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High affinity uptake of L-glutamate and γ-aminobutyric acid in *Drosophila melanogaster*

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Abstract. Preparations having properties resembling those of synaptosomes have been isolated from whole fly homogenates of *Drosophila melanogaster* using ficoll gradient floatation technique. These have been characterized by marker enzymes and electron microscopy and binding of muscarinic antagenist ^{3}H Quinuclidinyl benzilate. An uptake system for neurotransmitter, ã-Aminobutyric acid has been demonstrated in these preparations.

A high affinity uptake system for L-glutamate has also been studied in these subcellular fractions. This uptake of glutamate is transport into an osmotically sensitive compartment and not due to binding of glutamate to membrane components. The transport of glutamate has an obligatory requirements for either sodium or potassium ions. Kinetic experiments show that two transport systems, with K_m values $0{\cdot}33\times10^{-6}$M and $2{\cdot}0\times10^{-6}$M, respectively, function in the accumulation of glutamate. ATP stimulates lower affinity transport of glutamate. Inhibition of glutamate uptake by L-aspartate but not by phenylalanine and tyrosine indicates that a common carrier mediates the transport of both glutamate and aspartate. *β*-N-oxalyl-L-β *β*-diamino propionic acid and kainic acid, both inhibitors of glutamate transport in mammalian brain preparations, strongly inhibited transport of glutamate in *Drosophila* preparations

Comparison with uptake of ã-aminobutyric acid and glutamate in isolated larval brain is presented to show that the synaptosome-like preparations we have isolated are rich in central nervous system derived structures, and presynaptic endings from neuromuscular junctions.

Keywords. *Drosophila melanogaster,* γ-aminobutyric acid; glutamate; neurotransmitter uptake.

Introduction

Synaptosomes provide a useful system for the study of physiological processes underlying synaptic transmission (Wheeler, 1978). Since the first report of their characterization (Gray and Whittaker, 1962), they have been extensively employed to analyze diverse aspects of nerve cell structure and function. They have been used to study synaptic morphology (Whittaker, 1968; Korngluth *et al.,* 1979) and synaptic membrane components (Yoshida and Imura, 1979; Gilbert and Wythe, 1979) as also metabolism of nervous tissue (Bradford, 1970; Booth and Clark, 1979) and neuro-transmitter release (Whittaker *et al.,* 1972, Roberts *et al.,* 1979). Synaptosomal preparations have been employed with profit to identify uptake processes for

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Abbreviations used: AchE, Acetylcholinesterase; GABA, ã-aminobutyric acid; QNB, quinuclidinyl benzilate; ODAP, *β*-N-oxalyl-L-α, *β*-diamino propionic acid; CS, Canton special; SDH, succinate dehydrogenase.

putative neurotransmitters (Wheeler, 1979) and to unravel mechanism of action of certain neurotoxins (Tan and Abkowitz, 1979; Ramos *et al.,* 1979).

In a large majority of the above studies, synaptosomes have been isolated from mammalian nervous tissue. We have been attempting to use *Drosophila melanogaster* as a system to understand synaptic function. Behavioural genetic approaches have been very effective in identifying several locii in *Drosophila* genome which are likely to affect the function of the nervous system. Several of the behavioural mutants of *Drosophila* could have impaired synaptic transmission (Hall *et al.,* 1982). We have earlier been able to use a class of mutants to study the nature of the *Drosophila* acetylcholinesterase (AchE) (Zingde *et al.,* 1983). Neurochemical investigations *in vitro* of such mutants could lead to a clearer understanding of molecular events underlying synaptic function. It was therefore important to make synaptosomal preparations from *Drosophila* tissue. However, the conditions established for mammalian systems are not necessarily suitable for insect systems (Donnellan *et al.* 1976; Breer and Jeserich, 1980). To our knowledge, so far there have been only two reports of isolation of synaptosomes from insect nervous system. Donnellan *et al.* (1976) used separated total heads of house flees to isolate synaptosomes on discontinuous ficoll density gradients. Using dissected head and thoracic ganglia of *Locusta migratoria,* Breer and Jeserich (1980) have developed a procedure for microscale isolation of synaptosomes on a ficoll gradient. These procedures *per se* could not be adapted for *Drosophila* as large scale separation of heads in *Drosophila* requires drastic treatment such as freezing in liquid nitrogen which may destroy fragile synaptosomal structures.

There is increasing evidence that glutamate may function as a major excitatory neurotransmitter in vertebrate central nervous system and insect neuromuscular junctions (Johnson, 1978; Jan and Jan, 1979; Davies and Watkins, 1979; Puil, 1981). Presence of selective high affinity uptake system for glutamate in the synaptosomes currently constitutes strong evidence for its neurotransmitter role in the mammalian brain (Logan and Snyder, 1971; Bennett *et al.,* 1973; Biziere *et al.,* 1980). There is overwhelming electro-physiological evidence for the neurotransmitter role of glutamate in the invertebrate neuromuscular junction (Takeuchi and Takeuchi, 1964; Taraskevich, 1975; Usherwood, 1972). Evidence has been presented for the neurotransmitter role of glutamate in *Drosophila* larval neuromuscular junction (Jan and Jan, 1976a,b). However, selective high affinity uptake system for glutamate has not so far been reported in insect nervous system.

We have attempted to isolate synaptosome like structures from *D. melanogaster* whole flees using ficoll floatation technique which is an adaptation of the method of Breer and Jeserich (1980). In this paper, we describe the characterization and properties of such a preparation from *Drosophila.* We show that this preparation is associated with an uptake system for ã -aminobutyric acid (GABA), which is well established as an inhibitory neurotransmitter in the insect central nervous system (Pichon, 1974) and describe the properties of a sodium dependant, ATP stimulated high-affinity uptake system for glutamate.

Preliminary results of uptake studies with larval brains also show fast/high affinity uptake for glutamate and GABA.

