Stress induced taurine release in insects and its effect on the central nervous system.

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for the degree of Doctor of Philosophy

by

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ABBREVIATIONS

ACh,	Acetylcholine
ADP,	Adenosine diphosphate
AOAA,	Aminooxyacetic acid
ATP,	Adenosine triphosphate
DDT,	1,1,1-trichloro,2,2-bis(p-chlorophenyl)ethane
DMSO,	Dimethylsulphoxide
EGTA,	Etheylene glycol Bis-(β aminoethyl ether) N,N,N',N'-tetraacetic acid
GABA,	γ-aminobutyric acid
GDPH,	Glycerol-phosphate dehydrogenase
HEPES,	N-2-hydroxyethylpiperizine-N'-2 ethanesulphonic acid
NADH,	Nicotinamide adenine dinucleotide (reduced)
NEM,	N-ethylmaleamide
NTA,	Nitrilotriacetic acid
TAG,	6-aminomethyl 3-methyl-4H-1,2,4-benzthiaziazine-1,1-dioxide hydrochloride
TLC,	Thin Layer Chromatography
Tris,	Tris (hydroxymethyl)-aminoethane
TTX,	Tetrodotoxin

SUMMARY

The underlying cause of the increase in taurine concentration in the haemolymph and nervous system of stressed insects, and the effects of taurine on the nervous system, was studied in the locust (Schistocerca americana gregaria). The results may be summarised as follows.

1. Taurine was present in all tissues of the locust investigated, and was particularly concentrated in the flight muscle and eye.

2. When insects were poisoned with the GABA antagonist picrotoxin, a redistribution of taurine was observed in which the concentration of the amino acid was increased in haemolymph, nervous tissue, and fat body, and fell in flight muscle and eye. There was no gross change in the whole body content of taurine. Flying and insecticide poisoning also increased taurine concentration in the haemolymph.

3. Exogenous ^{14}C-taurine introduced into the haemolymph accumulated in flight muscle, eye and thoracic ganglia and was retained for a considerable period. No metabolism of ^{14}C-taurine by these tissues was observed. Clearance of ^{14}C-taurine from haemolymph was inhibited by low-temperatures and strucural analogues of taurine (hypotaurine, β-alanine and GABA), suggesting that this process requires energy and is carrier mediated.

4. Taurine appears to be biosynthesised via two possible pathways in the locust. From cysteine to cysteine sulphinic acid and thereafter via either hypotaurine or cysteic acid to taurine. Taurine biosynthesis is much greater during the first days of adulthood than in fully mature adult locusts. Flying and picrotoxin poisoning

both increase taurine biosynthesis in vivo in all locust tissues.

5. ^{3}H-Taurine was not taken up into locust synaptosomes (in contrast to what is seen in mammals), whereas ^{3}H-GABA was readily accumulated. Taurine and nipecotic acid both caused a concentration-dependent decrease in ^{3}H-GABA uptake into synaptosomes.

6. Locust synaptosomes were observed to accumulate $^{45}Ca^{++}$, and this was greatly increased when synaptosomes were depolarised by either veratridine or high K^{+} concentration. Taurine caused a concentration-dependent decrease in $^{45}Ca^{++}$ uptake into depolarised and resting synaptosomes, but was relatively less effective in the latter case. GABA and leucine did not reduce $^{45}Ca^{++}$ uptake into veratridine-depolarised synaptosomes, but verapamil and tetrodotoxin were both effective in this respect.

7. Taurine was observed to cause a concentration-dependent decrease in ^{3}H-ACh release from locust synaptosomes following veratridine or K^{+} depolarisation.

8. Taurine was observed to cause a concentration-dependent increase in ^{3}H-GABA release from synaptosomes. Since nipecotic acid had a similar effect it is suggested that this is due to inhibition of ^{3}H-GABA reuptake after depolarisation-induced release.

9. Release of both ^{3}ACh and ^{3}H-GABA from synaptosomes was observed to be calcium-dependent. Veratridine-induced release of transmitters was found to be abolished by tetrodotoxin.

10. Mitochondria isolated from flight muscle or thoracic ganglia readily accumulated $^{45}Ca^{++}$, and this was dependent on the external

phosphate concentration. In the presence of an inhibitor of mitochondrial calcium uptake (ruthenium red) or a strong calcium chelating agent (EGTA) a Na^+-requiring efflux of calcium was observed. Taurine had no effect on $^{45}Ca^{++}$ uptake into mitochondria, but was observed to decrease Na^+-dependent calcium efflux, and could thereby decrease intracellular free calcium concentration.

11. Taurine binding sites were detected using a membrane preparation derived from whole locust heads. Endogenous taurine (or some other substance which inhibited binding) was tightly retained by the preparation since ^3H-taurine binding was only observed after overnight dialysis of the membranes. Binding was absent at 0^oC and in the absence of Na^+. Washing the membrane preparation with distilled water after incubation with ^3H-taurine, indicated that the association of taurine with the preparation was not due to accumulation within vesicles.

12. Iontophoretically applied taurine and GABA onto locust somata hyperpolarised the cell and decreased membrane resistance, although GABA was considerably more effective in this respect. Both amino acids showed similar reversal potentials (about -75mV) when tested on somata held at a series of membrane potentials.

13. The pharmacology of taurine and GABA in locust somata was very similar. The effects of both compounds were reduced by TAG, a compound which has been proposed as a specific taurine antagonist. Responses to both amino acids were abolished by picrotoxin, and augmented by the benzodiazepine, flunitrazepam. These data suggest that taurine and GABA may be acting at a common site.

SECTION ONE

INTRODUCTION

1.1 General introduction

Taurine (2-aminoethanesulphonic acid) is widely distributed in both lower and higher organisms and frequently constitutes the most abundant component of the free amino acid pool (Jacobsen and Smith, 1968). However, because taurine is a 'non-essential' amino acid, is not incorporated into proteins, and is comparatively metabolically inert, its possible physiological functions have largely gone unstudied, although research in this area has now intensified. Evidence now strongly supports a role for taurine in the physiology and pathophysiology of excitable tissues (Phillis, 1978; Grosso and Bressler, 1976), amongst other postulated functions.

Many invertebrates have been shown to contain taurine, often at strikingly high concentrations (Jacobsen and Smith, 1968) and a role for taurine in osmoregulation of marine invertebrates has been proposed (Jacobsen and Smith, 1968). Within the class Insecta a number of studies have revealed the presence of taurine in a variety of insect species but the studies have been generally qualitative and only rarely has this been examined in specific tissues.

1.2 General anatomy of the locust and organisation of the nervous system

A simple diagram showing the dorsal veiw of a dissected locust is shown in Figure 1.1. The tissues indicated are those which have been used in the present study.

Although species differences exist, the insect nervous system broadly consists of cerebral ganglia and a ventral nerve cord composed of ganglia linked by paired interganglionic connectives (Lane, 1974).

Simplified diagram of dissected locust
showing those tissues sampled for taurine content

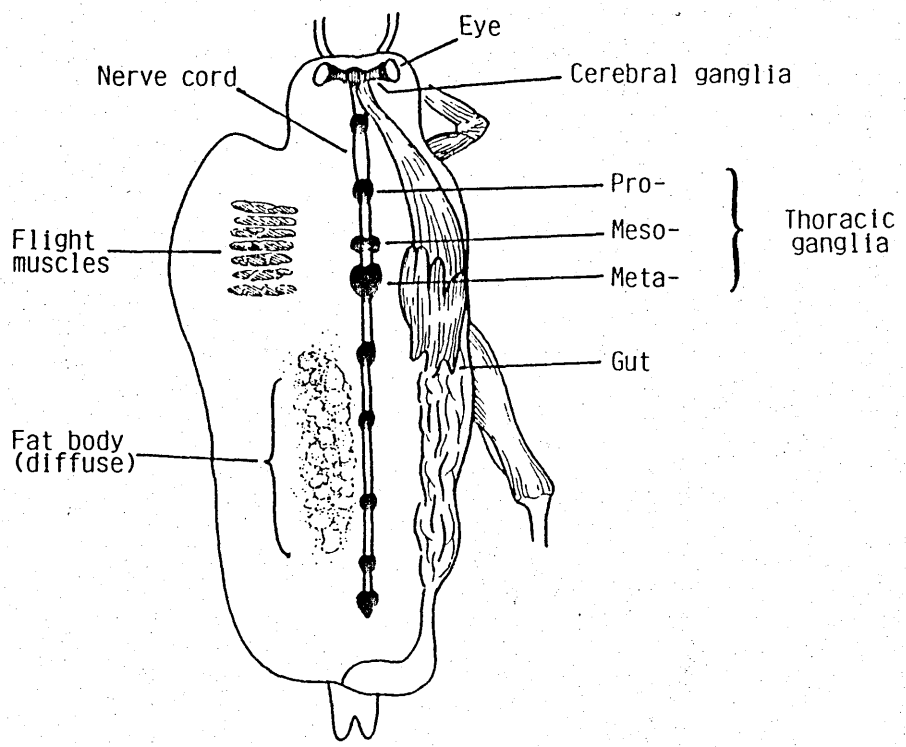


Figure 1.1 Anatomy of the locust.

The ganglia of the ventral nerve cord may be all seperate, as in the locust (Fig. 1.1), or may show varying degrees of fusion according to species.

The ganglia of the nerve cord are composed of a cortex of nerve cell bodies (somata), ensheathed by glial cells, surrounding a central neuropile, while the connectives consist only of axonal processes, invested by glial cells (Lane, 1974). The neuron is the most important cell type of the nervous system. The nucleus lies within the main part of the soma, or cell body. The dendrites, which may be highly branched, receive nervous input, while the axon propagates and transmits nervous signals to other neurons or effector cells.

The functional point of contact between neurons is the synapse. No synaptic contacts occur on the cell bodies of insect neurons, but do so within the neuropile of the ganglia, which is a mass of interwoven neural processes. Most synapses in the insect are chemically mediated with a synaptic cleft of 5-25 nm between the pre- and postsynaptic elements (Osborne, 1970). It is generally considered that the chemicals, or neurotransmitters, which mediate synaptic transmission are located in vesicles in the presynaptic terminal. The arrival of an action potential at the nerve terminal leads to the release of neurotransmitter into the synaptic cleft. The transmitter binds to specific receptors on the postsynaptic element and thereby elicits a response. This may take the form of openning an ion channel or activating second messenger systems in the postsynaptic cell. The release of neurotransmitter is generally considered to be preceeded by and dependent upon an influx of calcium ions in reponse to the depolarisation induced by the incoming action potential (Miledi, 1973; Llinas and Heuser, 1977).

The excitability of the nervous system is dependent upon the maintenance of ionic gradients across the neural membrane, and this in turn requires metabolic energy. The major ions which are involved are K^+, Na^+, Cl^- and Ca^{++} as well as organic anions located intracellularly. The ionic gradients across the membrane result in a low K^+, high Na^+ concentration outside the cell with the inverse holding true inside. The extracellular Cl^- concentration is much greater than the intracellular level while the opposite applies for Ca^{++}. The membrane is relatively permiable to K^+ but has much lower permeabilities to Na^+, Cl^- and Ca^{++}. These ionic gradients lead to a net potential difference across the nerve cell membrane with the inside of the cell being in the region of 100-150 mV negative compared with the outside. Selective channels exist within the nerve cell membrane through which specific ions can pass, and when these channels are opened (for example by a change in the membrane potential) the ion will naturally flow down its electrochemical gradient. The action potential is largely the result of a transient increase in the membrane permeability to Na^+ which leads to a rapid shift in the membrane potential towards and beyond 0 mV. This in turn leads to an increase in permeability to K^+ which terminates the action potential and leads to the repolarisation process. A prolonged increase in the permeability of the membrane to a given ion will result in that ion redistributing itself across the membrane until its electrochemical gradient is zero and there is no net driving force upon that ion. This generally occurs when the membrane potential has been shifted to such an extent that it overcomes any concentration

difference for a given ion across the membrane. The potential at which this occurs is described as the reversal potential for the ion in question. This varies according to the ionic composition of the cell.

Synaptosomes are a major preparation used in the study of the nervous system and have been used extensively in the present work. Synaptosomes are formed from the sheared off terminals of neurons obtained during homogenisation (Gray and Whittaker, 1962). The terminals then reseal to form a vesicular preparation which appears to retain many of the properties of intact nerve terminals. Thus synaptosomes are able to transport metabolically important substances such as choline (Marchbanks, 1968), which is the precursor of the neurotransmitter ACh within the synaptosome (Marchbanks and Israel, 1971). Furthermore, it is possible to obtain the release of neurotansmitter substances from synaptosomes. Indeed it has been observed that following electrical stimulation of synaptosomes those amino acids which are considered to be neurotransmitters are preferentialy released (Bradford, 1970). Recently the methods used to obtain mammalian synaptosomes have been applied to insect nervous tissue (Breer, 1981). These synaptosomes appear to retain many of their functional properties in much the same way as those of mammalian origin . They are able to accumulate choline and this is metabolised into ACh which can then be released under depolarising conditions (Breer and Knipper, 1984). This release of ACh is Ca^{++} dependent. GABA is also transported by insect synaptosomes (Gordon et al., 1982). An electronmicrograph of a synaptosomal preparation

obtained from the nervous system of Schistocerca gregaria is shown in Figure 1.2.

1.3 The stress response in insects

Since the observations by Sternberg and Kearns (1952) that the blood of DDT poisoned cockroaches contained toxins other than DDT many studies have been undertaken in an attempt to identify and characterise these toxic factors. Furthermore Beaumont (1958) found that stress induced by prolonged immobilisation of cockroaches also caused the appearance of toxins in the haemolymph, and that injection of this haemolymph into an unstressed roach caused the development of 'stress symptoms'. These symptoms are an initial period of hyper-excitability, followed by paralysis and eventual death. An apparently identical form of paralysis can be induced in the cockroach Naupoeta cinerea when a subordinate roach has an aggresive encounter with a dominant male (Ewing, 1967).

The source of the toxins involved in the stress response is unclear, although a number of studies have indicated the nervous system. After prolonged high frequency stimulation of the nervous system of Periplaneta americana in vitro a substance which was chromatographically similar to that found in the blood of DDT poisoned roaches appeared in the perfusate (Sternberg et al., 1959). The substance(s) affected nerve conduction in a manner very similar to that of the DDT-induced toxin; at low concentrations spontaneous activity was increased, while high concentrations (corresponding to those occuring in the haemolymph) caused a block in conduction. Cook et al. (1969) studied the distribution of the substance in

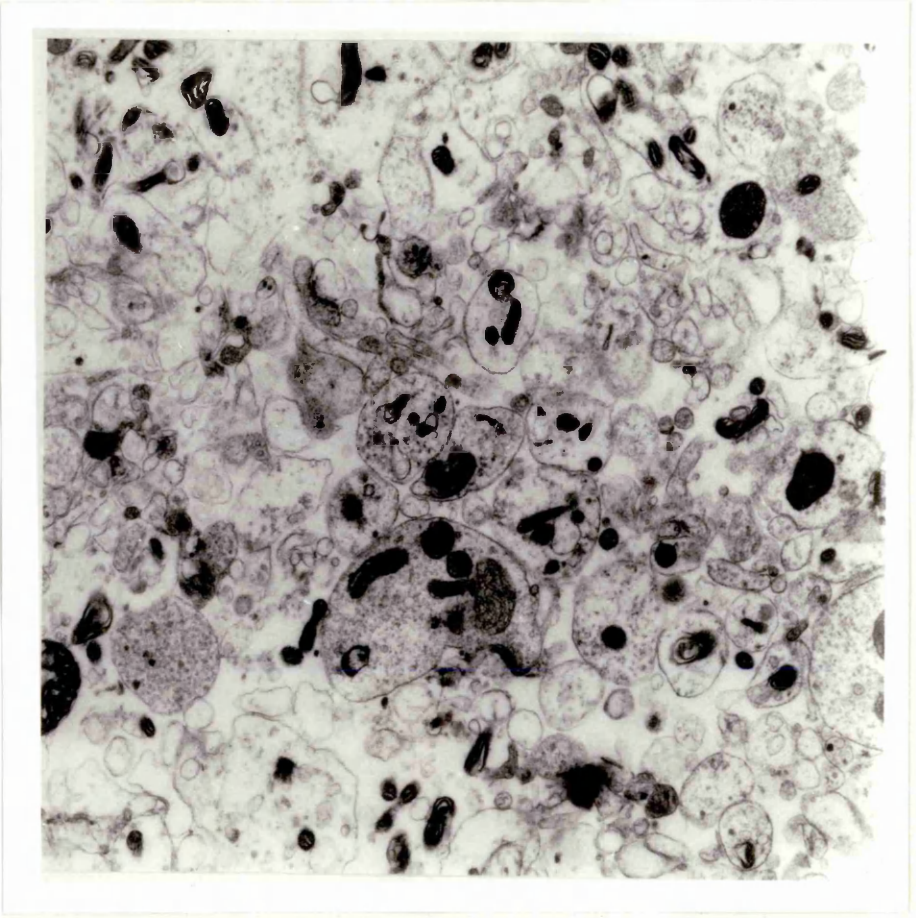


Figure 1.2 Electron micrograph of a synaptosomal preparation from S. gregaria. (Magnification X 32666, courtesy of Dr J. M. Pocock).

physically stressed P. americana and found that it occurred in decreasing concentrations in nerve cord, whole head, whole body (minus nerve cord) and legs respectively. Administration of the substance to Musca domestica produced a paralysis of rapid onset which lasted for a number of hours.

