two changes) and in double distilled water for 9 min (three changes). For controls, pre-immune control serum was substituted for antiserum in the above schedule. Sections were subsequently stained for 2 min in 2% w/v aq. uranyl acetate followed by 4 min in Reynolds lead citrate (Reynolds, 1963) before examination in a Phillips EM201 electron microscope.

Collagenase digestion and fractionation of flight muscle tissue

Flight muscle from 60 adult fleshflies was dissected as described by Donnellan *et al.* (1974) into 25 ml of digestion saline containing 140 mM NaCl, 10 mM KCl, 10 mM PIPES (pH 6.5), 10 mM EDTA, 5 mg collagenase (*Clostridium histolyticum*) and 37% w/v Percoll. The mixture was incubated for 1 h at 30°C with gentle agitation, filtered through nylon mesh (100 µm) and chilled for 10 min on ice before fractionation on Percoll gradients formed during centrifugation (23000 g for 20 min).

Enzyme assays

All assays were performed at 30°C and absorbances measured with a Unicam SP1800 spectrophotometer. Enzyme activities [1 U = 1 µmol/min] were the means of duplicate assays and were corrected for blank rates. Arginine kinase (EC 2.7.3.3), a marker for the muscle cell cytoplasm, was assayed as described by Blethen (1970). Fumarase (EC 4.2.1.2), a marker for the mitochondrial matrix (Tolbert, 1974), glutamine transaminase (EC 2.6.1.15), GS and protein were assayed as reported previously (Dowton and Kennedy, 1986).

Preparation of membranous fractions and flight muscle for examination by electron microscopy

Following Percoll density gradient fractionation of collagenase digested flight muscle, the membranous fractions were diluted 1:10 with saline (140 mM NaCl, 10 mM KCl, 10 mM PIPES pH 6.5) to decrease the Percoll concentration and make light sedimentation (3000 g for 10 min) possible.

The pellets were surface rinsed twice with saline, fixed in saline containing 2% glutaraldehyde (2 h at room temperature), washed with saline (3 times for 20 min), post-fixed in saline containing 1% OsO₄ (90 min at room temperature), washed in saline (3 times for 10 min), stained *en bloc* in saline containing 0.5% w/v aq. uranyl acetate (60 min at room temperature), dehydrated in a graded series of acetone (30% v/v, 50%, 70%, 3 times at 100%; each for 20 min), infiltrated with Spurr's resin (1:1 v/v 100% acetone: Spurr's resin for 10 h, 100% Spurr's overnight) and embedded in Spurr's resin at 65°C overnight. Sections of the embedded pellets were then cut using either a glass (bands 1 and 3) or diamond (band 2) knife with an LKB Bromma Ultratome and placed onto copper grids (200 mesh with a carbon-parlodion film). The sections were post-stained with 2% aq. uranyl acetate (5 min) and Reynolds lead citrate (5–7 min; Reynolds, 1963) before examination in a Phillips EM 201 electron microscope. Fleshly flight muscle was also prepared using the above schedule.

RESULTS

Detection of antibodies specific to fleshly flight muscle GS

The rabbit injected with biochemically pure GS was bled 8 times to obtain antisera. Rocket immunoelectrophoresis of flight muscle extract against these various antisera indicated that bleeds 2–5 contained a relatively high titre of antibodies to one flight muscle immunogen and a low titre of antibodies to a second immunogen. As bleeds 2–5 were most comparable, they were pooled and used for all subsequent investigations.

Antibodies to enzymes can often be detected by their inhibition of enzyme activity (e.g. Bollet *et al.*, 1962; Kennedy *et al.*, 1985). The immunoglobulin G fraction of the pooled antiserum from bleeds 2–5 inhibited the synthetase reaction of fleshly flight muscle GS by a maximum of 25–30% (Fig. 1). Inhibition was greatest after 10 min preincubation of GS and antiserum. The immunoglobulin G fraction of control serum had no effect on GS activity. In addition, when high levels of ADP affinity-purified GS (95 mU) were used as antigen during rocket