Experimental procedures

Materials

U-[^{14}C]-GABA (224 mCi/mmol), [^{3}H]-glutamic acid (43 Ci/m mol) and [^{3}H]-quinuclidinyl benzilate (QNB) (44 Ci/m mol) were obtained from Amersham, UK. U-[^{14}C]-glutamic acid (130mCi/m mol) was purchased from Bhabha Atomic Research Centre, Bombay. Ficoll and kainic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. β-N-oxalyl-L-α, β-diamino propionic acid (ODAP) was a gift from Prof. G. Padmanaban of Indian Institute of Science, Bangalore. All other chemicals were of the best analytical grade available.

Preparation of synaptosomal fractions

The canton special (CS) strain of *D. melanogaster* was used in all experiments. Flies were collected within two days of emergence and immediately chilled in ice. All operations were carried out in cold. The flies were homogenized in ice-cold 20 mM Tris buffer, pH 7·4 containing 0·32 M sucrose (extraction buffer), in a Braun homogenizer at a speed of 1000 rpm, using 6 strokes. The homogenate was filtered through a nylon net and centrifuged at 1100 *g* for 10 min in a sorvall RC5 centrifuge using SM24 rotor to remove the nuclear debris. The supernatant was gently layered over a cushion of 46 % sucrose and centrifuged at 16,000 *g* for 30 min in a HB4 swing-out rotor to obtain the mitochondrial pellet. The band above 46% sucrose which we call mitochondrial fraction was collected with a syringe and gently but thoroughly mixed with an equal volume of 24% ficoll (w/v) in 0·32 M sucrose Tris pH 7·4 and centrifuged thereafter for 90 min at 10,000 *g*. In addition to a pellet at the bottom of the tube, a pellicle was formed at the top of the gradient. The pellicle was collected with a syringe and centrifuged in 10 volumes of extraction buffer at 12,500 *g* for 20 min. The pellet thus obtained is relatively enriched in synaptosome like structures and is made into a suspension using 0·5 ml of incubation buffer (see below) per g of original tissue. This suspension (pellicle fraction) was used for further studies.

Uptake studies

The uptake of [^{14}C]-labelled GABA was measured by incubating the pellicle fraction in a modified Krebs-Henseleit solution (incubation buffer). All incubations were carriedout at 35°C. The modified Krebs-Hanseleit solution consisted of 127·2 mM NaCl, 5·0 mM KCl, 2·7 mM $CaCl_2$, 1·3 mM $MgSO_4$, 25 mM Tris-chloride (pH 7·4) and 11·1 mM glucose (Wheeler, 1978). The final concentration of GABA used was 5×10^{-6}M inclusive of radioactive GABA (0·25 μCi/ml) and that of glutamate was 1×10^{-6}M (0·125 μCi/ml reaction mixture). Incubation was started by adding 200 μl of pellicle fraction to 800 μl of incubation medium. At appropriate time intervals, 200 μl of the reaction mixture was withdrawn and filtered through Schleiser and Schull BA85 filters with a pore size of 0·45 μM, using a Hoeffer filtration manifold. The filters were washed twice with 5 ml of the incubation medium without radioactivity, left at room temperature, dried under an infra-red lamp and counted in a liquid scintillation counter after addition of 8 ml of toluene scintillation fluid. For

uptake studies with larval brains, 3rd instar larvae from a synchronous population were taken and the brains dissected out in the modified Krebs'-ringer solution and 5 brains were used for each sample. Final concentration of GABA used was ~ 5 μM and of glutamate ~50 nM. Incubations were done at room temperature (~26°C) for upto 50 min. The brains were collected after incubation by filtering through glass fibre filter paper (0·45 μ), washed and counted as done for synaptosomes. [^{3}H]-QNB binding by various fractions of *Drosophila* preparations was assayed by incubating 50 μl sample with H^3-QNB at a final concentration of ~3·8nM. Filtration assay was carried out as usual.

Electron microscopy

The sample was fixed in ice-cold 2·5% glutaraldehyde in 0·05M cacodylate buffer (pH 7·2) for 2 h. The fixed material was sedimented by centrifugation, post fixed in 1% osmium tetroxide for 2 h in the same buffer, dehydrated in graded ethanol and propylene oxide and finally embedded in Araldite. Ultra thin sections (60-90) nm) were stained with uranyl acetate and then lead citrate prior to examination in a Jeol electron microscope.

Enzyme assays

AchE (EC 3·1·1·17) was estimated by the procedure of Ellman *et al.* (1961). Succinate dehydrogenase (SDH, EC 1·3·99·1) was assayed according to procedure of King (1967). Protein was estimated by Hartree's method (1972).

Results

The fractionation procedure carried out using 12% ficoll resulted in 3 distinct fractions. The distribution of marker enzymes in each of the fractions is presented in figure 1. It would appear that pellet I, pellet II and pellicle correspond to mitochondria, broken membrane fragments and sealed membrane structures respectively. Pellet I has maximal activity of SDH and relatively less activity of AchE, while reverse is the case for pellet II fraction. Both the enzymes are enriched in the pellicle fraction. Table 1 shows binding of QNB to various fractions indicated, and shows that the synaptosome like fraction (pellicle fraction) has a substantial enrichment of binding in relation to the homogenate.