Attempts to identify the neuroactive substance(s) produced during stress have met with only limited sucess. Sternberg et al. (1959) found the compound to be chemically and chromatographically distinct from ACh, adrenaline, noradrenaline , histamine, GABA and 5-hydroxytryptamine, and subsequently suggested that an ester of a carboxylic acid might be the substance on the basis of chemical tests (Hawkins and Sternberg, 1963). However, Colhoun (1960) suggested that a catecholamine might be the neuroactive agent, while Tashiro et al. (1972, 1975) considered L-leucine and its metabolite isoamylamine to be probable candidates.

Recently taurine has been added to the list of proposed compounds. Following treatment with a variety of chlorinated hydrocarbon insecticides, as well as after physical stress, the taurine concentration in the haemolymph and nervous system of S. gregaria was markedly increased (Jabbar, 1982; Jabbar and Strang, 1985). Taurine content in the haemolymph of P. americana was also increased (Jabbar and Strang, 1985). It was not considered that the source of this taurine was the nervous system since the latter seemed to be unable to synthesise taurine in vitro when ^{35}S-methionine was used as precursor (Jabbar, 1982). The concentration of taurine occurring in the nervous tissue of S. gregaria is equivalent to concentrations which reduce

spontaneous activity in isolated nerve cord of P. americana (Jabbar and Strang, 1985).

Haemolymph taurine concentration is also increased under conditions which are within the normal physiological range. Van Der Horst et al. (1980) studied the effects of sustained flight on the level of free amino acids in the haemolymph of Locusta migratoria. After 2 hr of flight, taurine concentration had increased by as much as ten times, while a doubling was observed after as little as 15-30 minutes. Since a common factor in both physical and chemical stress is intense muscular activity, it seems possible that the musulature might be acting as a source of the taurine.

1.4 Occurence and distribution of taurine

In mammals taurine occurs at high concentrations in excitable tissues such as nerve and muscle (Sturman, 1973; Grosso and Bressler, 1796). Taurine distribution in insects, however, has received much less attention than has been the case in mammals.

Po-Chedly (1969) reported the presence of taurine at relatively low concentrations (0.2 μmoles/g) in the eggs of the beetle Tenebrio molitor, and taurine was found in the pupae of Calliphora erythrocephala but not in the pupae of Phalera bucephala (Agrell, 1949). Levenbrook and Dianamarca (1966) followed taurine concentration in the whole body of Phormia regina from the larval through to the adult stages. During this period taurine concentration rose from 5 μmoles/g to 10 μmoles/g wet weight of tissue. The quantity of taurine determined was not altered by acid hydrolysis, indicating it to be wholly in the free state. No role for taurine in the developmental process was suggested, and as will be considered later the increase was probably associated

with the development of adult structures in which taurine is highly concentrated. Clark and Ball (1952) investigated qualitatively the free amino acids in the whole body of a number of insect species. Taurine was present in the larvae of Estigmene acraea and the adult forms of the mosquitoes Culex tarsalis, Culex stigmatosoma and Culex incidens as well as in the adult cockroach Blatta orientalis . A number of reports have appeared in the literature concerning the presence of taurine in specific insect tissues. The paragonia of the fly Drosophila melanogaster (Chen and Diem, 1961) and the salivary glands of a number of diptera (Blummel, 1956) were found to contain taurine. Pratt (1950) found taurine to be present in the haemolymph of M. domestica, the larvae of Apis mellifera and the adult of Oncopeltus fasciatus , but not in the blood of the larvae of Prodernia endava, Gallina mellonella or the adult cockroaches P. americana or Blatella germanica. However, subsequent studies have shown taurine to occur in the haemolymph of P. americana (Stevens, 1961; Rakshipal and Singh, 1976; Jabbar and Strang, 1985). Taurine has also been found in the blood of the blowfly larva Calliphora augur (Hackman, 1956), in the larvae of Calliphora vicina (2.7μmoles/ml; Evans and Crossley, 1974), where it was observed to be located almost exclusively in the plasma fraction as opposed to the haemocytes which are known to contain a high concentration of some amino acids such as glutamate (Evans and Crossley, 1974). Bodnaryk (1981a) found the pupal blood of Mamestra configurata contained taurine at a concentration of 2.5μmoles/ml, while the adult locust S. gregaria, the insect of primary interest in the present study, has about 2μmoles/ml of taurine in the haemolymph (Jabbar and Strang, 1985). Very high concentrations of free amino acids (in the millimolar

range) in the haemolymph appears to be characteristic of insects (Wyatt, 1961). In contrast to this, the concentration of taurine in vertebrate blood is approximately 0.1 μmole/ml (Huxtable and Barbeau, 1976).

The tissue in which taurine generally occurs at the highest concentration is muscle. Taurine is by far the most abundant free amino acid in mammals and skeletal muscle accounts for about 75 % of the total body stores. Taurine concentrations in skeletal muscle are generally in the 5-15 μmoles/g wet weight range, while in cardiac muscle the concentration may be even greater, being, for example, about 30 μmole/g in the rat (Grosso and Bressler, 1976). Although few studies have considered taurine in insect muscle, those that exist indicate that in some cases the concentration may be very high indeed.

Kermack and Stein (1959) reported the presence of taurine in the thoracic muscles of L. migratoria; however, there must be some doubt as to the true identity of the compound as it was stated to be destroyed by acid hydrolysis in 6M HCl, conditions under which taurine is stable. Bodnaryk (1981a) investigated taurine distribution during pupal development of M. configurata and observed that in the diapausing pupae more than 90 % of the amino acid was in the haemolymph. However, when pupae start to develop adult structures these accumulate taurine from the haemolymph. In adult M. configurata more than 90 % of the taurine synthesised during metamorphosis occurs in the thorax and is almost certainly located in the large flight muscles (Bodnaryk, 1981b).

In L. migratoria taurine concentration in pure flight muscle has been estimated during the period from three days prior to the final

ecdysis up to twenty days after (Van Marrewijk et al., 1980). During this period taurine concentration rose from 6.6 μmoles/g to 54.5 μmoles/g wet weight. In contrast to this most other amino acids, with the exceptions of arginine and proline, declined in concentration (Van Marrewijk et al., 1980). Twenty days after the final ecdysis taurine accounted for about 40 % of the total free amino acid pool of the flight muscle (Van Marrewijk et al., 1980).

Taurine has been found in the nervous system of a number of insects. In L. migratoria the concentration in the brain and ventral nerve cord has been estimated as 22 μmoles/g wet weight (Osborne, 1971). In the nervous system of the stable fly Stomyxys cacitrans taurine concentrations of 37 and 110 μmoles/g dry weight were found in the cerebral and thoracic ganglia respectively (Holman and Cook, 1981). In S. gregaria taurine occurs in both the cerebral (0.49 μmoles/g) and thoracic (0.82 μmoles/g) ganglia (Jabbar and Strang, 1985). In the honey bee brain very high concentrations of taurine have been reported: 34 μmoles/g wet weight (Frontali, 1964).

Bodnaryk (1981a) studied changes in brain taurine content during pupal development of M. configurata. The taurine concentration rose from 0.6 μmoles/g at the start of metamorphosis to some 10.5 μmoles/g on day 16 of pupation, then fell to the adult level of about 1.5 μmoles/g at adult emergence after 32 days. The highest concentration of taurine in the brain corresponded to the period of intensive brain development, and Bodnaryk (1981a) therefore suggested a possible role for taurine in the developmental process.

By comparison, in mammals brain taurine concentration falls in the 2-5 μmoles/g range (see Grosso and Bressler, 1976), which is

similar to that found in some of the insect species studied. Taurine appears to be differentially distributed throughout the mammalian brain (Palkovits et al., 1986) and may be concentrated in nerve terminals (Kontro et al., 1980).

One part of the vertebrate nervous system in which taurine occurs at very high concentrations is the retina (Voaden et al., 1977). Interestingly, the large optic lobes of M. configurata contain a high concentration of taurine, especially during development (Bodnaryk, 1981a) but no other estimate of taurine concentration in insect eye appears to have been made.

The available data therefore indicate a qualitative similarity in the distribution of taurine in insects and in mammals. In general taurine occurs at highest concentrations in the so-called excitable tissues, muscle and nerve. This suggests that taurine may serve similar roles in terrestrial invertebrates and mammals.

1.5 Biosynthesis and metabolism of taurine

a) Biosynthesis

Biosynthetic pathways for the formation of taurine in vertebrates are reasonably well established (Jacobsen and Smith, 1968; Oja and Kontro, 1983) but very little comparable work has been performed in insects. The postulated pathways involved in taurine biosynthesis are shown in Figure 1.3.

M domestica administered either ^{35}S-methionine or ^{35}S-cysteine were observed to form cysteine sulphinic acid, cysteic acid, hypotaurine and taurine (Hilchey et al., 1958; Cotty et al., 1958), indicating that these insects are capable of forming taurine via two biosynthetic pathways (see Fig. 1.3). Henry and Block (1960, 1961) found ^{35}S-taurine after administration of ^{35}S-cysteine to the cockroaches

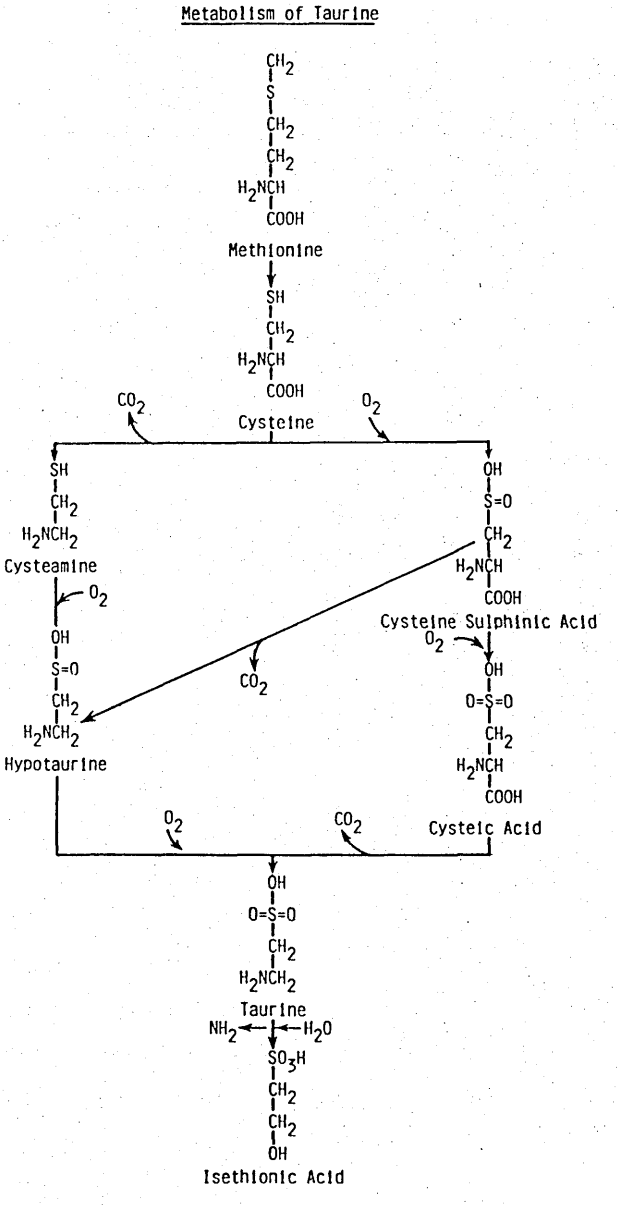


Figure 1.3 The proposed interelationships between the various compounds involved in taurine biosynthesis and metabolism.

P. americana and B. germanica, but the intermediates between cysteine and taurine were not indicated. Xenic (symbiont containing) cockroaches are able to incorporate the sulphur moiety of inorganic sulphate into a number of organic compounds including taurine, while cockroaches reared under aseptic conditions are unable to do so (Henry and Block, 1960; 1961). Chandler and Anderson (1976) observed that D. melanogaster fed ^{35}S-sulphate incorporated ^{35}S into cysteine, cysteic acid and taurine as well as a number of other compounds. These authors did not investigate any possible contribution from microbes in the incorporation of ^{35}S-sulphate into organic molecules.

The only study in insects indicating taurine biosynthesis via the 'cysteamine' pathway (see Fig. 1.3) is that of Bodnaryk (1981b) using the moth M. configurata. Following injection of a single dose of ^{35}S-cysteine into 2-day-old non-diapausing pupae, radioactivity appeared in cysteamine, hypotaurine and taurine, but no biosynthesis of either cysteine sulphinic acid or cysteic acid was observed (Bodnaryk, 1981b).

b) Metabolism

Only inorganic sulphate and isethionic acid (see Fig. 1.3) have been seriously considered as possible breakdown products of taurine (Jacobsen and Smith, 1968), and little work has been done on the subject.

Cotty et al. (1958) detected isethionic acid in whole body extracts of M. domestica after injection of ^{35}S-cysteine. In vitro metabolism of ^{35}S-taurine to isethionic acid by dog heart slices was observed by Read and Welty (1962), while rat brain and heart in vitro and in vivo were found to metabolise taurine to isethionic acid, but the rate of conversion was very slow (Read and Welty, 1962). Lahdesmaki and Kor-

honen (1978) found the metabolism of taurine into isethionic acid by rat brain *in vivo* and *in vitro* to be so small that they discounted any possibility that such metabolism could have any significant role in control of the brain taurine pool. This situation is made more complex by the observation of Fellman et al. (1980) that in many of the ion exchange procedures used to isolate isethionic acid the latter is eluted with contaminants such as aceyltaurine. Fellman et al. (1980) could find no metabolism of taurine to isethionate by mouse or rat tissues *in vitro*, but some metabolism occured *in vivo*. Since metabolism was not observed in germ free mice but could be demonstrated in the presence of gut microbes (*Pseudomonas sp.*) *in vitro* it was suggested that metabolism of taurine to isethionate is due to action of the gut flora (Fellman et al., 1980).

1.6 Effects of taurine on the nervous system

Taurine has become established as a strong candidate as an inhibitory neurotransmitter in both vertebrates and invertebrates (Phillis, 1978). Taurine was observed to have pronounced depressant actions on single neurons in cat spinal cord (Curtis and Watkins, 1960) and on reflex activity of toad spinal cord (Curtis et al., 1961). Taurine, like GABA, mediates a postsynaptic inhibition by enhancing chloride conductance (Sonnhof et al., 1975; Homma, 1979; Taber et al., 1986). The pharmacology of taurine is complex since its inhibitory actions are so similar to those seen with GABA and glycine as well as β-alanine. Strychnine has been found to antagonise the actions of taurine on frog spinal cord, while GABA and surprisingly glycine were unaffected (Barker et al., 1975a,b). However, picrotoxin and bicuculine blocked

the actions of GABA and taurine in frog spinal cord (Barker et al., 1975a), while bicuculine was found to block the actions of GABA but not taurine in neurons of cat brain stem (Haas and Holsi, 1973).

Taurine has a hyperpolarising effect on lobster axon and this shows a reversal potential at about -85 mV (Greuner and Bryant, 1975). These authors suggested that the response was due to increased Cl^- conductance in addition to an increase in K^+ conductance. Nistri and Constanti (1976) studied the action of taurine on lobster muscle fibre and frog spinal cord. In the former preparation taurine caused a chloride-dependent increase in membrane conductance without markedly altering membrane potential and a substantially similar effect to GABA was seen also. However, whereas the action of GABA was considerably reduced by picrotoxin, comparatively little reduction in the response to taurine occurred. In the frog preparation, on the other hand, taurine caused small Na^+-dependent depolarisations which were antagonised by both strychnine and picrotoxin in a non-competative manner (Nistri and Constanti, 1976).

Taurine has been found to reduce both spontaneous activity and evoked excitatory postsynaptic potentials (EPSPs) in the A6 ganglion of P. americana (Hue et al., 1978, 1981). The above authors found taurine to be equally effective in reducing the amplitude of inhibitory postsynaptic potentials. These actions of taurine appeared to be chloride-dependent (Hue et al., 1978). In contrast to this taurine does not appear to affect axonal transmission (Pelhate et al., 1978). After 30 min of superfusion with 20mM taurine no effect on evoked action potential amplitude or resting potential was seen in

the abdominal nerve cord of P. americana. Additionally, taurine had no effect on K^+ conductance in the axonal preparation (Pelhate et al., 1978). These results contrast with the effects of taurine on the axon in crustacea (Greuner and Bryant, 1975). Together the above data indicate that, at least in P. americana, the effects of taurine are entirely mediated at the synaptic level. Taurine has also been observed to cause a dose-dependent decrease in spontaneous firing of P. americana nerve cord in vitro (Jabbar and Strang, 1985).

Hue et al. (1979) have observed that taurine may act both pre- and postsynaptically to cause inhibition of neural transmission. Both taurine and GABA were found to cause a small presynaptic depolarisation, an effect which would decrease the amplitude of an incoming action potential (Davidson and Southwick, 1971; Gallagher et al., 1978). The responses of both taurine and GABA were sensitive to picrotoxin antagonism, while only taurine was antagonised by strychnine (Hue et al., 1979). As judged by monitoring EPSPs, taurine appears to reduce the release of ACh, a major excitatory neurotransmitter in insects (Florey, 1963; Pitman, 1971) in the A6 ganglion of P. americana (Hue and Chanelet, 1984). This effect was not antagonised by high external calcium, 4-aminopyridine or the putative taurine antagonist TAG, but was reduced by picrotoxin. Taurine has been observed to hyperpolarise isolated somata obtained from S. gregaria (two out of five cells responded) during bath application of the amino acid (Giles and Usherwood, 1985).

A major obstacle in establishing taurine as a neurotransmitter has been the lack of specific agonists or antagonists for taurine. Recently TAG has been proposed as a specific taurine antagonist

(Girard et al., 1982) since it reduces responses to taurine but not GABA or β-alanine in rat cortical and Purkinje neurons (Yarbrough et al., 1981). However, in the cat spinal cord the inhibitory actions of taurine, β-alanine, glycine, and to a lesser extent GABA, were all reduced by TAG (Curtis et al., 1982).