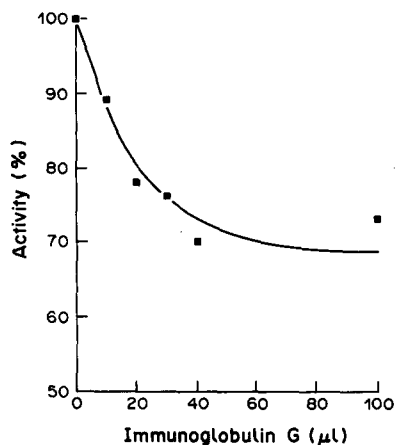


Fig. 1. Inhibition of the GS synthetase reaction by the immunoglobulin G fraction of bleeds 2–5. Immunoglobulins were pre-incubated with partially purified GS (see Materials and Methods) for 10 min before addition of substrates and measurement of residual GS activity using the biosynthetic coupled enzyme assay.

immuno-electrophoresis, the resulting immunoprecipitate could be stained for GS activity. The hydroxamate GS reaction product was observed in the agarose gel and correlated with the immunoprecipitate region of a duplicate gel stained for protein. Clearly, there was a substantial loss of GS activity during immunoelectrophoresis. These results indicate that the pooled antiserum from bleeds 2–5 contained antibodies specific to fleshly flight muscle GS.

Crossed immunoelectrophoresis of ultracentrifuged fleshly flight muscle extract against the pooled antiserum from bleeds 2–5 produced two immunoprecipitates, indicating that the antiserum was not monospecific. To prepare monospecific antiserum to GS, contaminating immunogen was prepared as described below for immunoadsorption of the non-GS antibodies.

Immunoadsorption of contaminating antibodies from the pooled antiserum of bleeds 2–5

To prepare immunogen for the adsorption of non-GS antibodies, GS from fleshly flight muscle was partially purified as described in Materials and Methods. Rocket immunoelectrophoresis of the various DEAE-Sephadex CL-6B chromatography fractions indicated the presence of non-GS immunogen in fractions eluting just after GS with no GS activity. Rocket-line immunoelectrophoresis of these fractions showed that this antigen cross-reacted with the upper of the two bands produced during rocket immunoelectrophoresis of crude flight muscle extract (Fig. 2a). This suggested that the upper band corresponded to the non-GS contaminant.

The non-GS immunogen was then bound to Affi-Gel 10 and non-GS antibodies adsorbed from the antiserum (pooled from bleeds 2–5) by incubation with the non-GS immunogen/Affi-Gel 10. When the antibodies that bound to the non-GS immunogen/Affi-Gel 10 were used as antiserum during rocket immunoelectrophoresis, only the upper band was observed, verifying that the upper band corresponded to the non-GS contaminant. The relationship of this immunogen to GS, if any, is unclear.

The antibodies that did not bind to the non-GS immunogen/Affi-Gel 10 inhibited the GS synthetase reaction and produced a single immunoprecipitate during rocket immunoelectrophoresis of crude flight muscle extracts (Fig. 2b). However, when this contaminant-adsorbed antiserum was studied by crossed immunoelectrophoresis, at least two immunoprecipitates were observed, each having similar electrophoretic mobility in the first dimension (Fig. 3a). The fact that the same antibody preparation cross-reacted detectably with only one flight muscle immunogen during rocket immunoelectrophoresis, but cross-reacted with more than one immunogen during crossed immunoelectrophoresis suggested that a single rocket immunoelectrophoresis-stable antigen was present, and that this antigen was unstable during extended immunoelectrophoresis. It has been previously shown that biochemically pure GS is unstable in an electric field, unless Mn²⁺ and a reduced sulphhydryl reagent are present (Downton and Kennedy, 1985). To investigate the possibility that the multiplicity of bands

formed during crossed immunoelectrophoresis was attributable to the instability of GS in an electric field, stability reagents were included in the electrophoresis buffers in both dimensions during crossed immunoelectrophoresis. Two closely associated immunoprecipitates were formed (Fig. 3b). The observation that the crossed immunoprecipitation pattern was altered in the presence of GS stabilizing reagents is consistent with the notion that the various immunoprecipitates are related to native GS. However, no conditions could be found in which a single immunoprecipitate was observed. It cannot therefore be concluded that the adsorbed antiserum is definitely monospecific. In order to produce antibodies to GS and be certain of monospecificity, monoclonal antibodies may have to be prepared.