Many cholinergic macromolecules are found exclusively in the central nervous system of *Drosophila.* It is hence likely that muscarinic receptor seen by QNB binding is also a marker for CNS derived structures. Our results presented in table 1 and the presence of uptake system for GABA and glutamate in larval brains (table 4), suggests that a substantial fraction of our *in vitro* preparation contain endings from the central nervous system of *Drosophila.*

Ultrastructurally, the pellicle fraction contained different populations of membrane bound structures. Large featureless membrane sacs were seen as well as organised profiles containing vesicles (figure 2). Some contained clear vesicles while others had both clear and dense vesicles. It appears that a heterogeneous population

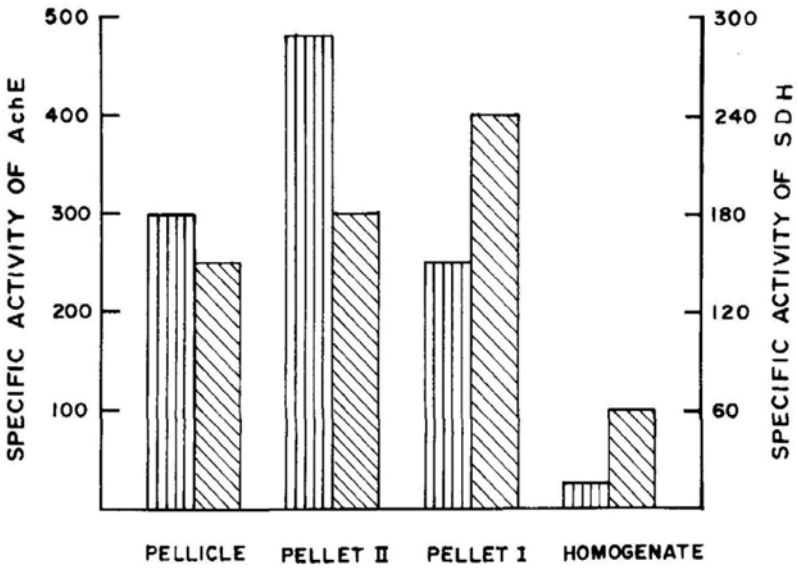

Figure 1. Enzyme activity profiles of final fractions. Vertical striped AchE and cross hatched, SDH. Activities are expressed in units of μ mol acetyl thiocholine hydrolysed/g/min or μ mol succinate oxidised/g/min.

The pellicle activities were measured in pellicles washed in isotonic buffer and resuspended in homogenesing buffer. Pellet I is the pellet at the bottom of the ficoll gradient.Pellet II is the homogeneously distributed particulate material in the ficoll gradient. After recovering the pellicle and pellet I fractions, the body of ficoll gradient was diluted 20fold by isotonic buffer and spun at 20,000 *g* for 45 min and a pellet of membraneous material that was obtained is called pellet II.

Table 1. Typical profile of QNB binding to different fractions of the *Drosophila* preparation.

Fraction	QNB-binding in fmol/mg protein
Homogenate	9·7
Post–3000 rpm supernatant	19·1
Synaptosome-like fraction (pellicle fraction)	36·2

containing a substantial number of nerve endings were collected in the pellicle. This was to be expected as whole flies were used for fractionation.

In view of the fact that GABA is well recognised as an inhibitory neurotransmitter in the insect central nervous system we looked for GABAergic uptake by the pellicle fraction which appeared to be relatively enriched in synaptosomes. When the pellicle fraction was incubated with [^{14}C]-GABA for various time intervals, there was a significant uptake of GABA which was linear for about twenty minutes (figure 3). The linear dependence of this uptake on protein concentration is shown in figure 4.

Table 2 shows that GABA uptake is dependent upon the simultaneous presence of external sodium and chloride ions. Replacement of chloride by acetate ions and

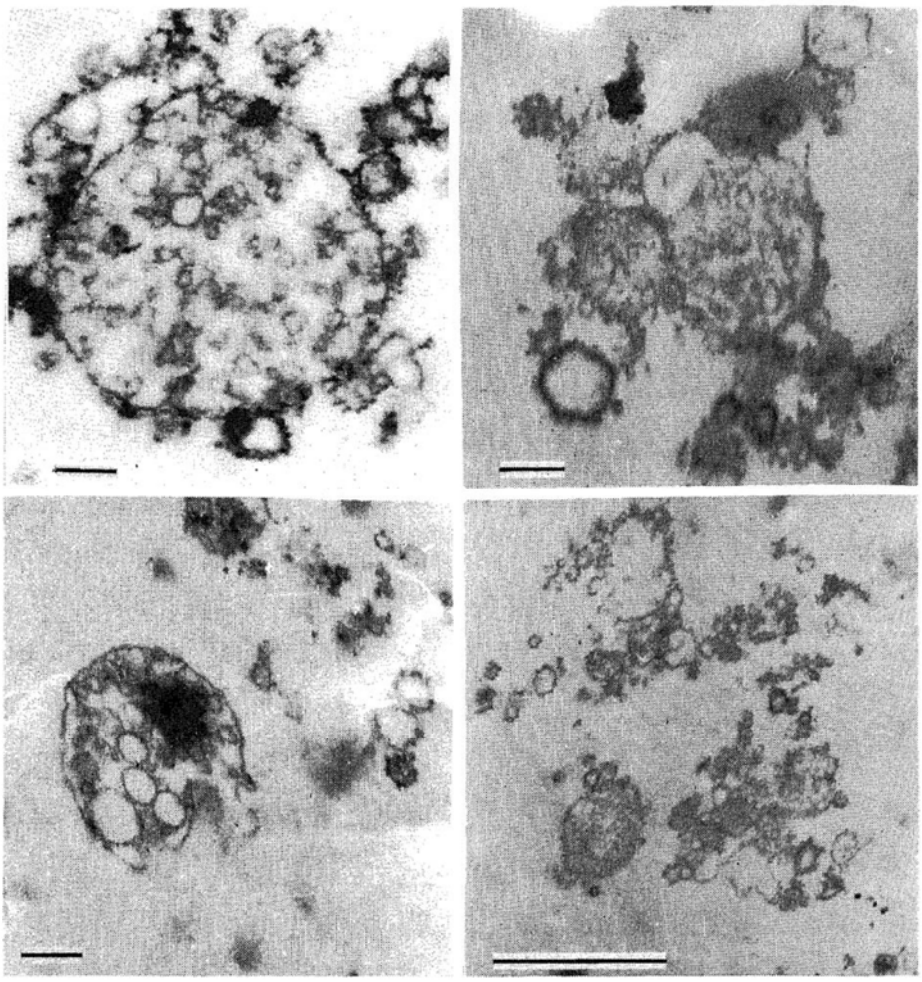

Figure 2. Montage of electron micrographs of the pellicle fraction obtained by floatation over 12% ficoll. Bars represent 0·25 μm.

replacement of sodium by Tris ions resulted in considerable decrease in the uptake of GABA. It may however be noted that in these experiments 25 mM chloride was already present as part of the incubation buffer and this may be one of the reasons why replacement of chloride by acetate ions did not result in greater decrease of GABA uptake.