In addition to the electrophysiological data there is a considerable body of biochemical data supporting a neurotransmitter or neuromodulator role for taurine (see Kuriyama, 1980; Barbeau et al., 1975; Oja and Kontro, 1978). Depolarisation-induced release of taurine from brain slices (Davison and Kaczemak, 1971; Collins, 1980) and synaptosomes (Placeta et al., 1986) has been observed. Taurine is enriched in synaptic vesicle fractions from bovine brain (Kontro et al., 1980) and a calcium-dependent efflux from rat cerebellum in vivo has been demonstrated (Bernadi et al., 1977). Taurine is also released from retinae under depolarising conditions (Pasantes-Morales et al., 1973; Salceda and Pasantes-Morales, 1975).

There is substantial evidence that taurine can modulate the release of neurotransmitters. Taurine has been found to reduce ACh release from a number of preparations. In rat superior cervical ganglion taurine inhibited K^+-evoked, but not spontaneous release of ACh, and similar results were obtained for ACh release from rat cortical slices (Kuriyama et al., 1978). Taurine, GABA and glycine were all found to reduce light-evoked release of ACh from rabbit retinae, and this effect was blocked by strychnine in the case of taurine and glycine but not GABA (Cunningham and Neal, 1983). Taurine has also been observed to antagonise the effects of evoked ACh release in guinea pig ileum. Increased contracture of the guinea pig ileum

following 4-aminopyridine (4-AP) perfusion was found to result from increased ACh release (Arzate et al., 1984) and this was antagonised by taurine in a concentration-dependent manner. On the other hand, taurine had no effect on responses to exogenously applied ACh indicating a presynaptic action (Arzate et al., 1984). In the same preparation GABA was similar in action but less potent than taurine, while glycine and β-alanine had no effect.

Taurine also appears to modulate catecholamine release. Kuriyama et al. (1978) found taurine to inhibit both the uptake and release of noradrenaline from rat cerebral cortical slices. Taurine produces a concentration-dependent decrease in dopamine release induced by 4-AP from rat striatal synaptosomes (Arzate et al., 1986). Both taurine and GABA were observed to decrease dopamine in the striata of unanaesthetised rats (O'Neil, 1986). Interestingly dopamine has been found to reduce K^+-evoked taurine release from rat retinae <u>in vitro</u> (Pycock and Smith, 1983).

Compared with its actions on other neurotransmitter systems, the affects of taurine on GABA release are less clear. Namima et al. (1983) reported an inhibitory effect of taurine on K^+-evoked GABA release from rat cerebellar slices. This effect of taurine was antagonised by bicuculine and the authors therefore suggested a modulatory effect of taurine on GABA autoreceptors which would presumably be located presynaptically (Namima et al., 1983). A previous study however, has indicated that taurine enhances K^+- or veratridine-induced release of GABA from rat cortical slices (Leach, 1979). This effect of taurine was not considered to result from an inhibitory action on GABA uptake or because of a

taurine-GABA exchange since in the absence of depolarising stimuli taurine had little effect (Leach, 1979). Nipecotic acid, an inhibitor of neuronal GABA uptake (Krogsgaard-Larsen and Johnston, 1975) was found to have a similar spectrum of action as taurine on GABA release, but was more potent (Leach, 1979). In vivo studies have shown that perfusion of rat hippocampus with saline containing millimolar GABA concentrations greatly increases extracellular taurine levels (Lerma et al., 1985), while perfusions with nipecotic acid in the saline increase both GABA and taurine concentrations in the extracellular fluid (Lerma et al., 1984). Additionally, taurine appears to modulate the GABA-benzodiazepine complex, increasing flunitrazepam binding in unwashed membrane preparations (De Robertis, 1984).

1.7 Transport of taurine in vivo and in vitro

Portman and Mann (1955) studied the distribution of ^{35}S-taurine in rats, 24 hr after administration directly into the duodenum. All tissues investigated accumulated taurine although there was great variation from one tissue to another, with muscle being the most enriched. Possible accumulation in the brain was not examined. After intravenous administration of ^{35}S-taurine in rats (Awapara, 1957) the amino acid was found to accumulate to a considerable extent in heart and spleen, less so in skeletal muscle, kidney, intestine, the gonads and brain, and very little in liver. However, while the level of ^{35}S-taurine had declined considerably in most tissues after 12 days, the amount remaining in heart, skeletal muscle and brain was proportionately much greater.

Similar results were obtained by Sturman (1973) after injection

of ^{35}S-taurine into rats. Most tissues, such as the visceral organs, accumulated taurine rapidly but this was followed by an equally rapid decline. In brain, as well as cardiac and skeletal muscle the accumulation of taurine was comparatively slow, and the subsequent decline in ^{35}S-taurine was less rapid than that observed in other tissues (Sturman, 1973). Attempts to disrupt taurine homeostasis, such as prolonged fasting, vitamin B-6 defficiency (the latter is a co-factor in taurine biosynthesis), or administration of large amounts of taurine, had almost no effect on tissue taurine concentrations (Sturman, 1973). Sturman (1973) concluded that two taurine pools must exist in the body: a small rapidly exchanging pool, and a much larger inaccessible pool with a much longer half-life.

These data are in good accord with those of Matsubara et al. (1985) who measured plasma ^{35}S- and ^{14}C-taurine after administration of an intravenous bolus to rhesus monky's. The results once again indicated the existence of two taurine pools, a small rapidly-exchanging pool and a large slowly-exchanging pool.

Numerous studies exist on the transport of taurine into the nervous system and preparations derived from it. Taurine is transported into rat brain slices (Oja, 1971) by saturable and non-saturable transport systems, the former of which is energy-dependent and competetively inhibited by structural analogues of taurine such as hypotaurine, β-alanine and GABA (Lahdesmaki and Oja, 1973). In some studies active transport of taurine into brain slices has not been inhibited in the presence of GABA (Kaczamarek and Davison, 1972). High affinity taurine uptake was observed in brain slices obtained from eight different areas of rat brain, with varying rates of uptake

which were not related to the endogenous taurine concentration (Collins, 1974).

Bodnaryk (1981a) studied the uptake of taurine into the brain of M. configurata after injection of a single dose of ^{14}C-taurine into the haemocoel of either diapausing or non-diapausing pupae. Only a very small fraction of the dose was accumulated in the brains of the non-diapausing pupae and even in the diapausing pupae only 0.2 % appeared in the brain (Bodnaryk, 1981a). No other publications on the uptake of taurine into insect nervous system in vivo appears to exist.

Taurine is transported into mammalian synaptosomes by a high affinity transport system (K_m 4.76μM) and this process appears to be entirely Na^+-dependent (Hruska et al., 1978). Taurine uptake is inhibited by a number of substances including GABA, hypotaurine and β-alanine, and most effectively by chlopromazine, although the latter is an antipsychotic drug and is not a structural analogue of taurine (Hruska, 1978). Other reports have indicated a higher K_m for taurine transport into synaptosomes (46μM; Kontro and Oja, 1978). Taurine transport has been found to be calcium-dependent in some systems (Wheler et al., 1979) and there is evidence that a specific population of synaptosomes from rat brain accumulate taurine (Sieghart and Karobath, 1974).

Much of the characterisation of taurine transport has been performed using either neuronal or glial cell cultures. Taurine is transported into neuroblastoma cells by a high affinity uptake system (K_m 10.1μM) and this is reduced by hypotaurine and GABA (Holopainen et al., 1983a).

Borg et al. (1979) studied the transport of taurine into a number of cells lines of both neuronal and glial origin. Many compounds were found to inhibit taurine uptake and the differences from one cell line to another were slight. Excluding metabolic poisons, hypotaurine and β-alanine were by far the most effective uptake inhibitors, with GABA being next most potent (Borg et al., 1979). Somewhat surprisingly, in one report GABA has been found to increase taurine uptake, into cultured mouse brain astrocytes, although β-alanine had a marked inhibitory effect (Schosboe et al., 1976).

In both neuronal and astrocyte cultures taurine, GABA and β-alanine were all found to be transported by high affinity uptake systems (Larsson et al., 1986). However, in glioma cells only taurine and β-alanine were observed to have high affinity uptake, while GABA was transported by a low affinity system (Martin and Shain, 1979). Since in both studies taurine, GABA and β-alanine were all found mutually to inhibit the transport of each other it was suggested that they share a common carrier (Martin and Shain, 1979; Larsson et al., 1986).

Taurine transport is Na^+-dependent (Borg et al., 1979) although high Na^+ concentrations (above 250 mM) reduce taurine uptake (Holopainen et al., 1983b). Taurine transport may be weakly dependent on K^+ up to 10 mM (Borg et al., 1979) but no requirement for Mg^{++} exists (Borg et al., 1979; Kontro, 1981; Kurzinger and Hamprecht, 1981). Reports differ as to the Ca^{++} dependency for taurine uptake; none was found by Holopainen et al. (1983b) using neuroblastoma cells, but some requirement in glial cells has been observed (Borg et al., 1979).

Taurine is actively transported into vertebrate retina (Pasantes-Morales et al., 1972; Lake et al., 1977), with characteristics similar to those reported into preparations derived from other parts of the nervous system (Kennedy and Voaden, 1976).

It has recently been observed that taurine uptake occurs into cultured neurons from P. americana (Beadle et al., 1987). Using autoradiography, taurine was observed to accululate in cell bodies, with little appearing in dendritic processes, and no taurine was taken up by glial cells in culture (Beadle et al., 1987).

1.8 Interactions of taurine with calcium

Taurine is considered to be a putative modulator of cellular calcium homeostasis. Many of the effects of taurine on nerve and muscle appear to be due to the effect of this amino acid on the intracellular free calcium concentration. Thus although most of the actions of taurine on the nervous system have already been considered, the actions of taurine which are specifically related to calcium will be described seperately.

Remtulla et al. (1979) observed that both taurine and GABA reduced passive calcium uptake into rat brain synaptosomes. In a subsequent study, using the same preparation, Pasantes-Morales and Gamboa (1980) studied the effects of taurine, GABA, glycine, glutamate and β-alanine of passive calcium accumulation. Of the compounds tested only taurine and β-alanine inhibited calcium uptake, while the others had no effect. These authors were unable to obtain an increase in calcium uptake under high K^+ depolarising conditions, and the effect of taurine on this parameter was therefore not studied. A previous report, however, had indicated no effect of taurine on

calcium accumulation in resting synaptosomes (Kuriyama et al., 1978). On the other hand, the inhibitory effect of morphine on calcium uptake has been found to be reversed by taurine in mouse brain synaptosomes (Yamamoto et al., 1981). However, the same authors observed that taurine reduced K^+-stimulated calcium uptake by 25 %, while GABA had no such effect (Yamamoto et al., 1981). Taurine has been observed to abolish calcium spikes in the dendrites of Purkinje cells, and it was suggested that this might be due to inhibition of calcium uptake into the cells (Okamoto et al., 1983a,b).

A number of reports exist on the effect of taurine on calcium transport in retinal preparations. At high calcium concentrations, in the millimolar range, taurine has been found to decrease calcium transport by retinal subcellular fractions (Pasantes-Morales et al., 1979; Lopez-Colome and Pasantes-Morales, 1981). On the other hand, when the calcium concentration is in the micromolar range, taurine appears to increase calcium accumulation with retinal subcellular fractions (Lombardini, 1983; Lombardini, 1985a,b). The latter effect appears to be quite specific for taurine since hypotaurine, β-alanine, GABA, cysteine sulphinic acid and isethionic acid do not stimulate such calcium uptake (Kuo and Miki, 1980).

Taurine also modulates calcium binding to muscle intracellular membranes. Taurine increases total calcium content of guinea pig heart (Dolara et al., 1973) and ventricular strips (Franconi et al., 1982). It has been observed that calcium binding to cardiac

sarcolemma is increased in the presence of taurine (Chovan et al., 1979; Chovan et al., 1980; Sebring and Huxtable, 1985). Taurine also increases calcium binding to skeletal muscle sarcoplasmic reticulum (Huxtable and Bressler, 1973; Dolara et al., 1986). Although it has been reported that taurine has no effect on calcium binding to sarcolemmal membranes (Remtulla et al., 1978), the evidence to the contrary appears overwhelming.

Finally, there is some evidence that taurine may affect calcium homeostasis in mitochondria. Mitochondria have an avid uptake system for calcium and can accumulate large amounts of the ion (Nicholls and Akerman, 1982). Insect mitochondria also transport calcium and this appears to be essentially similar to the situation found in vertebrates (e.g. see Dawson et al., 1971). Taurine was observed to increase energy-dependent calcium uptake into rat liver mitochondria at high calcium concentrations (Dolara et al., 1973). However, Kuriyama et al. (1978) found no effect of taurine on calcium uptake into rat brain mitochondria, but did observe a decrease in calcium efflux from the same preparation in the presence of taurine. The data concerning taurine, calcium and mitochondria are therefore sparse and equivocal.

1.9 Evidence for taurine binding sites

Taurine binding sites have been demonstrated in nervous tissue using preparations derived from brain and retina. Taurine binds to presynaptic membranes, but the number of sites, at least in calf brain, is very much less than is the case for GABA or glutamate

(Turpeenoja and Lahdesmaki, 1983).

Evidence exists for both sodium-dependent and sodium-independent binding of taurine. In preparations from calf brain taurine binding was found to be Na^+-independent, but was Ca^{++}-dependent (Lahdesmaki et al., 1977). Binding of taurine to mouse cerebral cortex has also been observed to be Na^+-independent (Kontro and Oja, 1986). In rat brain preparations both sodium-dependent and independent binding was observed, the former being considered as binding to transport proteins, and the latter to true 'receptors' (Marnela and Kontro, 1984).

Lopez-Colome and Pasantes-Morales (1980) reported Na^+-dependent taurine binding to chick retinal membranes. These authors did not consider this to be the result of interaction with transport sites, although displacement studies did not support the veiw that taurine was binding to a postsynaptic receptor. Sodium dependent binding has also been observed in rat retinal preparations, and this was considered to be composed of an interaction of taurine with true binding sites (i.e. 'receptors') as well as a contribution from transport sites (Lombardini and Prien, 1983). A general problem encountered in demonstrating taurine binding has been difficulty in removing sufficient endogenous taurine. Repeated washing is apparently insufficient (Lombardini and Prien, 1983), while repeated freezing and thawing or Triton X-100 treatment reveals low numbers of sites (Marnela and Kontro, 1984).

The other tissue which has been studied in some depth to detect possible taurine binding sites is cardiac sarcolemma and sarcoplamic

reticulum. Taurine binds to rat sarcolemmal preparations and this is pH- and temperature-dependent (Kulakowski et al.,1978). Both high and low affinity binding sites have been observed in rat and pig sarcolemma (Kulakowski et al., 1981) and it seems that these may modulate calcium binding (Chovan et al.,1980). Subcellular fractionation indicates that specific binding is greater in fractions which are enriched in sarcoplasmic reticulum, and this would be consistant with a calcium modulatory role for taurine in the heart (Quennedy et al., 1986).

1.10 Aims of the present study

The present work was stimulated by the observation that taurine concentration is elevated in the haemolymph of locusts and cockroaches stressed by insecticide poisoning or prolonged physical activity (Jabbar, 1982). The aim of the present study was in general terms to expand upon this observation in order to determine a) the origin of the taurine accumulating in haemolymph and ganglia and b) to study the effects of taurine on the locust nervous system.

It was recognised from the start, however, that no comprehensive study of taurine in insects has been undertaken, and what data exists is fragmentary due to superficial studies on many insect species. It was therefore important in the present study to obtain as much basic information concerning taurine in the whole insect as possible, to create a context into which later results could be assimilated. To an extent this required an approach in which taurine and its possible physiological functions

in the locust took precidence over simply seeing taurine as a substance of importance only under unphysiological conditions. Indeed, it was felt that an understanding of what role, if any, taurine might have in the stress response in insects could only be relevant against a background of greater understanding of taurine in insects in general. Much of the work in the present study has followed paths already taken by workers who have studied taurine in mammals. However, taurine is not a substance like GABA or ACh where a vast literature already exists from mammals. The present work clearly suggests that much of what applies to taurine in mammals also appears to hold for the locust, suggesting that a continuation of these studies might generate data in insects which might later be useful to mammalian physiologists, rarther than the reverse.

As stated the experimental approach to the study was initially to establish a background of basic information concerning taurine in the locust. This first required a study of the distribution of taurine in the locust and, as far as possible, its route of biosynthesis. With this information it became possible to evaluate the changes in homeostasis of taurine which occur during stress.

This was followed by a series of experiments to investigate effects of taurine on the locust nervous system. The latter experiments were all performed <u>in vitro</u> using synaptosomes, isolated somata, and mitochondria.

Finally, a study was taken to investigate possible binding sites for taurine in the locust. In this case it was decided to use a whole head preparation, which is quite common in insect work, rather than embark on the much more time consuming approach of

investigating specific tissues.

SECTION TWO

MATERIALS AND METHODS

2.1 Materials

Insects

Adult locusts, Schistocera americana gregaria of both sexes, were obtained from Larujon Locust Suppliers Ltd., Colwyn Bay, Wales, and were fed on bran and maintained at 30°C. Juvenile locusts, cockroaches (Blatta orientalis) and flour beetles (Tenebrio molitor), were kindly supplied by Dr Ann Lackie of the Zoology Department, Glasgow University, and were bred in that department. The latter were fed on cereal cakes and flour respectively.

Radiochemicals

All radiochemicals were purchased from Amersham International PLC, Amersham, Bucks.