Immunohistological localization of immunogen in fleshfly flight muscle

The adsorbed antiserum that was judged monospecific by rocket immunoelectrophoresis was used for immunohistological localization of GS in fleshfly flight muscle.

Flight muscle from the fleshfly *P. crassipalpis* was fixed in 8% *p*-formaldehyde for immunohistological localization of immunogen. The ultrastructure of *p*-formaldehyde-fixed *P. crassipalpis* flight muscle closely resembled that of the fleshfly *Sarcophaga bullata* (Neal, 1975), the fruitfly *Drosophila melanogaster* (Shafiq, 1964) and the fleshfly *P. crassipalpis* (present study) fixed in glutaraldehyde and OsO₄. In all of these dipteran flight muscles, the mitochondria appeared as large, dark irregular shaped organelles packed between the myofibrils. The cytoplasmic volume was low by comparison with the mitochondria and myofibrils. Tracheae were associated with the invaginations of the plasma membrane and tracheoles observed embedded in the muscle cell (see Shafiq, 1964). Sarcoplasmic reticulum was practically absent, but was evident around the tracheoles. Neuromuscular junctions have been reported to be present deep within the invaginations of the plasma membrane in both *S. bullata* (Neal, 1975) and *D. melanogaster* (Shafiq, 1964). However, in *P. crassipalpis*, neuromuscular junctions could not be identified with any certainty in either the *p*-formaldehyde or the glutaraldehyde/OsO₄ fixed tissue. This may be attributable to the general difficulty of fixing insect nervous tissue (Cobb and Pentreath, 1978).

Immunospecific labelling was observed in *P. crassipalpis* flight muscle fixed in 4 and 8% *p*-formaldehyde, but not in flight muscle fixed in 2% glutaraldehyde or 2% *p*-formaldehyde containing 0.2% glutaraldehyde. The labelling pattern observed was similar in 4 and 8% *p*-formaldehyde, but 8% was used routinely since it gave superior preservation of cell structure. Apparently, glutaraldehyde-fixed flight muscle lacked immunoreactive antigens. The immunospecific labelling was observed to be most intense (as particles/unit area) in the mitochondria and to a lesser but similar extent in the myofibrils and cytoplasm (Fig. 4a). On average, about 42% of the total immunogold was associated with mitochondria, 38% with the myofibrils and 20% with the soluble cytoplasm. Very little labelling was observed over the tracheae, tracheoles or in the invaginations of the

plasma membrane. By comparison, sections incubated with control serum contained very few gold particles, which were distributed at random (Fig. 4b).

The localization of immunogen in fleshfly flight muscle mitochondria and myofibrils was surprising in view of the previously reported subcellular localization of GS in the cytoplasm (Donnellan *et al.*, 1974; Dowton and Kennedy, 1986). In order to further investigate this, the distribution of GS in gently disrupted (collagenase digested) muscle tissue was studied using Percoll density gradient fractionation.

Distribution of GS in collagenase digested flight muscle

Figure 5a shows the distribution of GS in collagenase digested fleshfly flight muscle after Percoll density gradient fractionation. The muscle cell cytosolic marker arginine kinase was distributed evenly throughout the gradient as is expected for a soluble component in a gradient formed during centrifugation, together with some of the GS. The proportion of GS in these soluble fractions was greater than that of the mitochondrial marker fumarase. Both fumarase and GS were found to be concentrated in bands 2 and 3, although a higher proportion of GS was present in band 2. More complete separation of bands 2 and 3 revealed that GS was relatively equally distributed between the two bands (Fig. 5b), whereas the mitochondrial marker was found primarily in band 3.