The data presented in figure 5 illustrate the time course of [^{14}C]-glutamate uptake by a subcellular fraction (pellicle fraction) isolated from whole flies of *D. melanogaster*. The uptake was linear with time usually upto 8-10 min after which there was a sharp decline. All uptake experiments were therefore carried out for times of 8 min or less to avoid the declining phase of the uptake. To ascertain the proportion of radio-activity retained by non-specific absorption on filter papers and binding to membrane structures, the incubated fraction was osmotically shocked, filtered and

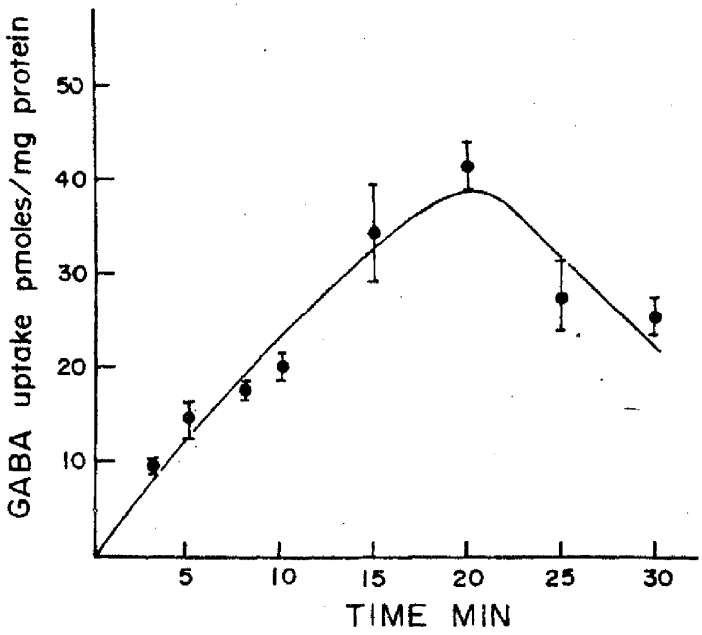

Figure 3. Time course of GABA uptake. GABA at a concentration of 5×10^{-6} M inclusive of [^{3}H]-GABA 0·25 μCi/ml was used. Incubations were started by the addition of 200 μl of synaptosomal preparation to 800 μl of the incubation medium. Aliquots of 200 μl were withdrawn at appropriate intervals, filtered and counted.

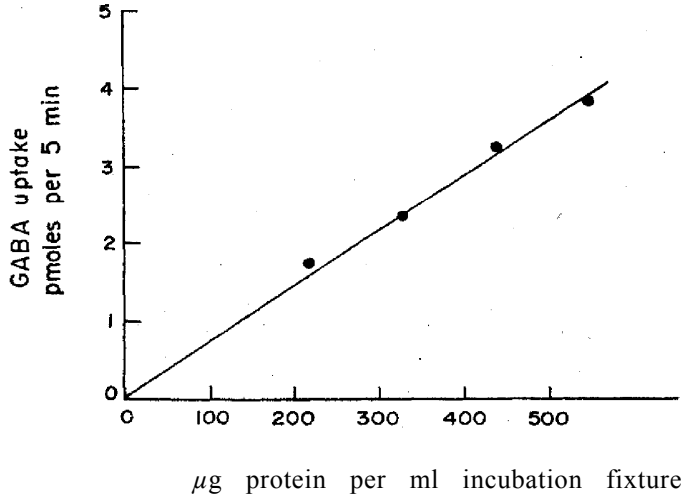

Figure 4. Dependence of GABA uptake on protein concentration. Uptake is calculated from the differences between counts retained after 0 min and 5 min incubations. GABA at a final concentration of 5×10^{-6}M and 0·25 μCi/ml was used in the incubation.

Table 2. Dependence of GABA uptake on sodium and chloride ions.

Incubation medium	GABA uptake (pmol/mg protein) for 8 min		
	Experiment 1	Experiment 2	Experiment 3
Standard incubation medium containing 127·2 mM sodium chloride	6·90	7·40	8·33
Standard incubation medium in which NaCl replaced by sodium acetate	3·51	4·16	3·79
Standard incubation medium in which NaCl replaced by Tris-Cl	5·18	5·74	5·64

Incubation to study GABA uptake were carried out as described in eperimental procedures'. 1·2 mg of pellicle protein was incubated with 5×10^{-6}M GABA. The uptake is calculated from the difference in cpm retained in filter papers of samples at 8 min and 0 min