^{45}Calcium (1Ci/mmol)

(methyl-^{3}H)Choline Chloride (80 Ci/mmol)

L-(^{35}S)Cysteine (47 mCi/mmol)

(G-^{3}H)-Dansyl Chloride (13.5 Ci/mmol)

(2,3-^{3}H)4-amino-n-butyric acid (64 Ci/mmol)

(^{3}H)-Inulin (22 Ci/mmol)

(U-^{14}C)Taurine (115 mCi/mmol)

(1,2-^{3}H)Taurine (29 Ci/mmol)

Fine Chemicals and enzymes

The following chemicals were obtained from Sigma Chemical Co., London, U.K.:-

Aminooxyacetic acid

Bovine serum albumin

Cysteamine

Cysteine

Cysteic acid

Cysteine sulphinic acid

Cystine

Ethylene glycol Bis-(β aminoethyl ether) N,N,N',N'-tetraacetic acid

N-ethylmaleamide

Glutathione (oxidised)

Glutathione (reduced)

N-2-hydroxyethylpiperizine-N'-2ethanesulphonic acid

Hypotaurine

Isethionic acid

Nipecotic acid

Ninhydrin

Picrotoxin

Protease type VII P-5255

O-phthalaldehyde

Taurine

Tetrodotoxin

Verapamil

Veratridine

The following chemicals were obtained from British Drug House Chemical Co., Poole, U.K.:-

Arginine

β-alanine

4-amino-n-butyric acid

Dansyl Chloride

Dihydroxyacetate phosphate

Folin and Ciocalteau reagent

Glycine

Hyamine hydroxide (10 % solution in methanol)

KCN

Nitrilotriacetic acid

Oxoglutarate

Proline

The following chemicals were obtained from Fisons Scientific Apparatus, Loughbrough, U.K.:-

Mercaptoethanol

Perchloric acid (72 %)

Sucrose

The following chemicals were obtained from the Boehringer-Manheim, Corporation, London, U.K.:-

Adenosine-5'-diphosphate

Adenosine-5'-triphosphate

Lactate dehydrogenase

Nicotinamide adenine dinucleotide (reduced)

Phosphoenolpyruvate

Pyruvic acid

Tris (hydroxymethyl)-aminoethane

6-aminomethyl 3-methyl-4H-1,2,4-benzothiaziazine-1,1-dioxide hydrochloride (TAG) was supplied by Merck Sharp and Dohme, Pennsylvania, U.S.A.

Methods

2.2 Extraction of taurine from insect tissues

Insects were immobilised by cooling at -20°C for 15 min, before the removal of tissue samples, which were immediately frozen in liquid nitrogen and then ground to a powder using pestle and mortar. The still-frozen samples were immediately transferred into preweighed plastic centrifuge tubes (Eppendorf) containing 0.5 ml of $HClO_4$ on ice. The tissues were then dispersed by homogenisation with a loose fitting teflon homogenisor, driven by an electric motor. The tube was then reweighed to find the weight of the tissue sample. When the amounts of tissue were too small to make this a practicable method, the wet weight of the tissue was estimated by protein content. A conversion factor was applied to determine the wet weight from protein content (Clement and Strang, 1978)

After centrifugation for ten min at 10,000g, the supernatant was removed and the pellet washed with a further 0.5 ml of $HClO_4$ in the same way. The resulting supernatants were combined and the acid neutralised by the addition of one tenth volume of 2M K_2CO_3. The precipitated potassium perchlorate was removed by centrifugation at 10,000g for 5 min, and the supernatant was then removed.

Haemolymph samples were taken according to the method of Clement and Strang (1980), and the protein precipitated with an equal volume of 0.3M $HClO_4$. The samples were then processed as

described above for tissue samples. The extraction and estimation procedure is shown in the form of a flow diagram in Figure 2.1.

2.3 Isolation of taurine

Taurine was isolated using ion exchange chromatography after the method of Garvin (1960). A 200μl portion of the $HClO_4$ extract was passed through the ion exchange column shown in Figure 2.2. The taurine was eluted using a total of 3.0 ml of distilled water. The first 0.5 ml of eluent was discarded since only the following 2.5 ml contain taurine. This method of taurine isolation is possible due to the very low pKa of the acid group on the taurine molecule (-0.3). The recovery of taurine was estimated using an internal standard, and was observed to be almost 100 %.

2.4 Confirmation of the purity of the isolated taurine

To check the purity of the taurine sample after passage through the ion-exchange column the eluent was reacted with an equal volume of dansyl chloride at pH 9.0 and subjected to two-dimensional chromatography on polyamide layers (British Drug House). The solvent used in the first dimension was 3 % formic acid and in the second dimension was either toluene: acetic acid (9:1 v:v) or ethyl acetate: 2-propanol: ammonia (10:4:3 v:v:v). After development the chromatogram was visualised under u.v. light to determine the position of the fluorescent derivatives.

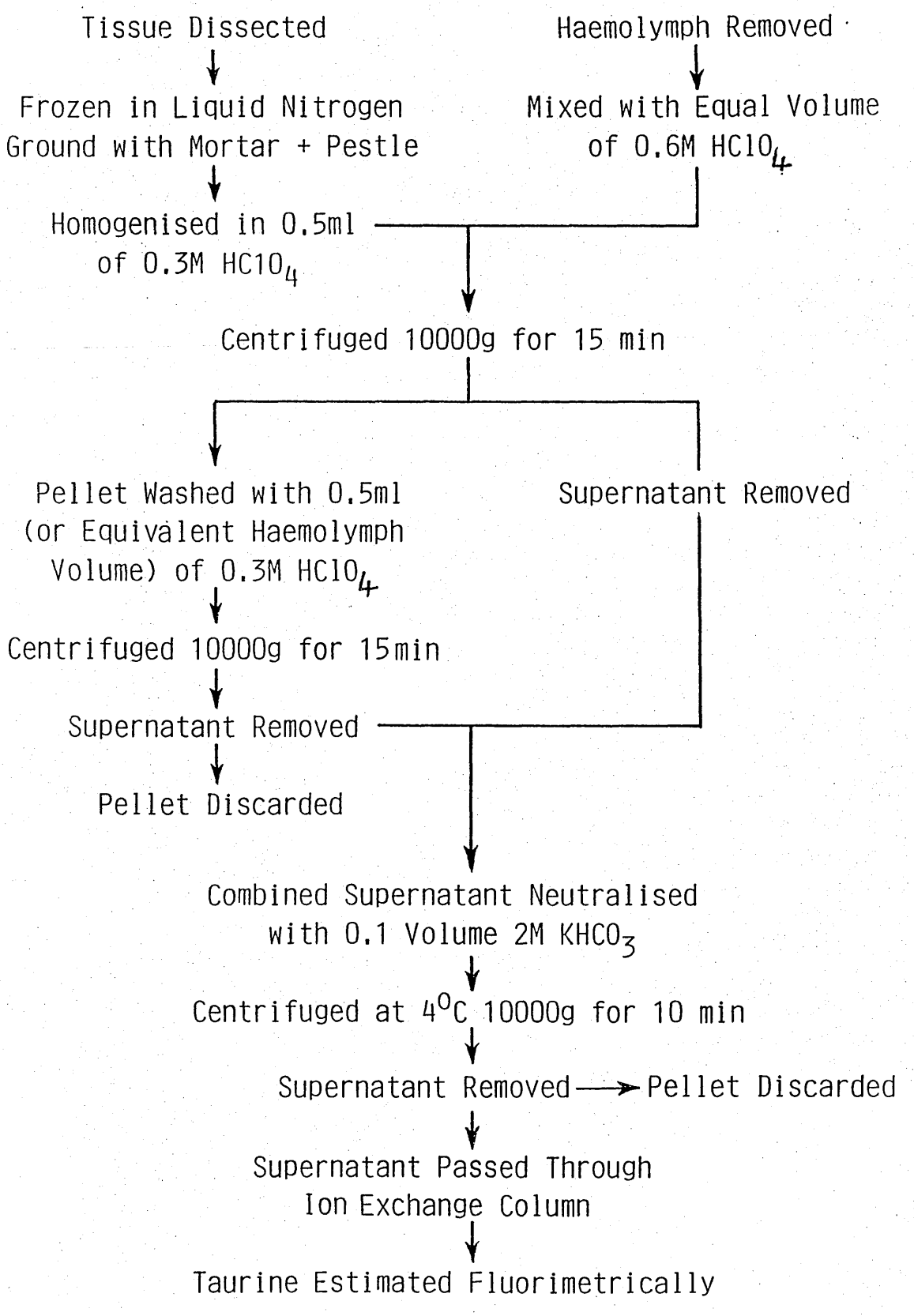


Figure 2.1 Flow diagram illustrating the procedure for the extraction, isolation and estimation of taurine.

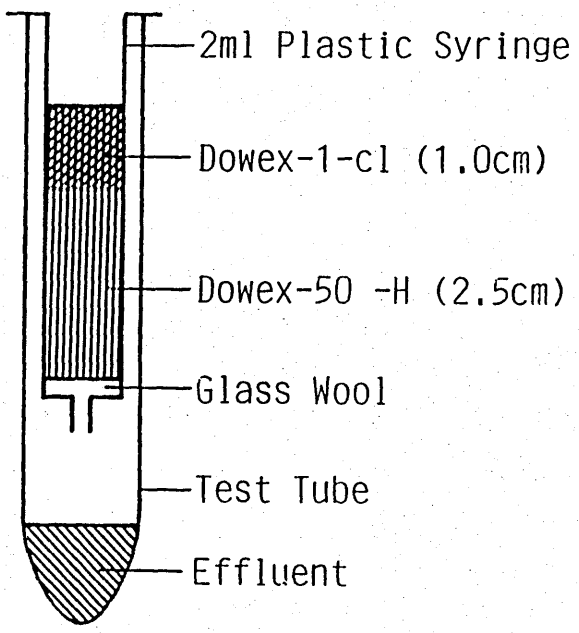


Figure 2.2 Ion exchnge column used for the isolation of taurine from other amino-containing compounds.

2.5 Estimation of taurine

Taurine was estimated by forming its o-pthalaldehyde derivative which was then quantified by spectrofluorimetry (Roth, 1972). A 200µl portion of the effluent from the ion exchange column was taken and mixed with 3.0 ml of the reagent mixture. The latter was composed of 0.05M borate buffer (pH 9.5) 90 ml of which was mixed with 1.5 ml of the o-pthalaldehyde containing solution (10 mg/ml in methanol) and 1.5 ml of mercaptoethanol. The sample was then left for 5 min prior to fluorimetric estimation.

2.6 Induction of stress in locusts

Lindane (a gift kindly supplied by Dr J. Donellan, Shell, U.K.) was dissolved in dimethylsulphoxide and given in a dose of 100µg per insect. Picrotoxin was dissolved in 0.9 % NaCl and given in a dose of 20µg per insect. Injections were made in a 10µl volume into the thorax. Controls received only solvent. After different periods of time up to 6 hr at room temperature tissue samples were taken.

Enforced flying was achived by freely suspending locusts by clips attached to the cuticle at the back in a stream of air at 30°C. At the end of 2 hr flying haemolymph samples were taken for analysis.

2.7 Assay for thoracic Ca^{++}-ATPase (myosin ATPase)

The method was a modification of one personally communicated to me by Dr J. C. Dow of the Biochemistry Department at Glasgow University. Locusts were immobilised by cooling, eviscerated and the wings and legs removed. Thoraces were finely chopped with

scissors before they were homogenised with a bladed overhead homogeniser. The tissue (1-3 thoraces) was homogenised for 10 sec in 10 ml of ice-cold 25 mM Tris buffer (pH 7.5) containing 1mM EGTA. This was sufficient to completely disrupt the muscle tissue, and longer periods of homogenisation were found to reduce the activity of the enzyme. Two 3 ml samples of this homogenate were then taken into conical flasks in ice containing 3 ml of 20mM Mg^{++} ATP. To the experimental flask was then added 3 ml of 100mM $CaCl_2$, while the blank received 3ml of water. After mixing, and while the flasks were still at $0^{o}C$, duplicate 0.5 ml samples were taken from each, and mixed with an equal volume of $HClO_4$. The assays were then incubated for 10 min at $30^{o}C$ and further samples taken. The acidified samples were centrifuged at 10,000g for 5 min and samples (100μl) taken to estimate the release of inorganic phosphate in a final volume of 700μl by a micro-modification of the method of Rockstein and Herron (1951). Blanks were found to have 20-30 % of the activity of the full assay.

2.8 Assay for arginine kinase

The method was amodification of that used to estimate creatine kinase activity in mammals, based on the production of ATP (Bernt and Bergmeyer, 1965). The reaction was followed by spectrophotometry at 340nm. The assay mixture had a volume of 1.2 ml and contained final concentrations of components as follows: 0.5M glycine buffer pH 9.0, 20mM Mg^{++} ATP, 1mM phosphoenolpyruvate, 0.2mM NADH, 20mM arginine, 70 U pyruvate kinase and 100 U lactate dehydrogenase. The activities of tissue extracts were noted before and after addition of arginine, and the net increase in rate gave

a measure of the activity of the arginine kinase. When muscle homogenates were being assayed, the mixture contained 10mM KCN to prevent adventicious oxidation of the NADH, but this was not found to be necessary with the haemolymph samples.

Haemolymph was sampled by carefully puncturing the ventral thoracic cuticle, and drawing the resultant drop of haemolymph into a graduated 5μl micopipette by capillary action only. More disruptive methods were found to cause tissue damage giving a falsely high activity of arginine kinase. Duplicate (1μl) samples were added to the assay mixture and the activity followed for 5 min before and after the addition of arginine.

To assay the activity of the soluble enzyme in the flight muscle, samples of muscle were taken from cooled locusts, weighed and then homogenised for 1 min in 1 ml of the glycine buffer containing 1mM mercaptoethanol and 0.3M sucrose (the latter to prevent any contamination of the cytoplasmic with mitochondrial arginine kinase). The homogenates were then centrifuged at 10,000g for 15 min, before removing the supernatant which was diluted ten-fold in the homogenising buffer before taking 1μl samples for assay.

2.9 Estimation of haemolymph volume

The volume of haemolymph in the living insect was estimated by the indirect isotope dilution procedure of Loughton and Tobe (1969). 1μCi of an aqueous solution of ^{3}H-inulin was injected into the thoracic cavity in a volume of 10μl. 30 min after injection, when the inulin had become distributed throughout the

body cavity, the thorax was punctured and a graduated 10μl micropipette used to take duplicate 10μl samples of haemolymph for determination of radioactivity by scintillation counting. The total haemolymph volume was calculated from the dilution of the isotope.

2.10 Uptake of U-^{14}C-taurine into locust tissues in vivo

^{14}C-taurine was given in a dose of 0.25μCi/g body weight of locust. ^{14}C-taurine had previously been diluted in insect saline (mM NaCl 214; KCl 3.1; $CaCl_2.6H_2O$ 2.05; $Na_2HPO_4.12H_2O$ 2.4; $NaH_2PO_4.2H_2O$ 0.2; $MgCl_2.6H_2O$ 6.6; glucose 16.7) so that a volume of 10μl/g was administered using an Hamilton syringe.

Haemolypmh was collected by the method of Clement and Strang (1980). Tissue samples were taken of eye, thoracic ganglia and flight muscle and taurine was extracted as described in section 2.2. Tissue weight was estimated by protein content. Supernatant was taken for scintillation counting.

2.11 Effects of structural analogues on U-^{14}C-taurine clearance from haemolymph

To study the effects of structural analogues on ^{14}C-taurine clearance from the haemolymph, isethionic acid, β-alanine, hypotaurine and GABA were injected in a dose of 1μmole/g in 10μl of insect saline using an Hamilton syringe, by puncture at the base of a leg.

All inhibitors were administered 5 min prior to injection

of ^{14}C-taurine. To study the effects of cold on taurine clearance from the haemolymph insects were immobilised by cooling in a deep freeze for 10 min before injection of ^{14}C-taurine, and were kept in ice thereafter. In all cases where the effects of inhibitors were studied haemolymph was collected 1 hr after injection of ^{14}C-taurine.

2.12 Metabolism of ^{14}C-taurine by locusts in vivo

Tissue extracts were taken into 75 % ethanol and resolved on cellulose thin layer chromatography (TLC) plates (Merck). The extracts were double-developed in one-dimension as desribed below. After resolution 0.5 cm bands were scraped from the plate so that the entire plate was analysed for radioactive spots. The position of taurine and isethionic acid on the plate was determined using standards. Taurine was visualised with 1 % ninhydrin in acetone, while isethionic acid was visualised using 0.1 % 2,6 dichloro-fluorescene. After scraping the plate the bands were then transfered to scintillation vials for counting.

2.13 Estimation of endogenous levels of taurine and its precursors

Estimation of taurine and its precursors was made using the procedure of Osborne (1973), using ^{3}H-dansyl chloride derivatives. Taurine and its potential precursors were extracted from haemolyph, eye, thoracic ganglia, flight muscle and fat body into 75 % ethanol. A 5μl portion of the supernatant was reacted with an equal volume of 1mM ^{3}H-dansyl chloride in acetone, buffered to pH 9.0 with bi-carbonate buffer. After allowing 30 min for the reaction, 5μl of

the mixture was then spotted onto a polyamide TLC plate and resolved in two dimensions as described in section 2.4. Visualisation of the spots under u.v. light was aided by the addition of carrier amounts of unlabelled dansyl-amino acids to the labelled extract. Identified spots were cut out, tranferred to scintillation vials containing 0.5 ml of scintillation grade Hyamine 10-X hydroxide, and left to elute overnight prior to scintillation counting. Quantification of the unknowns was made by comparing the counts incorporated into the samples with standards in which known ammounts of amino acid were reacted with ^{3}H-dansyl chloride.