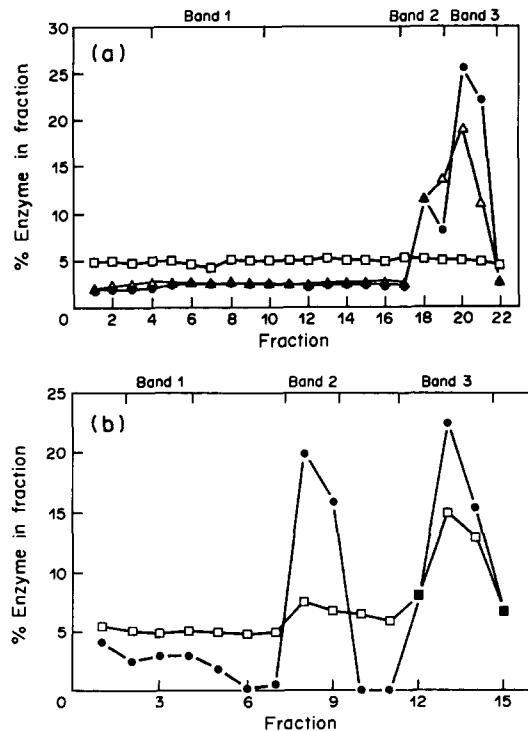


Fig. 5. Enzyme distribution profile after collagenase digestion of flight muscle and Percoll density gradient fractionation. (a) Using 30% w/v Percoll; □, arginine kinase, the muscle cytosol marker; ●, fumarase, the mitochondrial marker; △, GS, measured by the transferase assay. (b) Using 37% w/v Percoll; □, glutamine transaminase, an enzyme found in both the mitochondria and the cytosol (Dowton and Kennedy, 1986); ●, GS, measured by the transferase assay.

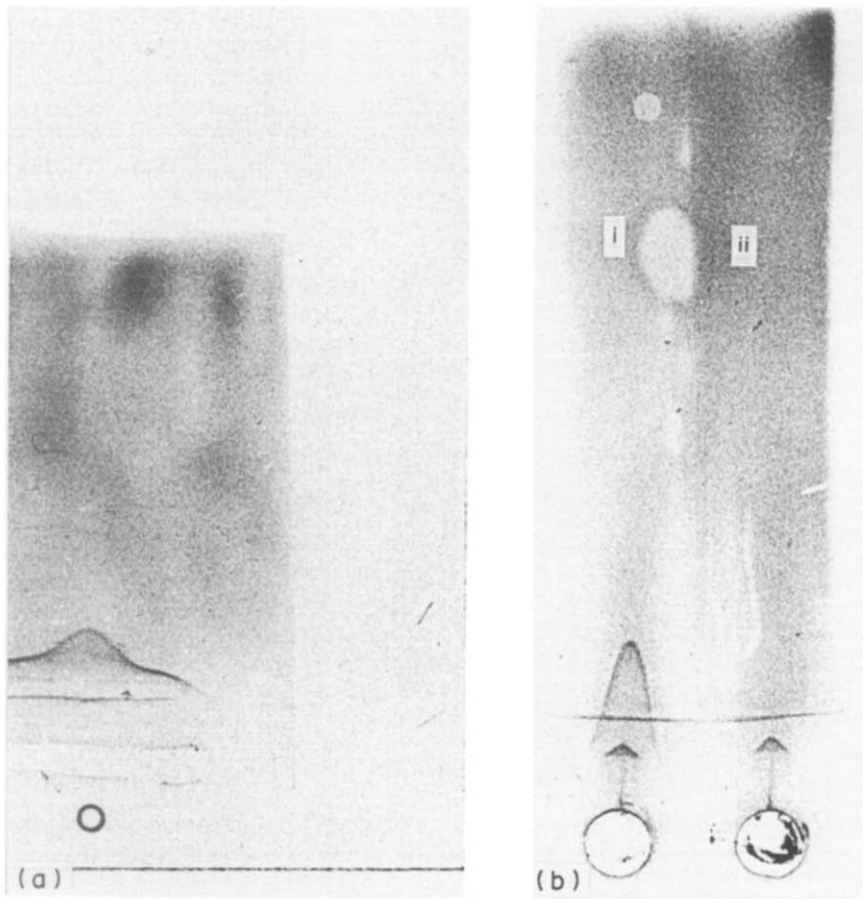


Fig. 2. (a) Rocket-line immunoelectrophoresis of crude flight muscle extract (in line) and 5 μ l of fraction 51 (in well) against the pooled antiserum from bleeds 2-5 (40 μ l/ml of agarose gel). (b) Rocket immunoelectrophoresis of 10 μ l of crude flight muscle extract against (i) pooled antiserum from bleeds 2-5, and (ii) antibodies that did not bind to the non-GS immunogen/Affi-Gel 10 matrix (both at 20 μ l/ml of agarose gel).