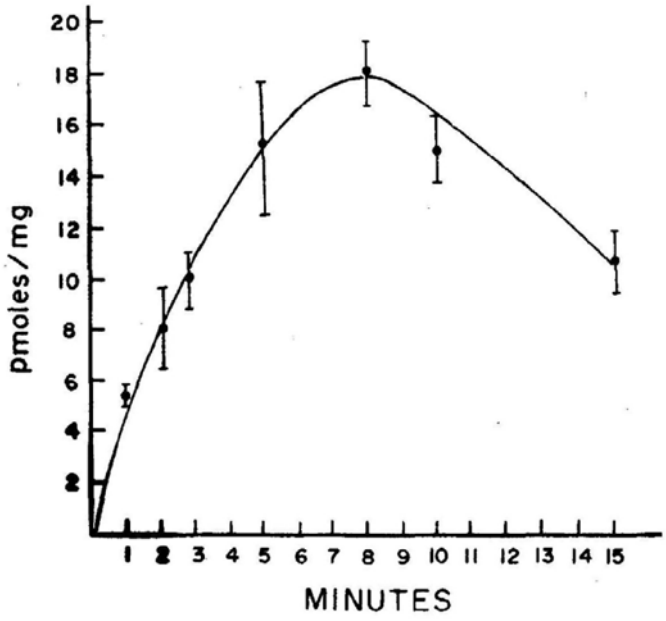

Figure 5. Time course of [^{14} C]-L-glutamic acid uptake by *Drosophila* pellicle fraction. The incubation mixture contained 1 mg protein of the preparation, 10^{-6}M L-glutamic acid inclusive of [^{14}C]-glutamic acid (0·125 μCi) in one ml of Krebs-Henseleit solution [127·2mM NaCl, 5·0 mM KCl, 2·7 mM $CaCl_2$, 1·3 mM $MgSO_4$ 25 mM Tris-chloride buffer (pH 7·4) and 11·1 mM glucose]. Incubations were started by the addition of the pellicle fraction to the rest of the incubation mixture and aliquots withdrawn, filtered, washed and counted. Values are corrected for zero time background.

counted. The counts thus retained were equal to those in the blank, showing that the radioactivity was taken up in an osmotically sensitive compartment (figure 6). Zero time values after the addition of pellicle fraction were essentially the same as the blanks where no protein was added.

Figure 7 shows the dependence of glufamate on Na^+ concentration. Two curves, showing glutamate uptake for 3 min (figure 7a) and 5 min (figure 7b) respectively are shown. It is clear that uptake of glutamate in this fraction is absolutely dependent on Na^+ concentration and that sucrose cannot replace Na^+. In another experiment Na^+ concentration was varied and osmolarity difference made up either with sucrose (figure 8a) or with Tris-chloride (figure 8b). It is seen from figure 8 that maximum uptake of glutamate is obtained when the Na^+ concentration is around 100 mM. Experiment described in figure 8 also showed that chloride had no effect on glutamate uptake as Tris-chloride was ineffective. Sodium acetate and potassium chloride were also as effective as sodium chloride in stimulating the uptake.

Figure 9 shows the dependence of glutamate uptake on protein concentration in the incubation mixture under the conditions of our experiments. The uptake of glutamate was maximal at a protein concentration of 0·8 mg/ml. However the usual concentration of protein employed in other experiments was of the order of 0·3-0·4 mg/ml.

Figure 10a shows the effect of glutamate concentration on its uptake by the pellicle fraction. Two uptake systems are evident. One with high affinity has a K_m of

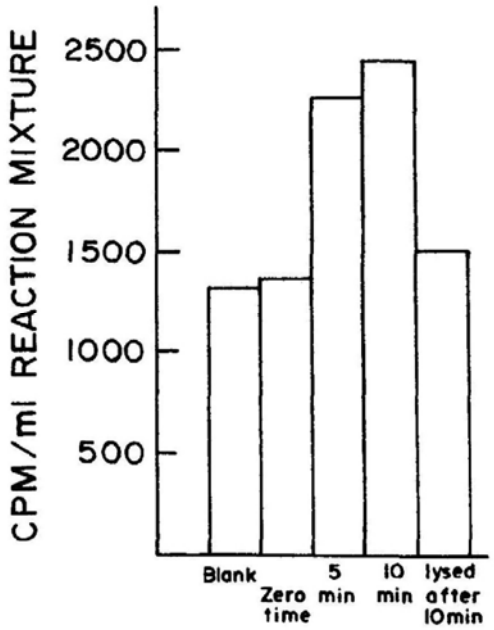

Figure 6. Retention of radioactivity (glutamate) on filter papers. Comparison of blank, zero time and lysed samples. Lysed samples are 10 min samples to which a 20 fold volume of ice cold water was added and after a short vigorous shaking the samples were filtered. In some cases lysis was done by washing filtered pellicle fraction with ice cold water in place of isotomic buffer.

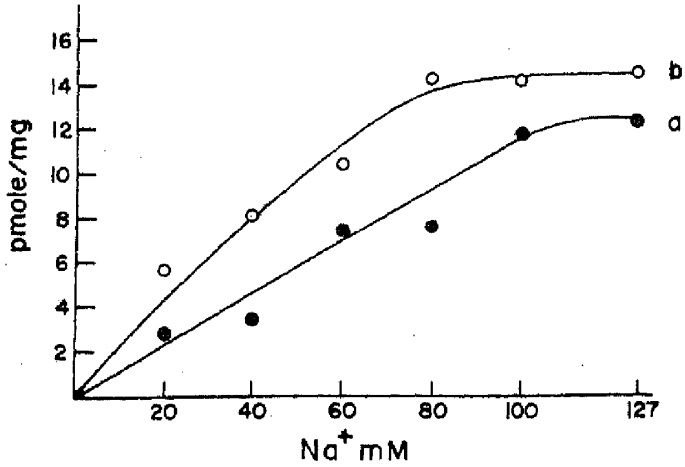

Figure 7. Uptake in the presence of increasing concentration of NaCl. In the Krebs-Henseleit solution, NaCl was replaced by equiosmolar sucrose to obtain lower NaCl concentrations. (a), uptake for 3 min (b), uptake for 5 min. Zero time blanks were subtracted in each case.