2.14 Metabolism of ^{35}S-cysteine in vitro

Samples of eye, thoracic ganglia, flight muscle and fat body were taken and transferred to tubes containing 0.3 ml of insect saline with 10μCi of ^{35}S-cysteine. Samples were incubated at 30^{o}C for 6 hr with a fine stream of 95 % O_2/ 5 % CO_2 continuously passed through the medium. In order to prevent evaporation of the saline the gas mixture was first passed through water at 30^{o}C. Samples were extracted into 75 % ethanol containing 10mM N-ethylmaleamide. The latter reacts with free SH groups and enhances the resolution of cysteine and cysteamine from other sulphur containing compounds during TLC (Cotty et al., 1958).

2.15 Two-dimensional chromatography and autoradiography

Supernatant from studies in vitro of ^{35}S-metabolism was resolved using two-dimensional chromatography. Cellulose TLC plates were cut to give an 8 cm run in both dimensions. 5μl aliquats of supernatant were applied to the plate. The chromatagram was double-developed in

the first dimension using butanol:acetic acid:H_2O (11:6:3 v:v:v) as solvent and then once in the second-dimension using methanol:pyridine: H_2O (25:1:5 v:v:v) as solvent.

The plates were then autoradiographed to locate the radioactive spots. The plates were taped onto cut sheets of aluminium and, in a dark room, lit only by a safety lamp, Kodak X-omat AR film was then taped into position over the TLC plate and sandwiched between another cut sheet of aluminium. The sheets were then tightly clamped together and covered with light proof wrapping and left for up to 9 days. At the end of this period films were unwraped under safety lamp and developed for 5 min in developer and 5 min in fixer. Compounds were identified by comparison with standards visualised with ninhydrin.

2.16 Metabolism of ^{35}S-cysteine in vivo

Locusts were injected with a dose of 20μCi of ^{35}S-cysteine in 10μl of insect saline, and at times thereafter samples were taken of haemolymph, eye,thoracic ganglia, flight muscle and fat body. Tissues were homogenised in 75 % ethanol (0.5 ml) containing 10mM N-ethylmaleamide. After homogenisation the samples were centrifuged at 10,000g for 5 min, and the supernatant was then taken for application on to cellulose TLC plates. 10μl of supernatant was spotted on to each plastic backed cellulose TLC plate. Each sample was run in triplicate along side each other to maximise the amount of material recovered. The TLC plate was cut so that each run was exactly 8 cm. The plate was double-developed in butanol:acetic acid: H_2O (11:6:3 v:v:v) and after drying was dipped into a 1 % solution

of ninhydrin in acetone and heated for 5 min in an oven at 100°C to reveal amino-containing material. At either side of each TLC plate a set of standards was run in order to locate the bands containing taurine and its precursors. The bands corresponding to taurine and its precursors were then scraped into scintillation vials for counting.

To study the effects of picrotoxin and flight on ^{35}S-cysteine metabolism locusts were treated as described in section 2.6. In the case of flown locusts, ^{35}S-cysteine was administered 4 hr prior to the 2 hr of flight.

2.17 Preparation of synaptosomes

Synaptosomes were prepared using a modification of the method of Breer (1981). Thoracic and cerebral ganglia were homogenised in an hand held homogeniser in 5 ml of 0.25 M sucrose buffered to pH 7.5 using 100mM Tris adjusted with HCl. After centrifugation at 500g for 10 min the supernatant was collected and the pellet was rehomogenised in a further 5 ml of buffered sucrose and centrifuged again. This was repeated once more giving a combined total of 15 ml of supernatant which was then centrifuged at 15,000g for 45 min. The resulting synaptosomal pellet was then very gently resuspended in buffered sucrose. Preparation was performed in a cold room at 4°C.

2.18 Uptake of ^{3}H-taurine and ^{3}H-GABA into synaptosomes

Synaptosomes were incubated in insect saline containing 2μCi of labelled amino acid and in the case of the GABA experiment 50μM AOAA to prevent GABA metabolism. About 100μg of synaptosomal pro-

tein was incubated with 200μl of insect saline at 30^{0}C. The incubation was stopped by the addition of 2 ml of ice-cold saline, followed by rapid filtration through 25mm diameter filters (0.45 μm pore size). The filters were then washed with a further 4 ml of cold saline, and were then placed in a scintillation vial containing 1 ml of 2 % Triton X-100 for 1 hr prior to counting.

The concentrations of taurine and GABA were 340 and 170nM respectively. In one experiment however, 1mM unlabelled taurine was present in addition to the ^{3}H-taurine.

2.19 Effect of taurine and nipecotic acid on synaptosomal GABA uptake

Synaptosomes were incubated for 2 min in insect saline containing 2μCi of ^{3}H-GABA and the incubation was stopped as descibed above. Taurine concentration was in the range of 1-12mM, while nipecotic acid was present at concentrations between 0.01-1.0mM.

2.20 Uptake of $^{45}Ca^{++}$ into synaptosomes and effect of inhibitors

Synaptosomes were incubated under the conditions described in section 2.18, with 2μCi of $^{45}Ca^{++}$ in the saline. In experiments other than those in which the time course of $^{45}Ca^{++}$ uptake into resting synaptosomes was studied, the incubation time was 3 min. Synaptosomes were depolarised using either K^{+} (100mM) or veratridine (100μM) the latter of which was dissolved in a small volume of dimethylsulphoxide prior to being added to the saline. An equal volume of DMSO was added to the saline of control samples. Taurine was present in the 5-20mM range, GABA and leucine were present at 10mM, tetrodotoxin at 1μM and verapamil at 10μM.

2.21 Release of ^{3}H-ACh and ^{3}H-GABA from synaptosomes

Experiments in which synaptosomes were perfused were performed using insect saline which contained 50μM AOAA when ^{3}H-GABA was used. In order to preload the synaptosomes the entire resuspended synaptosomal pellet was preincubated for 5 min in 800μl of insect saline. This was then transferred to a tube containing 12.5μCi of either ^{3}H-choline or ^{3}H-GABA in 50μl of saline, and was incubated for a further 15 min at 30^{o}C. Locust synaptosomes incubated with ^{3}H-choline have been observed to release ^{3}H-ACh when stimulated with high K^{+} or veratridine (Breer and Knipper, 1984). The synaptosomes were then loaded on to Whatman GF/B filters (about 90μg synaptosomal protein per filter) in the apparatus used for superfusion shown in Figure 2.3. The flow rate was set at 1 ml/ min and 20 ml of saline was passed over the synaptosomes to allow the preparation to stabilise, after which 2 ml fractions were collected using a Watson-Marlow fraction collector. Synaptosomes were depolarised by challenging with either high K^{+} concentration (100mM) or veratridine (100μM) at the times indicated in the Figures in the results section. The collected fractions were then transferred to scintillation vials for counting.

Drugs were added by carefully pouring insect saline containing a known drug concentration into the syringe mounted on each filter unit (Fig. 2.3). Since the residual volume of the filter unit was only 180μl, the time between adding the drug and its arrival at the synaptosomes was very small. Taurine was present in the 5-20mM range, nipecotic acid was used at 1mM, and tetrodotoxin was used at a concentration of 1μM. When release of neurotransmitter was

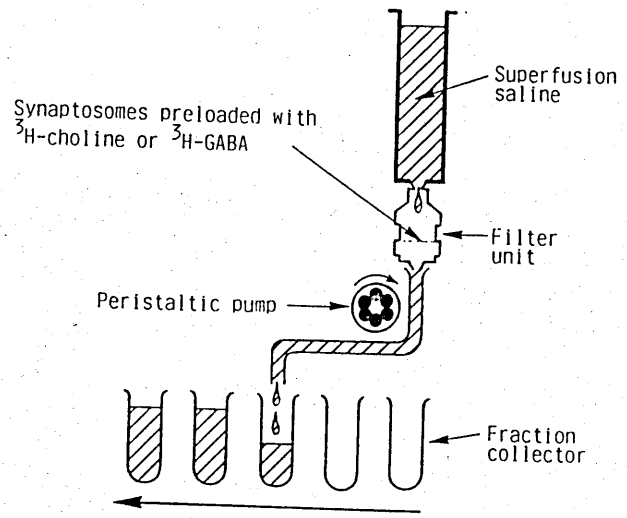


Figure 2.3 Apparatus used for superfusion of synaptosomes.

studied in the absence of Ca^{++}, EGTA was present at 1mM.

2.22 Preparation of flight muscle and nervous tissue mitochondria

Mitochondria from flight muscle were prepared from the combined flight muscles of 12 locusts, dissected on ice, wiped clean of adhering fat body, and transferred into 20 ml of ice-cold medium (0.25M sucrose, 5mM Tris, 1mM EGTA) at pH 7.4. 5 ml of the same medium containing 3.5 mg of protease (Sigma, type VII, P-5255) was added to the isolated flight muscle which was digested for 12 min on ice using an overhead homogeniser with a very loose-fitting pestle to stir and disaggregate the mixture every 2 min. The mixture was then filtered through gauze and centrifuged for 10 min at 10,000g and the supernatant discarded. The pellet was carefully resuspended in 10 ml of medium and centrifuged at 800g for 5 min. The supernatant was removed and centrifuged at 10,000g to sediment the mitochondrial pellet which was then resuspended in 0.6 ml of 0.25M sucrose buffered with 5mM Tris at pH 7.4. The entire procedure is illustrated in the flow diagram shown in Figure 2.4.

Mitochondria from nervous tissue were prepared by homogenising the thoracic ganglia of 20 locusts in 1 ml of medium (as described above) for 1 min at 1000 rpm, in an all glass homogeniser. The supernatant was then taken and a further 9 ml of medium was added to it and this was centifuged at 800g for 5 min. The supernatant was removed and centrifuged at 10,000g for 5 min to sediment the mitochondrial pellet, which was

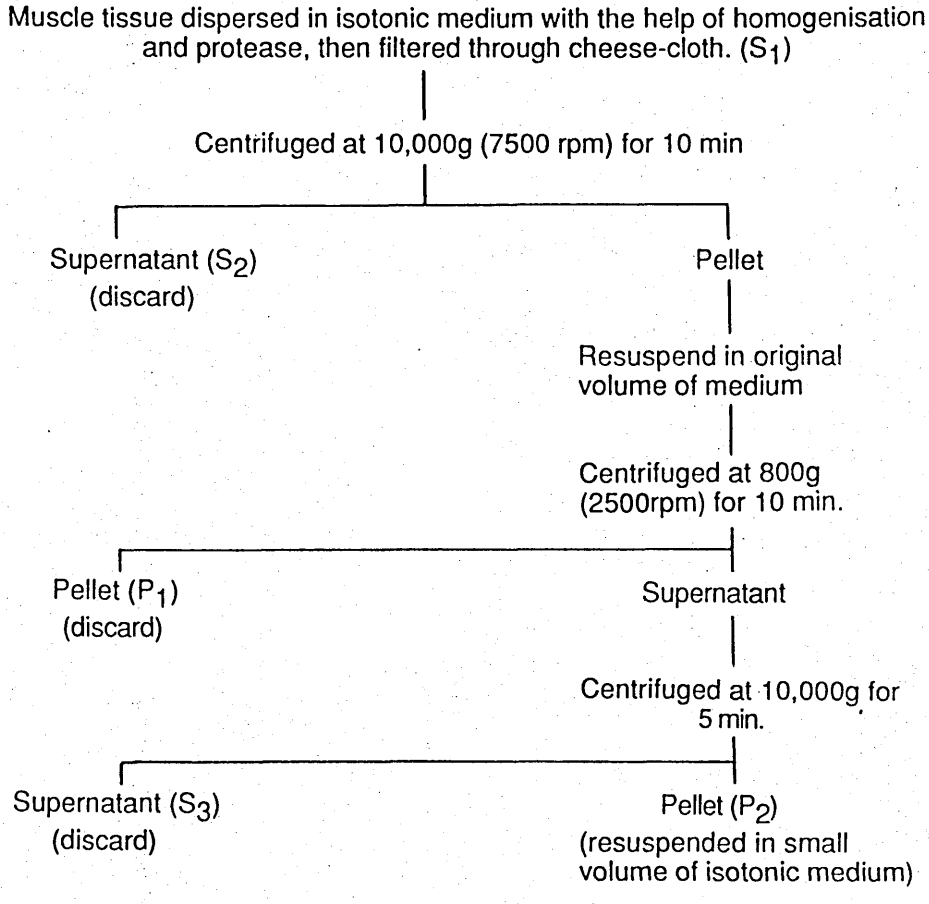


Figure 2.4 Flow diagram illustrating the procedure for the preparation of mitochondria from locust flight muscle.

resuspended as described for flight muscle mitochondria. All preparative procedures for both muscle and nervous tissue mitochondria were performed at 0-4^{o}C.

2.23 Estimation of the purity of the flight muscle mitochondria

Since the methodology for obtaining mitochondria from ganglia and the enzymic characterisation of the preparation was performed in our laboratory previously, the latter was not repeated in the present work. This did not apply to the flight muscle preparation, however, and this was therefore characterised by assaying the enzymes NAD-linked-glycerol-phosphate dehydrogenase (GPDH), which is resticted to the cytoplasm, and glutamate dehydrogenase, which is found only in the mitochondrial matrix.

Both enzymes were assayed by following the oxidation of NADH by the decline in absorbance at 340nm. Enzymes were assayed in a medium of 0.3M sucrose buffered at pH 7.4 (50mM Tris adjusted with HCl) containing 10mM KCN and 0.2mM NADH. Assays were performed at 30^{o}C. The final concentrations of other components were as follows: in the case of glutamate dehydrogenase the assay also contained 10mM oxoglutarate and 2mM ADP, while the GPDH assay contained 0.5mM dihydroxyacetone phosphate.

After initial rates of activity had been established for at least 5 min, Triton X-100 was added to the cuvette to give a final concentration of 0.2 %. This released any occluded enzyme, and the new activity was assayed for a further 5 min.

2.24 Incubation of mitochondria

Mitochondria were incubated in a medium of the following composition: 0.25M sucrose, 10mM KH_2PO_4, 5mM proline, 1mM pyruvate, 1mM nitrilotriacetate (a relatively weak buffer of free calcium), 16μM bovine serum albumin, 1mM ADP and 0.2μCi of $^{45}Ca^{++}$, at 30^{o}C. To study the effect of taurine on efflux of $^{45}Ca^{++}$ from mitochondria, they were first loaded with $^{45}Ca^{++}$ by incubation for 2 min, at which point further calcium accumulation was prevented by the addition of either ruthenium red (an inhibitor of mitochondrial calcium uptake) or EGTA to give final concentrations of 5μM and 500μM respectively. NaCl was added to a concentration of 10mM in order to activate Na^{+}-dependent Ca^{++} efflux from the mitochondria. Taurine was present at 20mM concentration. All incubations were performed in 200μl of medium to which 20μl (about 70μg of protein) of the mitochondrial suspension were added. Incubations were stopped as described for synaptosomes in section 2.18, after which filters were placed in vials containing 1 % Triton X-100 to lyse the mitochondria for 1 hr prior to scintillation counting.

2.25 Preparation of membranes from whole locust head

Taurine binding was studied using membranes prepared from whole locust heads. The heads of 30 locusts were removed, the antennae cut off, and then transferred into 50 ml of ice-cold Van Harrevald's saline (composition (mM): NaCl 205; KCl 5.4; $CaCl_2.6H_2O$ 13.6; $MgCl_2.6H_2O$ 2.6; Tris 5.0 adjusted to pH 7.7 using HCl) and homogenised using an overhead bladed homogeniser for 30 sec

in a cold room at $4^{o}C$. The homogenised heads were then filtered through gauze and the filtrate was then rehomogenised using ten strokes of an all glass motor driven homogeniser. The filtrate was then centifuged at 5000g for 10 min and the supernatant was then filtered again through cotton wool. The filtrate was then centrifuged at 15,000g for 30 min, after which the supernatant was discarded and the pellet resuspended in 20 ml of saline and centrifuged again at 15,000g for 30 min. The pellet was resuspended in 20 ml of saline and dialysed overnight against a large volume of Van Harrevald's saline to remove the endogenous taurine that had not been removed by the washings. After dialysis the preparation was centrifuged again at 15,000g for 30 min, and then resuspended in 10 ml of saline.

2.26 Incubation of the membrane preparation

0.5 ml of the membrane preparation in Van Harrevald's saline were incubated with 2μCi of ^{3}H-taurine, alone or along with 1mM unlabelled taurine. Most incubations were performed at room temperature ($25^{o}C$), except when the temperature-dependency of binding was studied, in which case the experiment was performed at $0^{o}C$. To study the Na^{+}-dependency of binding the NaCl in the saline was replaced by the same concentration of choline chloride. Most incubations were terminated by centrifugation in which 0.4 ml of the incubation mixture was transferred to 0.4 ml tubes with an elongated tip to receive the pellet (Starstedt, 72702 tubes, Leicester, U.K.) and then centrifuged at 15,000g for 30 min. The supernatant was removed by suction and discarded and the tip of the tube was cut off exactly at the point of the membrane pellet. The tip was then cut transversely, through the membrane pellet,

into three parts which were placed in a scintillation vial containing 0.5 ml of soluene to solubilise the pellet prior to counting. In one experiment incubation was terminated by the addition of 2 ml of ice-cold saline and rapid filtration through Whatman GB/C filters. The filters were then washed with either 5 ml of cold saline or distilled water. Filters were then placed in scintillation vials containing 1 ml of 2 % triton overnight prior to counting. The latter experiment was performed in order to assess whether association of taurine with the membrane preparation was the result of uptake into vesicles formed by the preparation.

2.27 Preparation of dissociated locust somata for intracellular recording

Thoracic ganglia from locusts were desheathed and dissociated mechanically, without enzyme treatment, by repeated passage through a pasteur pipette. Ganglia were dissociated, and subsequent recordings were made in a saline of the following composition (mM): NaCl 214; KCl 3.1; $CaCl_2$ 9.0; Hepes 5.0 adjusted to pH 7.0 with 1m Tris. Dissociated somata were plated on to plastic petri dishes (5 cm diameter) in a final volume of 1.5 ml per dish, and were allowed to stand for at least 10 min to allow the cell bodies to adhere to the dish.