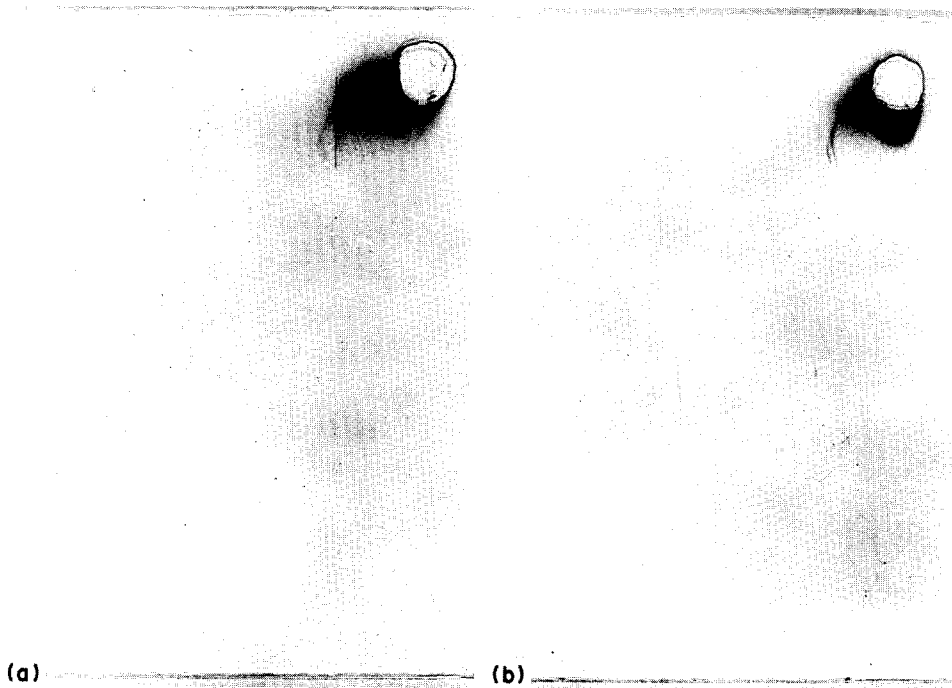


Fig. 3. Crossed immunoelectrophoresis of 25 μ l of crude flight muscle extract against the antibodies that did not bind to the non-GS immunogen/Affi-Gel 10 matrix (12 μ l/ml of agarose gel). Electrophoresed in the first dimension at 280 V \times 1.5 h. The buffer used in (a) was Tris/barbital buffer, and in (b) was Tris/glycine containing the GS-stabilizing reagents 2-mercaptoethanol (10 mM) and MnCl_2 (2.5 mM).