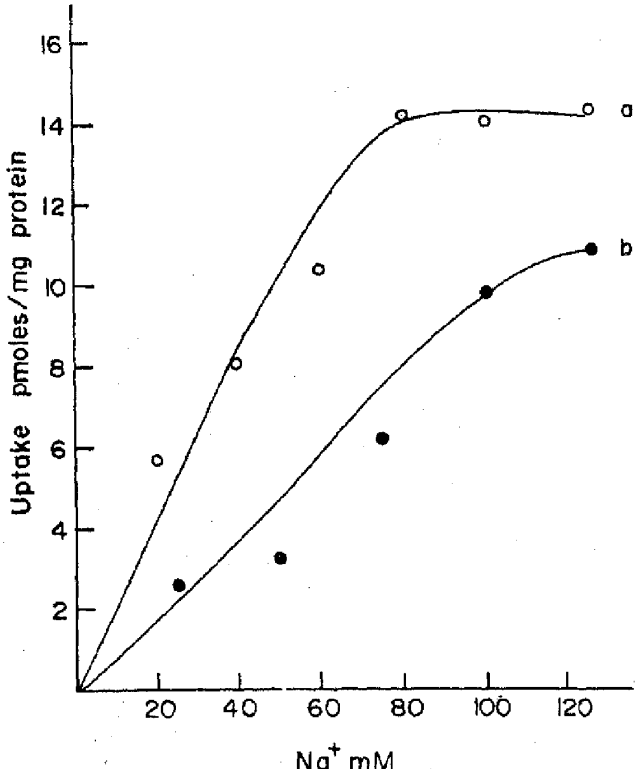

Figure 8. Uptake in the presence of increasing concentration of NaCl. Uptake is expressed as p mol/mg protein/5 min. Zero time blanks were corrected for in each case. (a), NaCl replaced by sucrose (b), NaCl replaced by Tris-chloride. In experiments where KCl or Na-acetate were used, in place of NaCl, the results were identical.

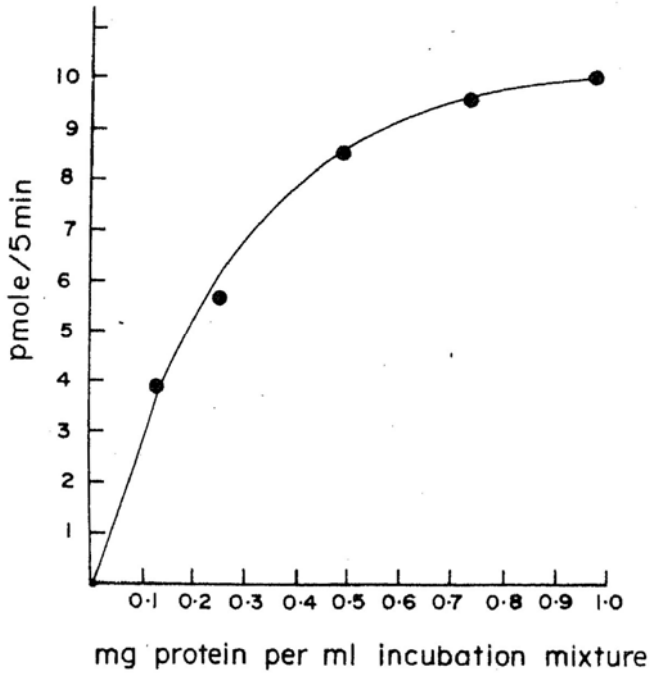

Figure 9. Dependence of [^{14}C]-L-glutamic acid uptake on protein concentration. [^{14}C]-glutamic acid at 10^{-6}M (0·125 μCi/ml) was incubated with varying amounts of the pellicle fraction. The uptake for 5 min was calculated from the difference in cpm between zero time and 5 min samples for each protein concentration.

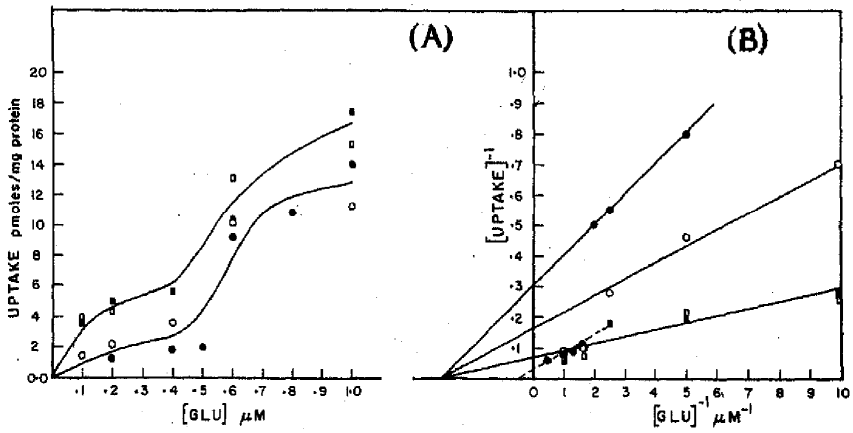

Figure 10. A. Dependence of uptake on glutamate concentration. L-Glutamate concentration varied from 10^{-7} M to 1×10^{-6} M in the Krebs-Henseleit medium. The radioactivity was also varied from 0·0125 μCi/ml to 0·125 μCi/ml. When higher concentrations of glutamate were used, the radioactivity was retained at 0·125 μCi/ml. Circles represent uptake for 3 min and squares represent uptake for 5 min. The values are for different preparations. B. Double reciprocal plot of the uptake data. The two foci correspond to K_m :0·33 μM and 2 μM.

$0{\cdot}33 \times 10^{-6}$ M and the other one with a relatively lower affinity has a K_m of $2{\cdot}0 \times l0^{-6}$ M (figure 10b).

In mammals glutamate is taken up by the same system as transports aspartate but different from the one that takes up GABA, glycine and other amino acids (Balcar and Johnston, 1972). In such a case high concentrations of aspartate should inhibit the uptake of glutamate. To study the specificity of the glutamate uptake system in *Drosophila* preparations, phenylalanine, tryptophan and aspartic acid were added to the incubation mixture. Figure 11 shows that while phenylalanine and tryptophan did not have any effect, aspartate even at 10^{-5}M concentration inhibited the glutamate uptake completely. This indicates that glutamate and aspartate are transported by the same system as is the case with mammals (Bonnum *et al.,* 1981).