2.28 Intracellular recording from locust somata

Petri dishes were mounted on the stage of an inverted optics microscope using phase contrast optics to locate the cell bodies. Cell diameters ranged from 10-100μm, and in general somata with diameters in the 30-70μm range were used for impalement. Intra-

cellular electrodes were pulled from thin walled, fibre-fused, glass capilliaries which were back-filled with 1M potassium acetate at pH 7.0. The electrodes were mounted on a micromanipulator (Carl Zeis Jena, D.D.R.) and connected via a silver/ silver chloride wire to the headstage of a high input resistance preamplifier (Winston 1090/ BR1, Winston Electronics Co., U.S.A.). The amplifier was equipped with an active bridge circuit which permitted passage of current through the recording electrode to polarise the cell. Stimulating pulses were generated using a Grass S44 stimulator. Potentials were monitored on an oscilloscope (Iwatsu SS-5702) and hard-copy recordings were obtained using a BBC SE120 pen recorder.

2.29 Application of taurine and GABA.

Taurine (0.5M, pH 8.0) and GABA (1M, pH 4.5) were iontophoresed through 'recording pipettes' (section 2.28) using the current pulses from a model 160 micro-iontophoresis programmer (W.P.I. Inc., U.S.A.). Pipette tips were positioned approximately 4-5μm from the cell under test. To prevent diffusion of the amino acid from the pipette tip negative holding currents of 40-50nA were applied.

Since the ejection currents for taurine and GABA were found to differ considerably to obtain a given response from the cell an estimate of the quantities of amino acid ejected was made using labelled amino acids. ^{3}H-taurine and ^{3}H-GABA were ejected into petri dishes containing 1.5 ml of saline using ejection currents of 400 and 40nA respectively for a 5 min period. The saline was then transferred to scintillation vials for counting.

2.30 Drug application to locust somata

Potential effectors of taurine or GABA mediated responses were applied by a simple bolus perfusion method. Drugs were prepared at twice the stated concentration and loaded in disposable syringes, connected via a perfusion line to the saline bath. Gentle positive pressure was applied to expel the drugs and a volume sufficient to double the bath volume was added. Drug application was therefore all-or-none, and no wash-out facilities were available using this procedure.

2.31 Protein estimation

After solubilisation of samples in 0.1M NaOH, containing 1 % sodium dodecylsulphate, protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.32 Scintillation counting

Samples for scintillation counting were mixed with either Ecoscint or Instagel and counted on a Beckman LS 6800 scintillation counter. To obtain efficiencies, in order to derive dpm, a channels ratio method was used. The efficiencies were approximately 40 % for ^{3}H and 70-80 % for ^{14}C, ^{35}S and $^{45}Ca^{++}$.

SECTION THREE

RESULTS

3.1 Separation and estimation of taurine

After passage of tissue extracts through the ion-exchange column all amino-containing material was retained on the column with the exception of taurine. This was confirmed by making dansyl derivatives of extracts before and after passage through the column. The latter were resolved by TLC on polyamide plates which were visualised under u.v. light (Fig. 3.1).

A range of taurine concentrations over which fluoresence was directly proportional to concentration was established using standards (Fig. 3.2), and tissue extracts were estimated within this range.

3.2 Distribution of taurine in the adult locust

Table 3.1 shows the concentration of taurine in various tissues of *S. gregaria*. The amino acid was found in all tissues examined, and was present at particularly high concentration in the flight muscle (25μ-mol/g). The concentration in the eye was also relatively high, but was considerably less than that in flight muscle (Table 3.1).

3.3 Distribution of taurine in juvenile locusts

For the sake of simplicity individual tissues were not investigated, but the taurine concentration was measured in head, thorax and abdomen. Concentrations of taurine in the abdomen and head varied little at different stages between hatching and adulthood, averaging 1.7μmol/g in abdomen and 2.1μmol/g in the head. The concentration

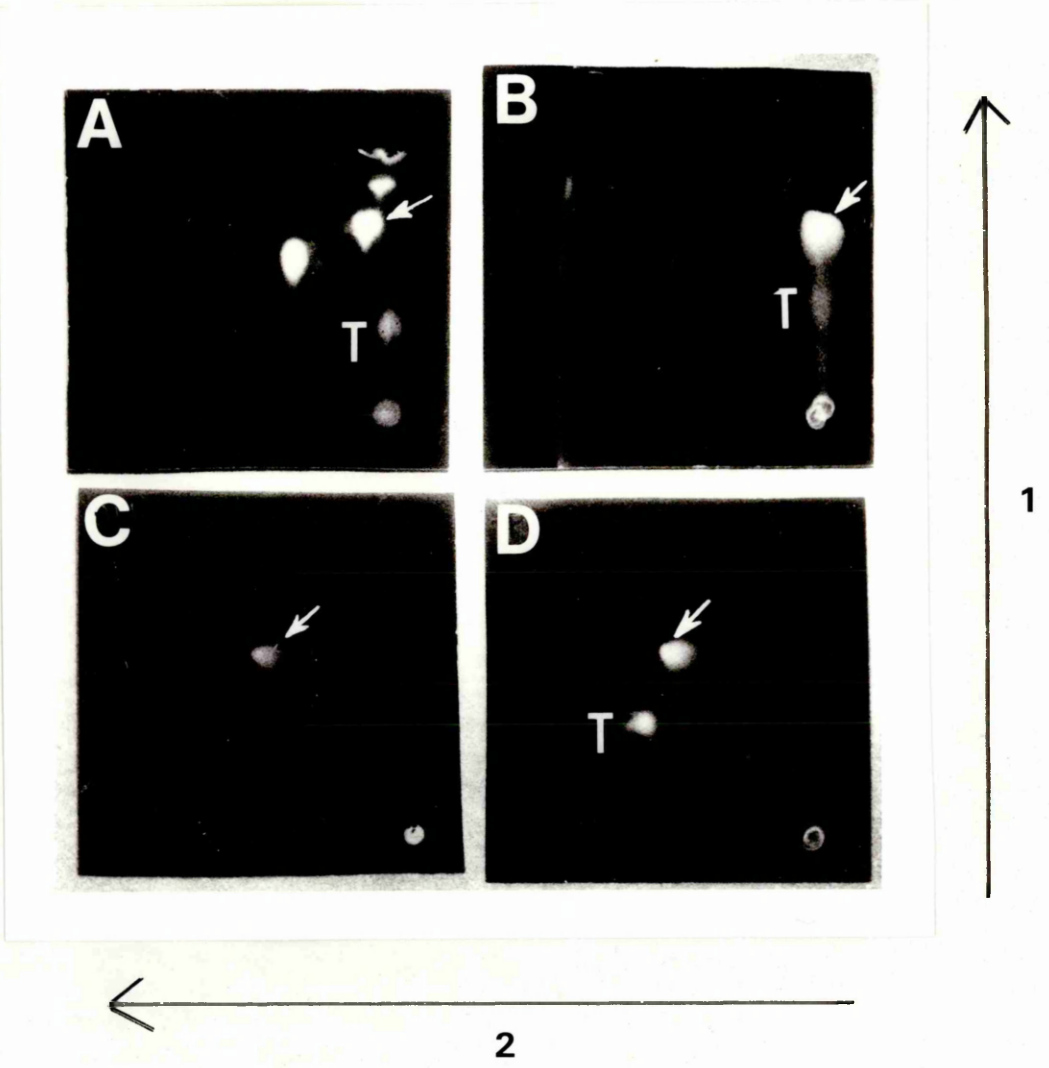


Figure 3.1 Chromatograms on polyamide layers of dansylated extracts of whole locust body before (A) and after (B-D) passage through the ion exchange column. Chromatograms were photographed under u.v. light. In all cases the first dimension (1) was run in 3 % formic acid. The second dimension was run in either toluene:acetic acid (9:1 v:v) for A and B, or ethyl acetate:88 % ammonia:2-propanol (40:30:100 v:v:v) for Cand D. Dansyl taurine is indicated by the letter T, while the arrow points to dansyl hydroxide.

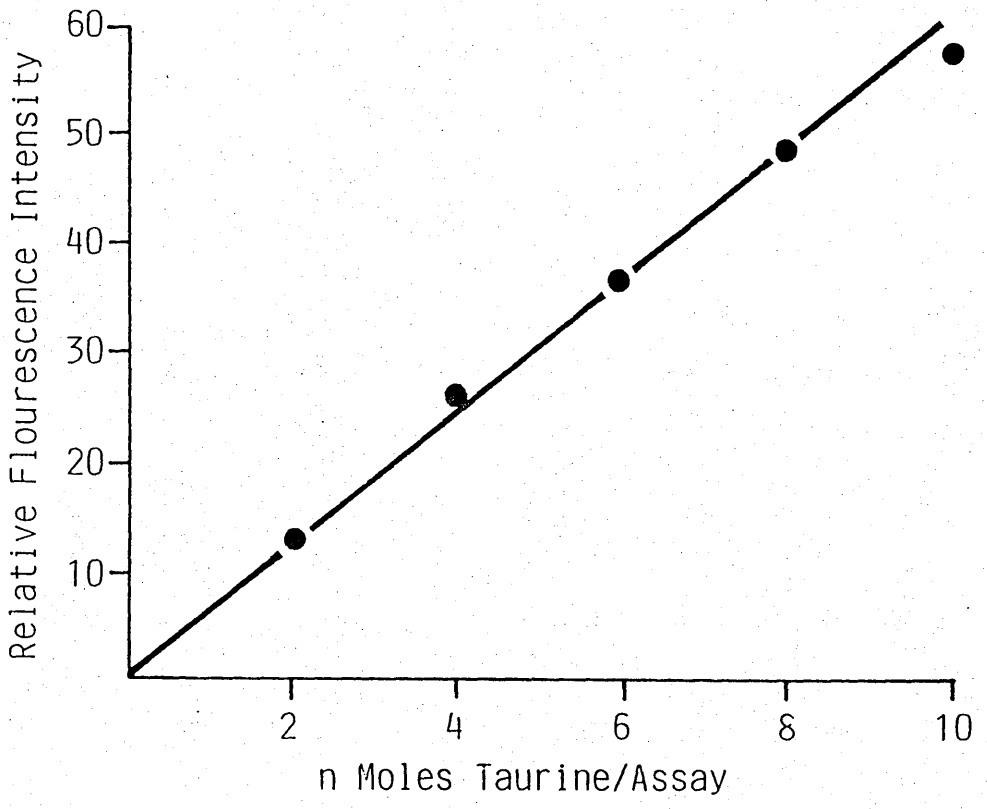


Figure 3.2 Standard curve for the fluorimetric assay for taurine.

Table 3.1 Taurine distribution in the adult locust

Tissue	Taurine concentration (μmol/g wet weight) ± S.D.
Whole body	6.20 ± 0.84
Haemolymph	1.49 ± 0.56
Gut	0.72 ± 0.21
Fat body	1.01 ± 0.31
Eye	7.17 ± 1.31
Cerebral ganglia	1.51 ± 0.17
Thoracic ganglia	2.31 ± 0.31
Flight muscle	25.63 ± 3.17

Results are the mean of 4-6 locusts in each case

Table 3.2 Relationship of taurine concentration to the activity of myosin ATPase in the locust thorax

Instar	Specific activity of ATPase (mmol Pi released/g wet weight per/min) (a)	Taurine (μmol/g) (b)	Ratio (a/b)
5	23	3.3	7.0
6 (1 day after)	38	4.4	8.7
6 (25 days after)	50	11.3	4.4

Values are the averages of duplicate estimates of myosin ATPase. Taurine concentrations taken from Fig 3.3

in the head showed some tendency to rise as the locust became more mature, giving an adult concentration of 3.7 ± 1.9 μmol/g compared with 1.6 ± 0.3 μmol/g in the first instar. In the case of the thorax there was a slow increase during the larval stages, with a final dramatic increase during the first three weeks of adult life (Fig. 3.3). The greatest development of flight muscle capacity, and the increase in thoracic taurine concentration both take place between the fifth instar and adulthood in the locust. Taking Ca^{++}-myosin-ATPase activity as an estimate of muscle mass, the greatest increase in taurine concentration lagged slightly behind the increase in muscle mass (Table 3.2), but the two are obviously related.

3.4 Distribution of taurine in the body segments of the cockroach, beetle and adult locust

Table 3.3 shows the distribution of taurine in the main body segments of *B. orientalis*, *T. molitor* and *S. gregaria*. These insects were chosen beacause of their differences in flight capacity. The flour beetle is flightless, the male of *B. orientalis* has quite well developed wings while those of the female are vestigial, and the locust is a powerfull flying insect capable of long migratory flights.

There were no significant differences in the taurine contents of head and abdomens of the three species studied, but clear differences were observed in the thoraces.

3.5 Effects of stress on taurine concentration in locust tissues

Figure 3.4 shows the effect of Lindane poisoning on the taurine concentration of haemolymph over a period of 6 hr. Taurine concentra-

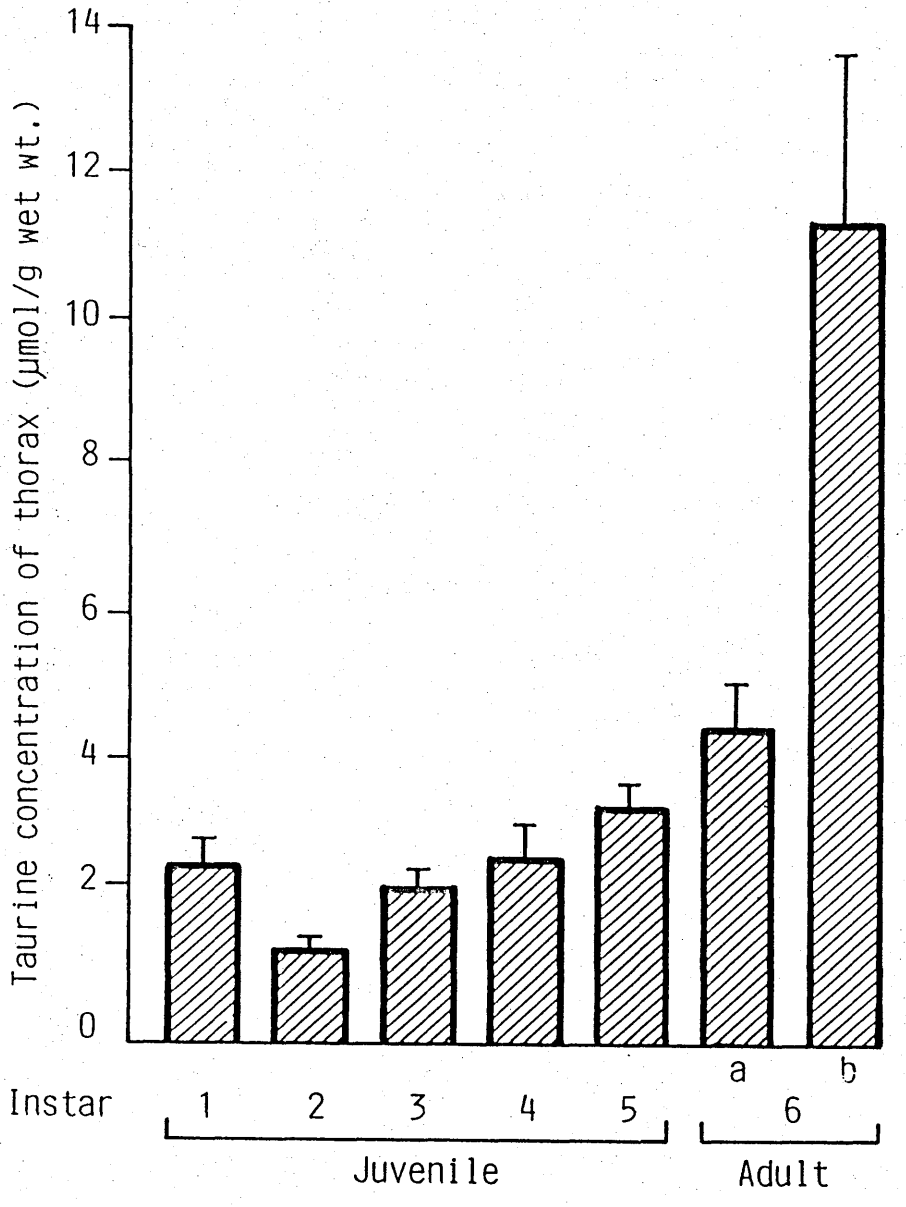


Figure 3.3 Taurine concentration in the thoraces of locusts throughout their development. Adult samples were taken at (a) 1 day, and (b) 25 days after final ecdysis. Error bars represent s.e.m. n=4.

Table 3.3 Taurine concentration in body segments of adult insects

	Taurine concentration (μmol/g wet weight)		
Species	Head	Thorax	Abdomen
S. gregaria	3.63 ± 1.74	11.33 ± 2.41	1.85 ± 0.48
B. orientalis (male)	3.93 ± 0.35	10.48 ± 1.95	0.99 ± 0.65
B. orientalis (female)	3.55 ± 0.12	4.61 ± 0.63	0.74 ± 0.63
T. molitor	5.92 ± 2.20	1.79 ± 0.16	1.65 ± 0.05

Results are the mean ± S.D. of 4-6 insects in each group

Table 3.4 Effect of picrotoxin on taurine distribution in locusts

	Taurine concentration (μmol/g wet weight	
Tissue	Control	Treated
Whole body	6.20 ± 0.84	5.88 ± 0.37
Haemolymph	1.10 ± 0.32	5.76 ± 1.59*
Cerebral ganglia	1.51 ± 0.17	4.05 ± 1.22*
Thoracic ganglia	2.31 ± 0.31	5.09 ± 1.57*
Fat body	1.01 ± 0.31	2.88 ± 0.27*
Eye	7.17 ± 1.31	5.32 ± 0.99*
Flight muscle	24.51 ± 2.95	19.47 ± 1.67*

Results are the mean ± S.D. of 4-6 adult locusts in each group.