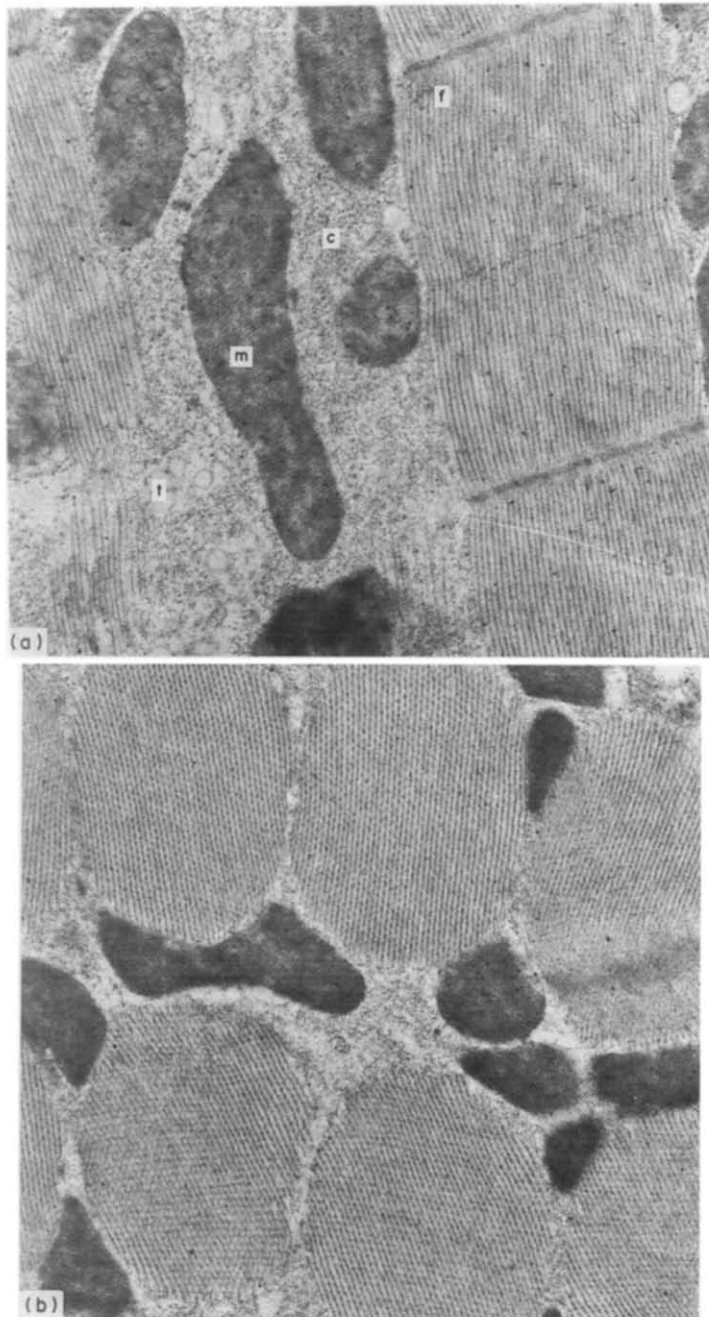


Fig. 4. (a) Immunospecific labelling of flight muscle (fixed in 8% *p*-formaldehyde and embedded in Lowicryl K4M) using antibodies that did not bind to the non-GS immunogen/Affi-Gel 10 matrix (diluted 1:25); c, cytosol; f, myofibril; m, mitochondrion; t, tracheole. Magnification $\times 8893$. (b) Background gold labelling of flight muscle sections incubated in pre-immune (control) serum (diluted 1:25) and processed as described in the Materials and Methods section. Magnification $\times 8893$.