ODAP is a neurotoxin isolated from the seeds of *Lathyrus sativus* which causes neurolathyrism. ODAP was shown to be a potent inhibitor of high affinity glutamate uptake system in rat brain synaptosomes (Lakshmanan and Padmanabhan, 1974). Figure 12a shows the effect of ODAP on glutamate uptake by *Drosophila* pellicle

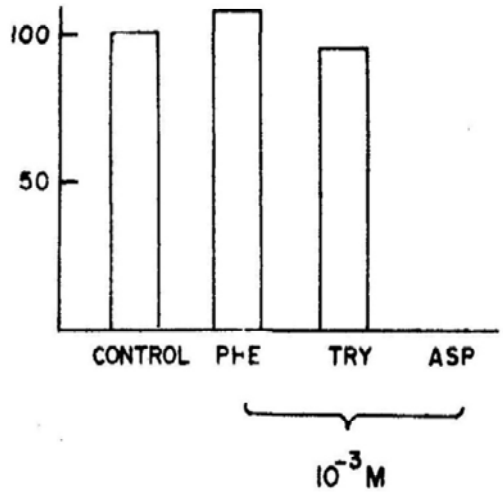

Figure 11. Inhibition of uptake by amino acids. Phenylalanine, tyrosine and aspartate were present in the respective samples at a concentration of 10^{-3} M in the Krebs-Henseleit solution. Aspartate concentration 10^{-5}M, 10^{-4}M and 10^{-3}M show a similar profile. Glutamic acid was 10^{-6}M ($0{\cdot}125\mu$Ci/ml).

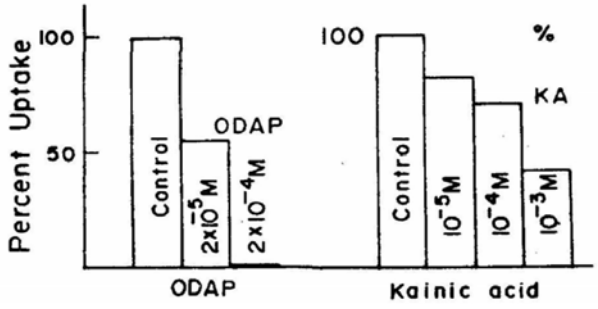

Figure 12. Effect of kainic acid and ODAP on uptake of L-glutamic acid by *Drosophila* pellicle fraction.

fraction. While 2×10^{-5} M ODAP brought about 40% inhibition of glutamate uptake, 2×10^{-4} ODAP resulted in complete inhibition of uptake. Figure 12b shows a dose dependent inhibition of glutamic acid uptake by another glutamate antagonist kainic acid (Simon *et al.*, 1976).

Table 3 shows the effect of sodium and ATP on glutamate uptake by the pellicle fraction from *Drosophila.* ATP stimulated the uptake of glutamate in the presence of Na^+ nearly two fold. The uptake of glutamate in the absence of Na^+ could also be stimulated by ATP. Thus ATP is able to at least partly restore glutamate uptake in the absence of sodium ions.

Table 4 shows results of GABA and glutamate uptake studies with isolated larval

Table 3. Role of ATP and sodium in L-glutamate uptake.

		Uptake (p moles/mg protein) in 3 min	5 min	8 min
a*	Control + Na − ATP	2·79	4·80	5·86
	+ Na + ATP 2 mM	5·30	9·40	21·70
	− Na + ATP 2 mM	2·06	3·26	4·93
	− Na − ATP	Nil	Nil	Nil
		Uptake (p moles/mg protein/5 min)		
b.+	0·2 μM Glu		2·41	
	0·2 μM Glu + ATP		2·37	
	1·0 μM Glu		3·68	
	1·0 μM Glu + ATP		15·59	
		Uptake (p moles/mg protein) in 3 min	5 min	8 min
c.*	Control	3·07	5·28	5·86
	+ ATP	6·40	7·88	16·30

Uptake studies were performed in Krebs-Hensleit medium and corrected for zero time values.
+The concentration of radioactivity was maintained at 0·025 μCi/ml at 0·2 μM Glu and 0·125 μCi/ml at 10 μM Glu.
*Glutamic acid concentrations were 10^{-6}M (0·125 μCi/ml) in Krebs-Hensleit medium. NaCl was replaced by equi-osmolar sucrose for studies in the absence of sodium.

Table 4. Typical set of GABA and glutamate uptake studies in isolated larval brains.

Time (min)	Uptake in fmoles/brain GABA	Glutamate
0	230	1·07
40	1010	9·38
40, (0°C)	440	2·35
40, 2 mM aspartate*	—	4·35
40, 1 mM picrotoxin*	1180	—

GABA final concentration was ~ 5 μM.
Glutamate final concentration was ~ 50 nM.
*Final concentration in the assay mixture.

brain. Values are expressed as uptake per larval brain. The average protein per larval brain in *D. melanogaster* is approximately 1 0 μg.

Discussion

Our early attempts to prepare synaptosomes from separated heads were not successful. The reason could be that nerve endings being fragile get disrupted and synaptosomes are not formed at all after the drastic treatment of freezing the flies in liquid nitrogen prior to separation of heads.

The procedure described above, using whole flies, yields intact, sealed membrane structures a substantial number of which are similar to synaptosomes. As with Breer and Jeserich (1980) this could be attributed to mild homogenization procedure involving less damaging liquid shear forces. Maintenance of cold conditions all through and using young flies appear to be very critical in obtaining the synaptosome like structures in pellicle. However, inspite of all these precautions, the success rate is only about 75-80% and the preparations vary considerably in qualitative and quantitative recovery of these structures.