* denotes significant difference from controls ($P < 0.05$).

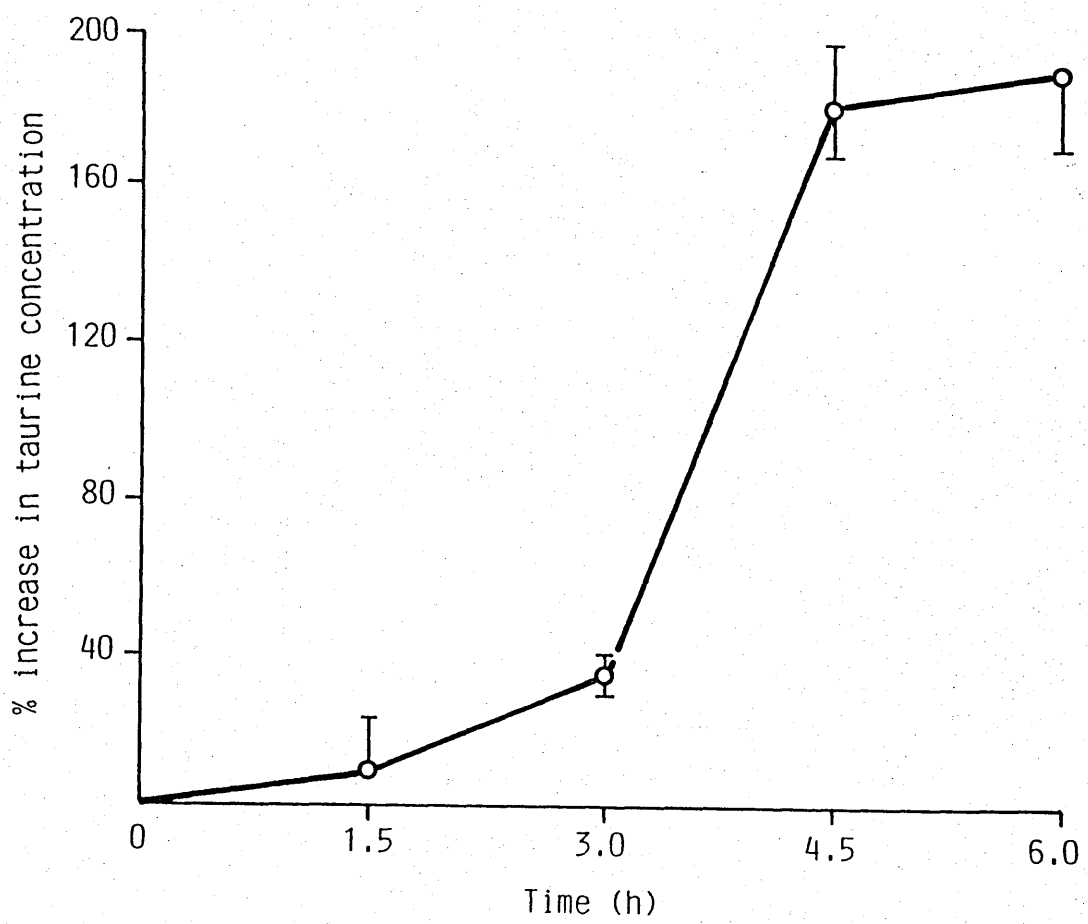


Figure 3.4 Effect of Lindane on the concentration of taurine in the haemolymph of locusts. Doses of 100μg Lindane per insect were injected in 10μl of dimethylsulphoxide. Controls received only solvent. Vertical bars indicate s.e.m. Concentration differences were significantly different from control at 4.5 and 6 hr ($P < 0.01$).

tion increased as the symptoms of poisoning progressed, and reached a plateau after 4.5 hr. The greatest increase took place after 1.5 hr when the insects muscular tremors had become most pronounced.

To confirm the association of violent muscular activity with the increase in taurine concentration in the haemolymph, insects were treated with picrotoxin, an antagonist of GABA (Olsen, 1981), the main inhibitory neurotransmitter in insects. Picrotoxin did indeed produce violent muscular activity in locusts, resulting in pronounced changes in taurine distribution, without any increase in the whole body concentration of the amino acid (Table 3.4). The increases in the haemolymph, nervous tissue and fat body were accompanied by decreases in flight muscle and eye of about 20 % in each.

Flying also increased the taurine concentration of haemolymph. After 2 hr continuous flight taurine concentration had risen from 1.1 ± 0.43 to 2.03 ± 0.3 μmol/ml (mean $\pm$ S.D., $P < 0.05$). This also indicates the association between muscular activity and taurine increase in the haemolymph.

3.6 Release of arginine kinase activity as a result of stress

As shown in table 3.5 there is, as expected from analogy with the mammal, a very high activity of arginine kinase in the cytoplasm of the flight muscle, but little in the haemolymph of resting locusts. Although both flying and picrotoxin-treatment slightly increased the average activity of the enzyme in the haemolymph, in neither case was this significant. This suggests that

Table 3.5 The activity of arginine kinase in locust tissues

Tissue	Treatment	Specific activity (μmol/g or ml/ min)
Flight muscle	none	1008 ± 190
Haemolymph	none	1.6 ± 0.9 (0.6-3.1)
	Picrotoxin	2.7 ± 1.7 (0.8-4.0)
	Flying	2.7 ± 2.4 (0.6-5.8)

Results are the mean ± S.D. of four locusts. Assays were made at 30°C. Insects treated with picrotoxin were injected 6 hr before sampling. Females only were used. The figures in parentheses indicate the range of individual insects.

Table 3.6 Haemolymph volumes in stressed and control locusts

Treatment	Haemolymph volume (μl)
Control	385 ± 80
Picrotoxin	493 ± 85
Flying	800 ± 250*

The value for the flown locusts (*) is significantly different from the control at the level of $0.05 < P < 0.10$. The volumes are those of female locusts only. Results are the mean ± S.D. of four insects.

the release of taurine into the haemolymph is not simply the result of tissue damage.

3.7 Changes in haemolymph volume as a result of stress

Since one of the main features of the stress response observed in the present work was pronounced muscular activity the possibility was conidered that the increase in haemolymph taurine concentration could be due to a reduction in haemolymph volume due to desiccation of the insect. As shown in Table 3.6 the procedures which caused increased taurine concentration actually tended to elevate the volume of the haemolymph, and in the case of flown locusts this did reach a level of statistical significance. This might indicate some tissue damage in the insect, although the results on arginine kinase activities (section 3.6) indicate that if this is the case the tissue is not muscle.

Studies on the fate of ^{14}C-taurine injected into locusts

3.8 Clearance of ^{14}C-taurine from haemolymph and uptake into tissues

The clearance of ^{14}C-taurine from the haemolymph over 6 hr after injection is shown in Figure 3.5. The clearance of ^{14}C-taurine from haemolymph is best described by a two compartment model indicated by two exponentials, one rapidly declining componant, and one slowly declining componant.

Uptake of ^{14}C-taurine into tissues in which this amino acid is concentrated was studied over a long time period (Fig 3.6). As would be expected, the initial specific activity of ^{14}C-taurine

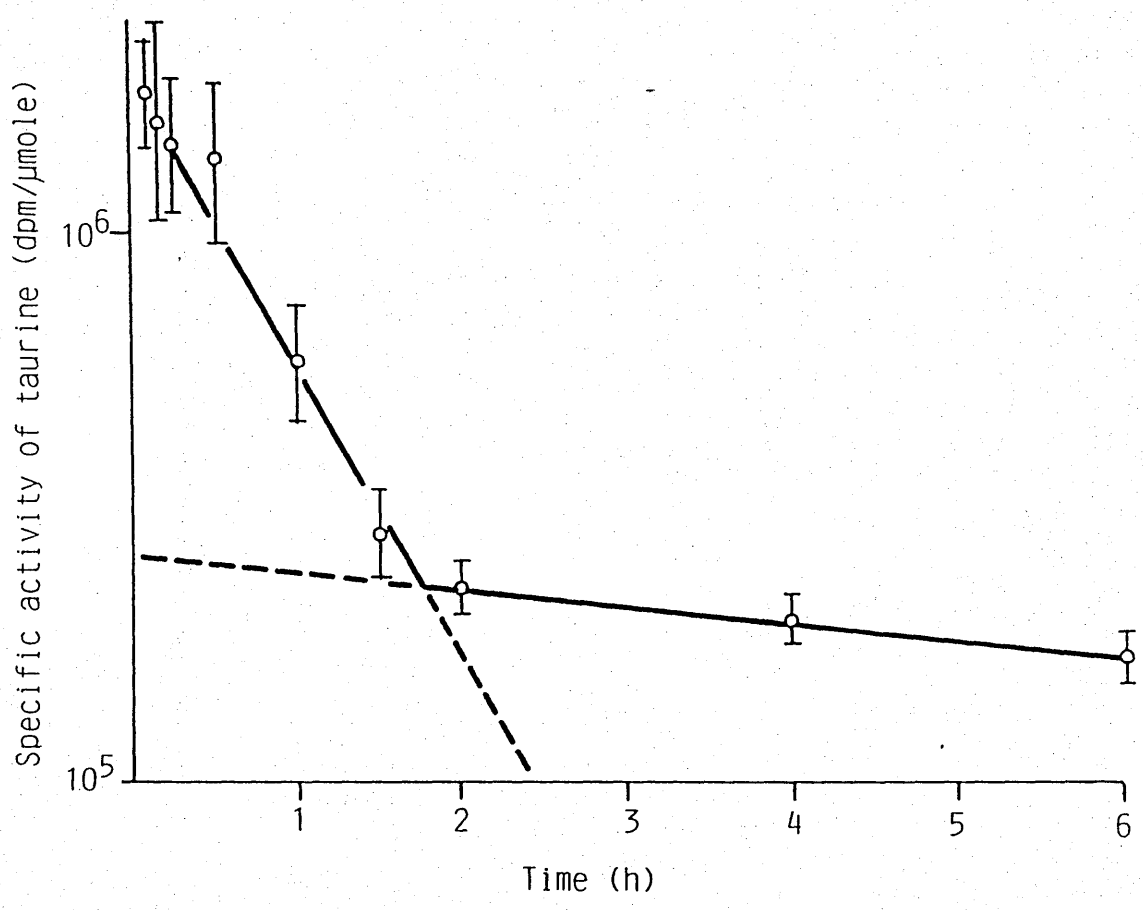


Figure 3.5 Clearance of ^{14}C-taurine from locust haemolymph. The results are the mean ± s.e.m. of four locusts.

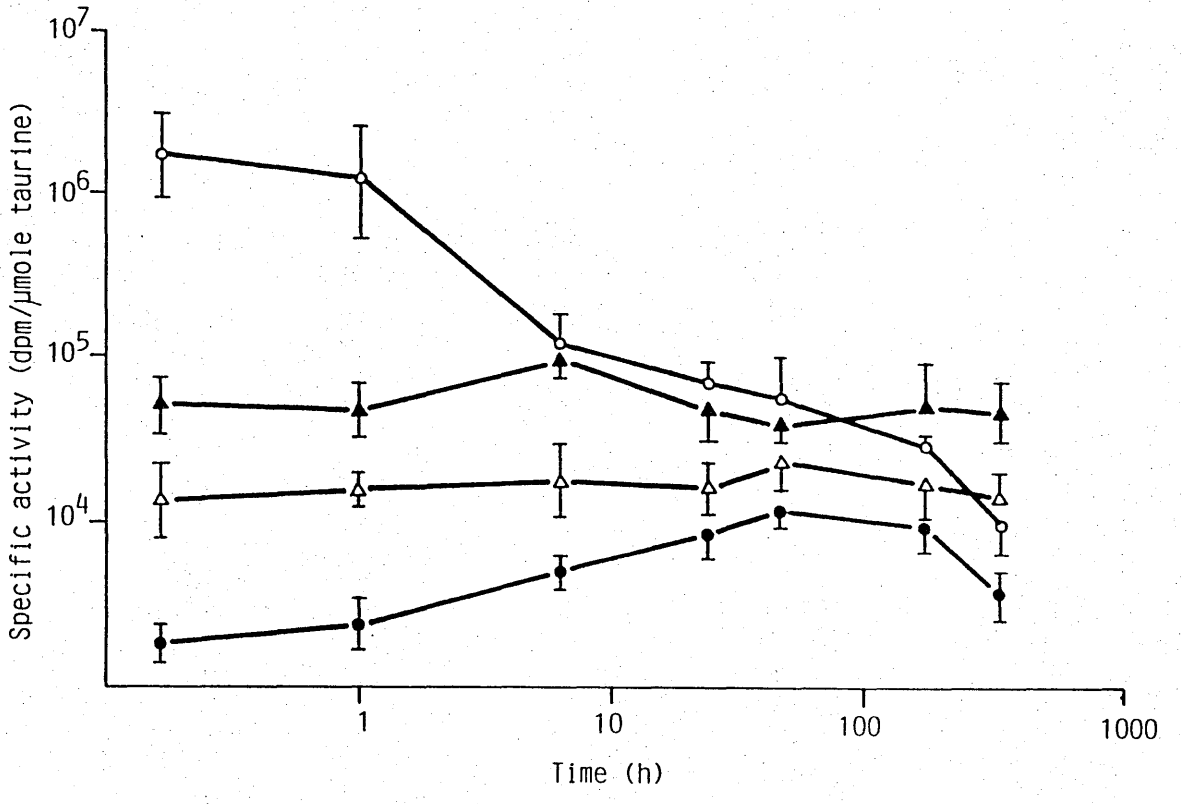


Figure 3.6 Specific activity of taurine in locust tissues after injection of ^{14}C-taurine into the haemolymph. Tissues studied were haemolymph (o—o), flight muscle (●—●), thoracic ganglia (▲—▲) and eye (△—△). Results are the mean ± s.e.m. of four locusts for each point.

in the haemolymph was much greater than in any of the tissues. Both eye and thoracic ganglia accumulated taurine too rapidly to follow, and were at a maximum at the first time point studied (10 min). In contrast to this, muscle accumulated ^{14}C-taurine very slowly, reaching a maximum specific activity after 2 days, and slowly declining thereafter.

3.9 Effect of structural analogues and temperature on ^{14}C-taurine clearance from haemolymph

Table 3.7 shows the effect of structural analogues and temperature on ^{14}C-taurine clearance from haemolymph. When locusts were maintained at 0^{o}C during the experiment taurine clearance was greatly reduced. Hypotaurine, GABA and β-alanine all reduced ^{14}C-taurine clearance, but isethionic acid had no significant effect.

3.10 Metabolism of ^{14}C-taurine in the locust

In order to investigate possible metabolism of ^{14}C-taurine in the locust duplicate samples from the above mentioned experiment (section 3.8) were extracted into ethanol and resolved by TLC. After scraping the TLC plate in 0.5 cm bands radioactivity was only detected in the band corresponding to taurine, which was indicated by the addition of carrier quantities of taurine, visualised with ninhydrin. There was therefore no evidence for taurine metabolism in the locust. Taurine is however excreted in the faeces. Taurine concentration was measured in locust faecal material using the method for taurine estimation

Table 3.7 Effect of temperature and structural analogues on ^{14}C-taurine clearance from locust haemolymph

Treatment	Specific activity of taurine (dpm/μmole x 10^{-6})	% Change
Control	0.71 ± 0.12	100
Cold (0°C)	2.33 ± 0.34*	328
Isethionic acid	0.61 ± 0.02	86
β-alanine	0.94 ± 0.10*	132
Hypotaurine	1.27 ± 0.14*	179
GABA	1.36 ± 0.11*	192

Haemolymph was sampled 1 hr after the injection of ^{14}C-taurine. Results are the mean ± s.e.m. of 6-8 locusts in each group. * denotes significant difference from control ($P < 0.05$).

described in sections 2.2-2.5. The taurine concentration of in locust faeces was 0.14 ± 0.01 μmoles/g. It may be therefore that taurine turnover in the locust is dependent upon excretion of the unmetabolised amino acid.

Studies on the biosynthesis of taurine

3.11 Separation of taurine and its precursors using TLC

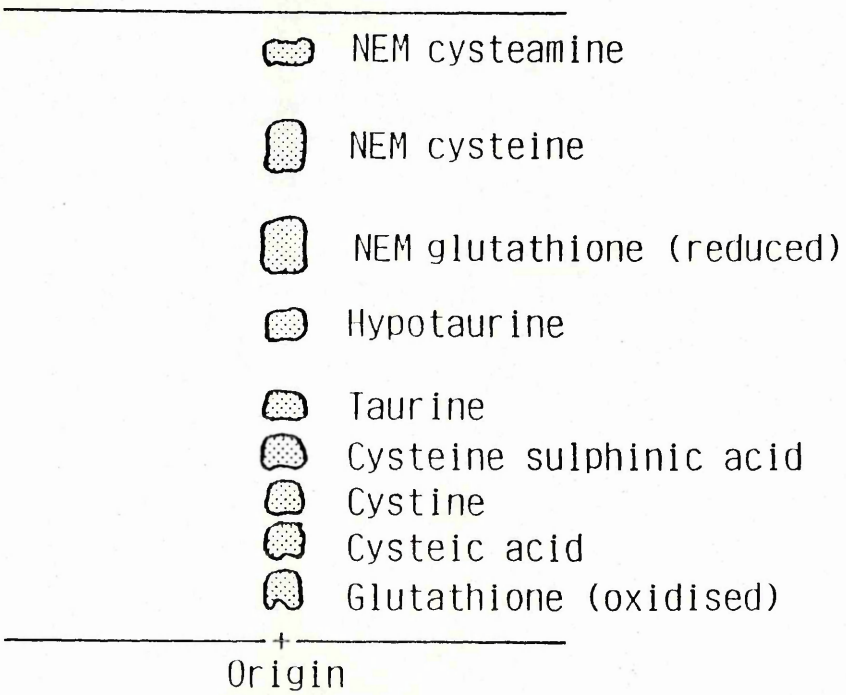
Figure 3.7a shows the separation of taurine and its biosynthetic precursors using one-dimensional chromatography, and this is depicted in a drawing in Figure 3.7b. Figure 3.8 shows the same compounds developed in two dimensions. Oxidised and reduced standards of glutathione were included since these are known to incoroporate ^{35}S after administration of ^{35}S-cysteine to insects (Cotty et al.,1958).