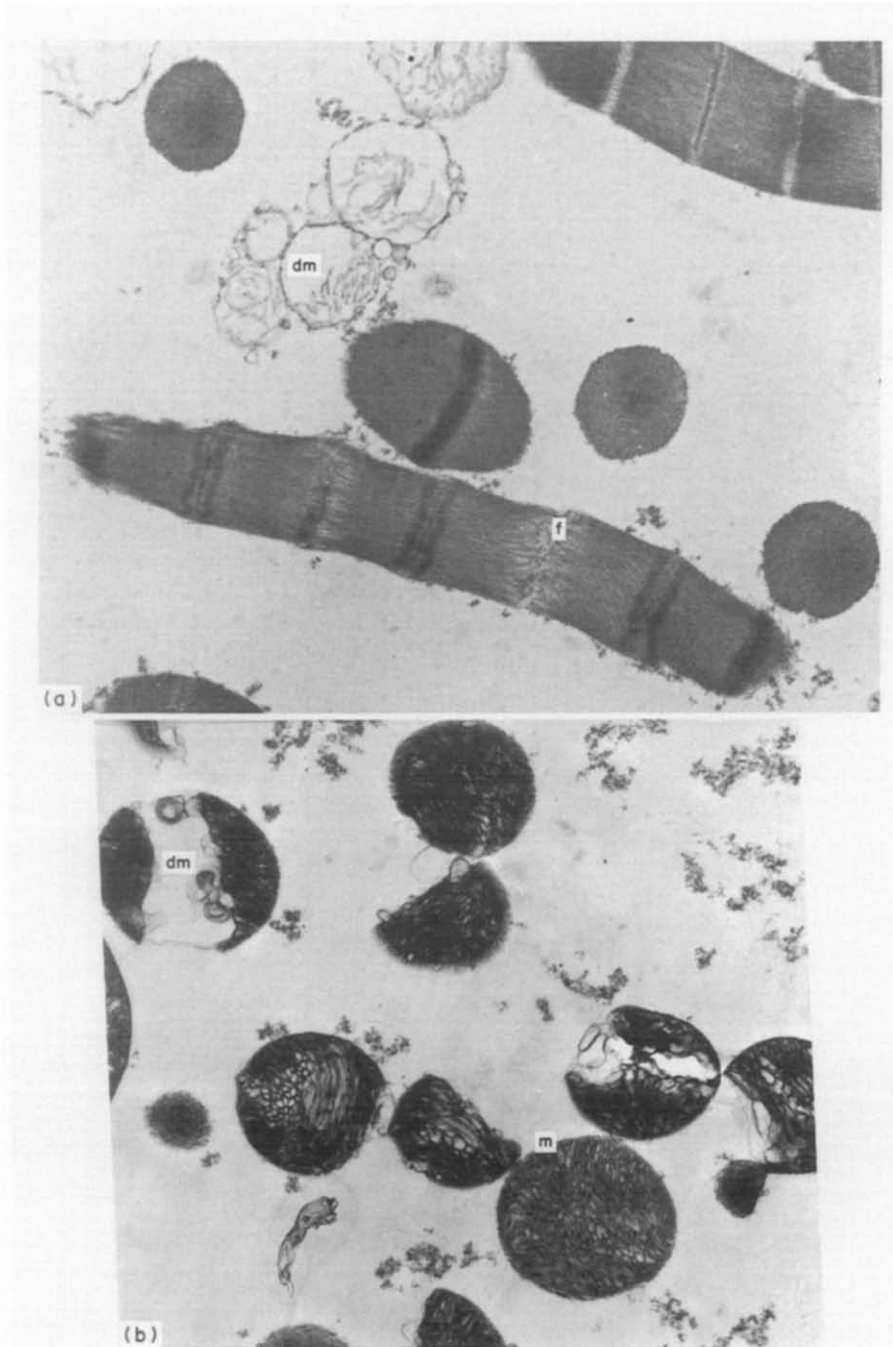


Fig. 6. Electron micrographs of pelleted material from (a) band 2 and (b) band 3. Pellets were fixed in 2% glutaraldehyde and embedded in Spurr's resin. dm, damaged mitochondrion; f, myofibril; m, mitochondrion. Magnification $\times 6104$.

The muscle cell compartments present in the various bands were subsequently examined by electron microscopy (Fig. 6). Band 1 contained a high proportion of membrane vesicles with no internal structure. These were probably vesicles of sarcoplasmic reticulum and vesicles of other muscle or nerve cell membranes. Band 2 was comprised primarily of myofibrils, although some damaged mitochondria were also observed. As was expected from the distribution of the mitochondrial marker enzyme, band 3 was comprised primarily of mitochondria. However, many appeared to have been damaged by either the incubation conditions or preparation of the material for electron microscopy.

The results of the subcellular distribution investigation correlated well with the immunohistological results. GS was localized primarily in the mitochondria and to a lesser extent in the myofibrils and cytoplasm in both studies.

DISCUSSION

The distribution of GS in fleshly flight muscle reported in the present investigation was unexpected given the previously reported cytoplasmic localization of this enzyme (Donnellan *et al.*, 1974; Downton and Kennedy, 1986). Although the purified antiserum was possibly not completely monospecific, the antiserum used did contain specific antibodies to GS as indicated by its inhibition of the GS synthetase reaction and the demonstration of GS activity in the immunoprecipitate. Furthermore, it gave a single immunoprecipitate on rocket immunoelectrophoresis. Thus the observed immunohistological labelling should indicate the distribution of GS. The close similarity between the immunohistological labelling and the distribution of GS in gently disrupted (collagenase digested) flight muscle provides independent evidence that GS is located in the mitochondria, as well as in the myofibrils and the cytoplasm. This similarity also supports our assumption that the antiserum was effectively monospecific for GS.