Regarding the experiments on GABA transport described here, it is necessary to emphasize that we are dealing with GABA translocated into osmotically sensitive structures and not with GABA bound to membranes. Thus when the pellicle fraction incubated with GABA was osmotically shocked, filtered and counted, the counts retained were equal to those in the blank, proving that the radioactivity was transported into osmotically sensitive compartments.

The GABA transport system in *D. melanogaster* is found to be dependent on both Na^+ and Cl^- ions. This has also been observed with synaptosomal preparations from mammalian tissue (Kanner, 1978) and synaptosomes and membrane vesicles from locust ganglia (Gordon *et al.,* 1982). GABA uptake is found to be linear upto 20 min.

The experiments indicate the presence of a high affinity glutamate uptake system in the pellicle fraction from *D. melanogaster.* This seems to need monovalent cations and is stimulated by ATP. Whereas glutamate uptake declines rapidly; GABA uptake is linear to about 15 min which probably indicates that the structures which contribute to the two uptake systems are different. This is also indicated by the relatively poorer levels of glutamate uptake in isolated brains compared to *in vitro* fractions while GABA uptake levels are similar in both systems. It may be noted that glutamate is known to be a neuromuscular junction transmitter in *Drosophila* (Jan and Jan, 1976b). Isolated brains are unlikely to carry these synapses with them, whereas the synaptosome-like fraction from whole flies may contain these structures.

Because of the fragility of synaptosomal structures and variation in absolute uptake rates specially with respect to glutamate, it is important that comparative studies are carried out with the same preparation. For example figure 3 is average of studies where the absolute optimum uptake was somewhat similar; whereas table 2 shows another set of results which are close. However the trends in the results have always been the same. Inspite of some of these problems, the procedure presented in this paper led to viable preparations which could be used to study some of the properties associated with the synaptic system of *Drosophila.*

One intriguing feature of glutamate uptake is that it is not linearly dependent on protein concentration but reaches a plateau at higher protein concentrations. On the

other hand GABA uptake is proportional to the quantity of protein present. The saturable nature of glutamate uptake with respect to protein content may perhaps be due to further metabolism of glutamate in the preparations leading to loss of radio-label. This suggestion is supported by our preliminary results (not shown) that α-ketoglutarate and GABA are the products formed when glutamate is taken up by these preparations.

In our experiments with glutamate uptake, background values where no pellicle ra tion is added are substantially high (see figure 6) as compared to background values in case of GABA uptake which are quite low. Several attempts were made to reduce the background by increasing the number of washes and by presoaking the filter papers in cold glutamate but to no effect. However, all the conclusions have been derived from experiments in which glutamate uptake was measured at various time intervals and zero time values subtracted. Also, counts taken up by the pellicle fraction could be released by lysis of the preparation. We therefore consider our glutamate uptake experiments reliable and conclusions drawn therefrom meaningful.

On the analogy of sodium dependent glutamate uptake in other systems (Kenner and Sharon, 1978), it is possible that in the system under investigation, glutamate is cotransported by the carrier along with the sodium ions, the downhill transport of which energizes the uphill transport of glutamate (Krnjevik, 1974). This suggestion is strengthened by the fact that ATP can replace sodium ions in supporting transport of glutamate (table 3).

The requirement for sodium ions does not appear to be specific, and can also be met by potassium ions. It is quite likely that the closed structures in our preparations are low in concentration of monovalent cations. Hence either sodium or potassium ions are likely to move inwards cotransporting glutamate along with them. We are not certain about the conditions of membrane potential in this system and hence do not know what its contribution to glutamate transport would be.

The requirement for ATP in the glutamate transport system of our preparations appears to be complex. The dependence of glutamate uptake on its concentration, itself is biphasic (figure 10). The uptake observed at higher concentrations of glutamate alone is stimulated by ATP (table 3). Also the stimulation by ATP at a given glutamate concentration becomes obvious only at longer time intervals. All these observations indicate that two transport systems for glutamate are operating in our preparation. One surmise is that there is a vesicular transport system for glutamate functioning inside the structures. It is known that vesicular transport of catecholamines requires ATP transport (Apps, 1982). The stimulation by ATP of glutamate transport observed at longer times of incubation supports this idea since glutamate and/or ATP concentration has to build up inside the sealed membrane structures for vesicular transport to take over. It is also possible that the biphasic dependence of uptake on glutamate concentration is a specific stimulation of lower affinity uptake by endogenous ATP.

Some of the intriguing features of glutamate transport described here such as biphasic dependence on glutamate concentration and complex nature of ATP requirement could be ascribed to contamination by glial cells which are known to transport glutamate in other systems (Fonnum, 1978). At the present stage of our investigations it is not possible to rule out this possibility. However two observations argue against substantial glial cell contamination. One is the dose dependent inhibition by kainic acid which is known not to inhibit glutamate transport by glioma cells

in culture (Nicklas, 1981). Also our preliminary experiments showed that t e incubation of our preparations with glutamate produced GABA and α-ketoglutarate but not glutamine. Since glutamine synthetase is mainly localized in glial cells (van der Berg *et al.,* 1975), the lack of detectable amounts of glutamine in the present case indicates the absence of glial cells in our preparations.

The preliminary results from larval brain uptake of glutamate and GABA indicate that this could also be a useful method of measuring neurotransmitter uptake. Lack of any efect of Picrotoxin, a known GABA receptor antagonist, on GABA uptake in larval brains ruled out receptor binding as the cause for increase in radioactivity with the incubation time. Inhibition of glutamate uptake by aspartate is in line with the known efect of aspartate on glutamate transport system (Fonnum *et al.,* 1981). Difficulties in the use of this system, however arise in replacing incubation media and conditions conveniently. We also notice that the synaptosome-like fraction made from whole flies may have a larger population of glutaminergic nerve endings compared to isolated brain. Isolated brain in the process of picking up may lose most of the synaptic endings at the neuromuscular junction. Hence the *in vitro* preparations described in this paper could still be more useful in such studies.

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