A possible explanation for the previously observed distribution of fleshly flight muscle GS exclusively in the cytoplasm is that myofibrillar GS is easily solubilized by mechanical disruption of the tissue. This is not surprising considering that the myofibrils have no limiting membrane. Alternatively, GS may partition between the cytoplasm and the myofibrils *in vitro*, such that the observed localization depends on the medium in which the tissue is fractionated. In addition, in both of the previous studies, GS was found partly associated with the membranous fractions. This association may correspond to the mitochondrial GS found in the present study. In fact, if the data for the distribution of GS and marker enzymes from the study of Donnellan *et al.* (1974) are plotted (Fig. 7), their results are also consistent with both a mitochondrial and cytosolic localization of GS.

It is difficult to speculate on the participation of fleshly flight muscle GS in neurotransmitter metabolism due to the lack of ultrastructural definition of the neuromuscular junction in the present study. From reports in the literature on the ultrastructure of

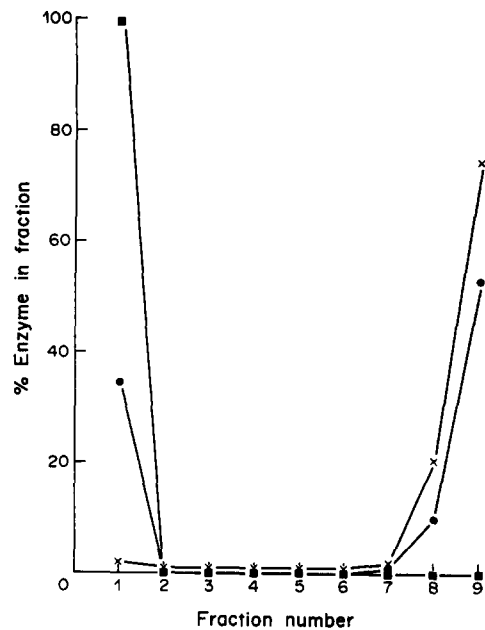


Fig. 7. Enzyme distribution profile of fleshly (*S. barbata*) flight muscle homogenates after sucrose density gradient fractionation, the data from Donnellan *et al.* (1974); ■, GPDH, a cytosol marker; ×, GDH, a mitochondrial marker; ●, GS, measured by the hydroxylamine-substituted synthetase reaction (Donnellan *et al.*, 1974).

other closely-related dipteran flight muscles, it is expected that the neuromuscular junction of *P. crassipalpis* lies deep within invaginations of the plasma membrane, with no glial sheath. At these types of junctions, inactivation of neurotransmitter glutamate must involve a mechanism other than that of active uptake into glia. Perhaps uptake of glutamate by muscle cells (i.e. internalization), such as that observed by van Marle *et al.* (1985), contributes to neurotransmitter inactivation at such junctions. Biochemical evidence of such an uptake of glutamate by insect muscle cell membranes was reported by Briley *et al.* (1982). These investigators isolated a fraction from locust thoracic muscle with the characteristics of plasma membrane and associated sarcoplasmic reticulum. The fraction showed a specific and high affinity binding and uptake of glutamate. If the muscle cell membrane is involved in neurotransmitter uptake, myofibrillar and cytoplasmic GS may be involved in the metabolism of this neurotransmitter.

Mitochondrial GS is unlikely to be involved in neurotransmitter metabolism due to the relatively impermeable nature of this organelle to glutamate in diptera (Van den Bergh, 1964). Instead, the NH_4^+ -scavenging property of fleshly flight muscle GS (Downton and Kennedy, 1985) may be involved in the detoxification of NH_4^+ in mitochondria. In many diptera, proline is utilized as a source of energy at the onset of flight (for a recent review, see Beenackers *et al.*, 1985). Proline is first converted to glutamate and then to 2-oxoglutarate by glutamate dehydrogenase (EC 1.4.1.3) or glutamate:pyruvate transaminase (EC 2.6.1.2). Glutamate dehydrogenase activity produces NH_4^+ which would disrupt the proton gradient between the mitochondrial matrix and the inter-

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