3.12 Autoradiography of resolved tissue extracts

In order to estimate potential ^{35}S containing compounds that might interfere with a quantitative estimate of the specific activity of taurine and its precursors, ^{35}S incorporation was assessed using autoradiography. Figures 3.9-3.12 show autoradiographs of resolved extracts from flight muscle, thoracic ganglia, fat body and eye respectively, after *in vitro* incubation with ^{35}S-cysteine. All of the postulated precursors in taurine biosynthesis were detected (see Fig. 1.3), although not in every tissue, with the exception of cysteamine which was totally absent. The autoradiographs indicated that of the bands resolved using one-dimensional chromatography cystine, cysteine sulphinic acid, taurine and NEM-cysteine would be

a

b



Figures 3.7a,b Resolution of taurine, its putative precursors and glutathione, after reaction with NEM, which enhances separation, by two-dimensional chromatography on cellulose TLC plates. After resolution, chromatograms were stained with ninhydrin.

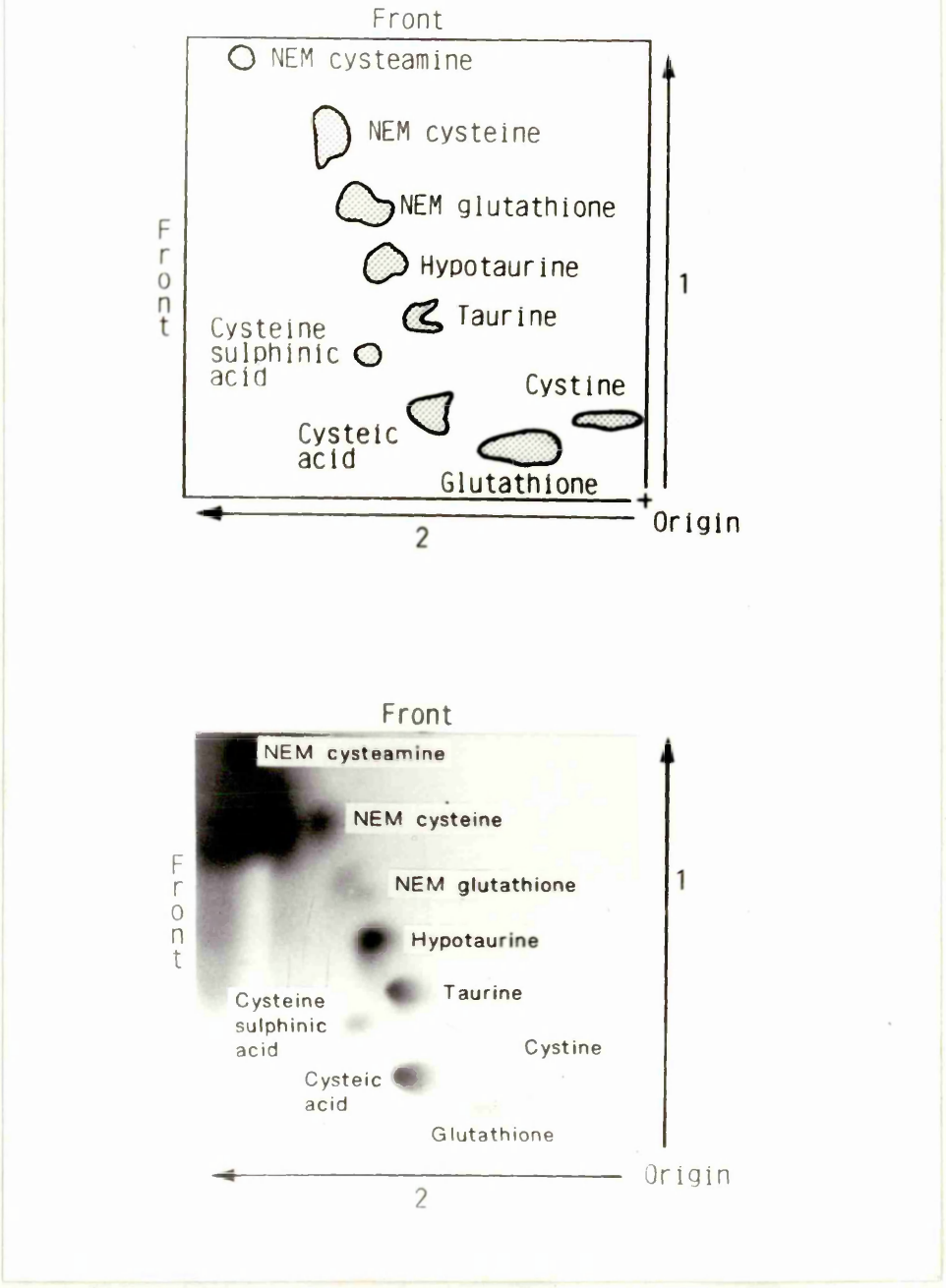


Figure 3.8 Resolution of taurine, its putative precursors and glutathione in two dimensions on cellulose TLC plates after reaction with NEM. The plate was run twice in the first dimension in butanol: acetic acid: H_2O (11:6:3 v:v:v), and once in the second dimension in methanol: pyridine: H_2O (25:1:5 v:v:v) before staining with ninhydrin.

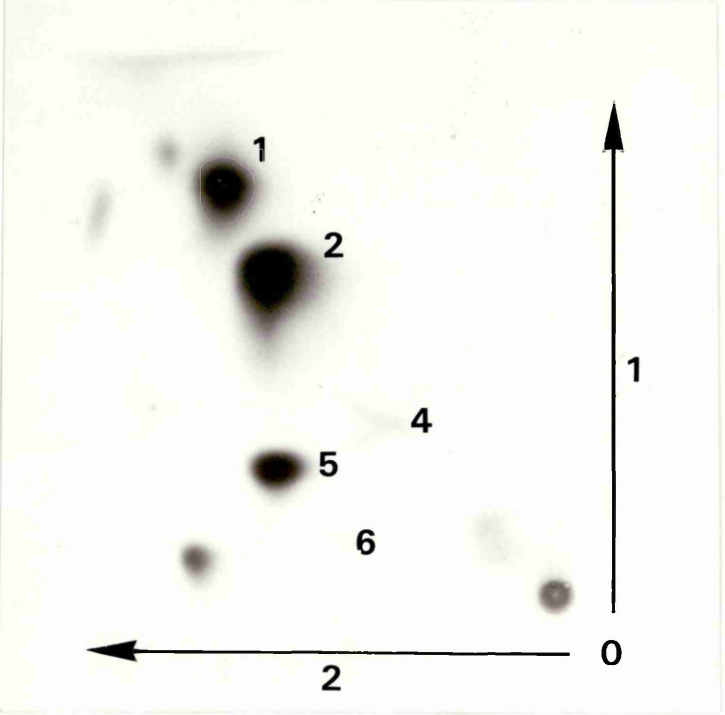
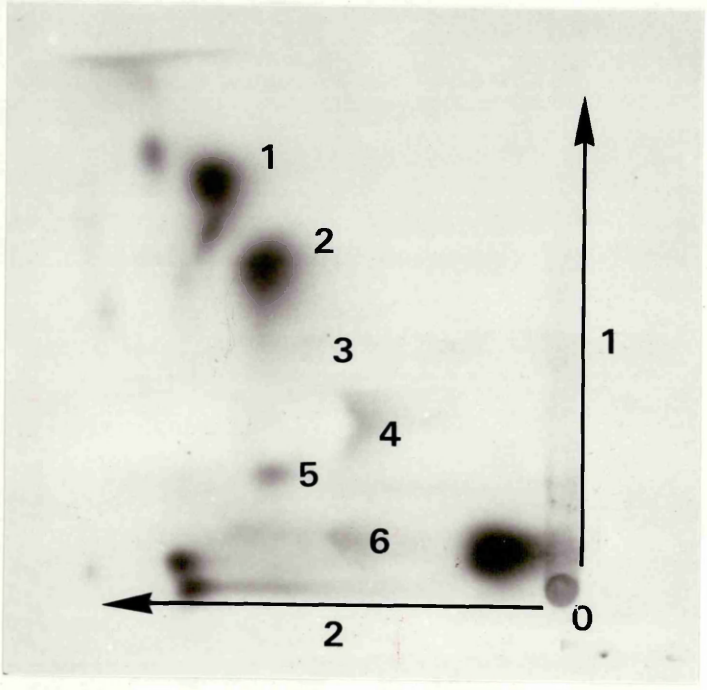


Figure 3.9 Autoradiograph of resolved ^{35}S labelled compounds after 6 hr <u>in vitro</u> incubation of locust flight muscle with ^{35}S-cysteine. Muscle was extracted and resolved as described in the methods section. The TLC plate was then subjected to autoradiography for 2 days. Labelled compounds were identified by reference to standards. 1 = NEM cysteine, 2 = NEM glutathione, 4 = taurine, 5 = cysteine sulphinic acid, 6 = cysteic acid.



3.10 Autoradiograph of resolved ^{35}S labelled compounds after 6 hr <u>in vitro</u> incubation of thoracic ganglia with ^{35}S-cysteine. Thoracic ganglia were extracted and the extract resolved as described in the methods section. The TLC plate was then subjected to autoradiography for 7 days. Labelled compounds were identified by reference to standards. 1 = NEM cysteine, 2 = NEM glutathione, 3 = hypotaurine, 4 = taurine, 5 = cysteine sulphinic acid, 6 = cysteic acid.

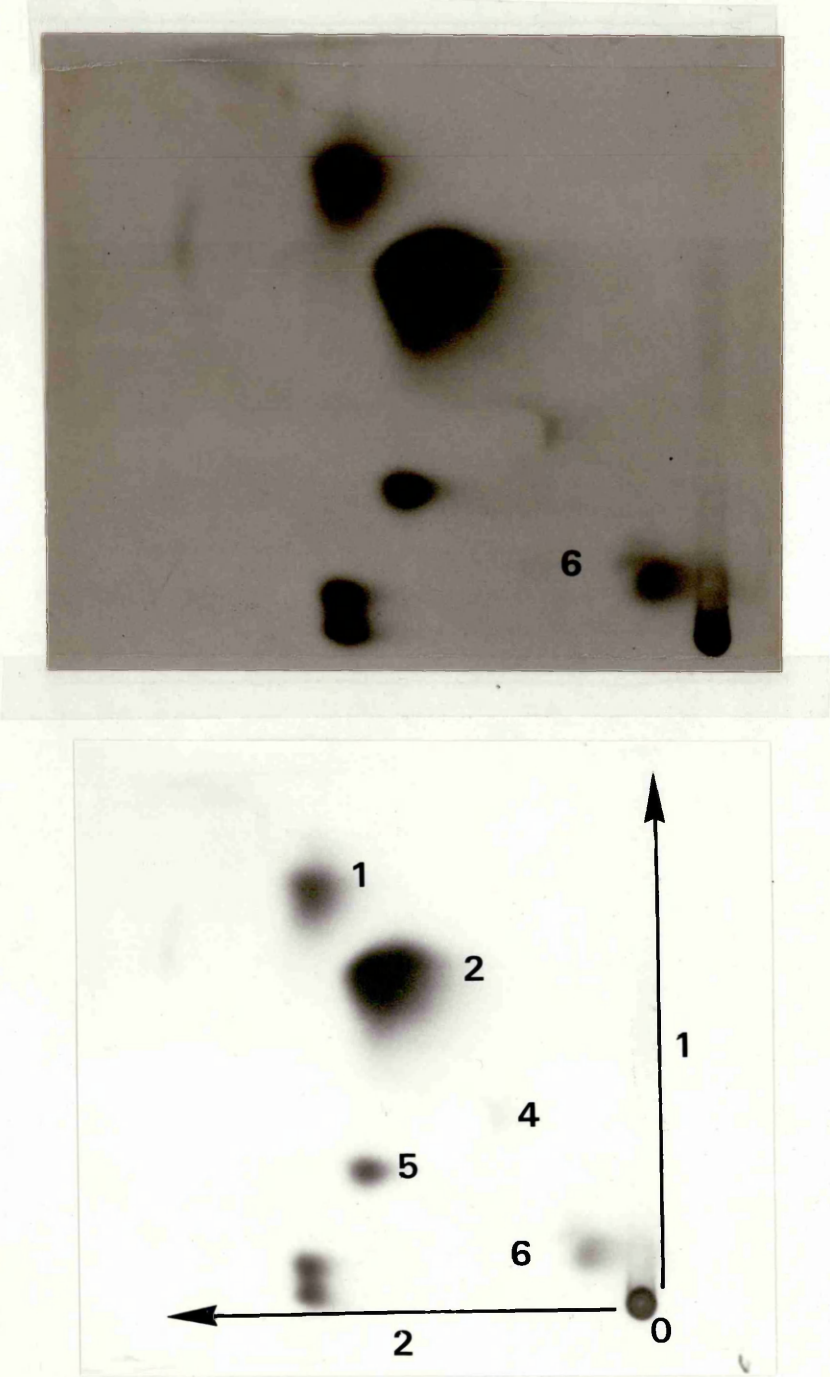


Figure 3.11 Autoradiograph of resolved ^{35}S labelled compounds after 6 hr <u>in vitro</u> incubation of fat body with ^{35}S-cysteine. Fat body was extracted and resolved as described in the methods section. The TLC plate was then subjected to autoradiography for 9 days. Labelled compounds were identified by reference to standards. The original is shown in this case due to the extreme faintness of cysteic acid. 1 = NEM cysteine, 2 = NEM glutathione, 4 = taurine, 5 = cysteine sulphinic acid, 6 = cysteic acid.

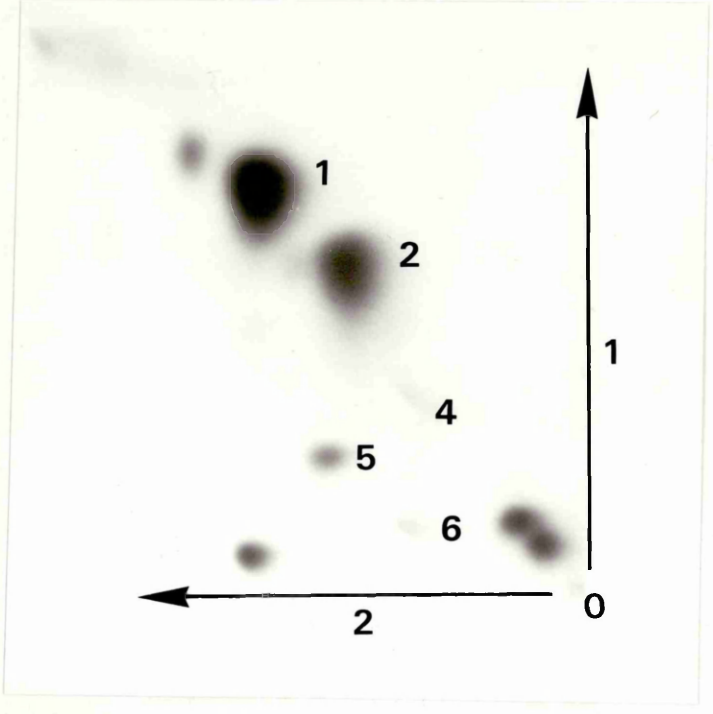


Figure 3.12 Autoradiograph of resolved ^{35}S labelled compounds after 6 hr incubation of locust eyes with ^{35}S-cysteine <u>in vitro</u>. Eyes were extracted and the extract resolved as described in the methods section. The TLC plate was then subjected to autoradiography for 3 days. Labelled compounds were identified by reference to standards. 1 = NEM cysteine, 2 = NEM glutathione, 4 = taurine, 5 = cysteine sulphinic acid, 6 = cysteic acid.

uncontaminated by other ^{35}S labelled compounds, but this could not be said with certainty for either hypotaurine or cysteic acid. The incorporation of label into the latter two compounds was assessed therefore simply qualitatively on the basis of the autoradiograms.

3.13 Estimation of endogenous levels of taurine and its precursors

The endogenous concentration of taurine and its putative precursors in the tissues of mature (25 days after final ecdysis) and young (1 day after final ecdysis) adult locusts is shown in Table 3.8. In general compounds were present at higher concentations in mature than young adult locusts. Cysteamine was not detected in any of the tissues studied. The endogenous concentrations of the compounds were estimated to calculate specific activities after ^{35}S-cysteine administration. Figure 3.13 shows an illustration of the resolved compounds, while Figure 3.14 shows standard curves used to quantify the amount of material present in the samples.

3.14 Metabolism of ^{35}S-cysteine in vivo

Figures 3.15-3.19 show the time course of incorporation of ^{35}S from ^{35}S-cysteine into taurine and those precursors in which a specific activity could be accurately quantified. In no tissue was cysteamine detected. It can be seen that in all tissues as well as haemolymph taurine retained a high specific activity throughout the course of the experiment. Indeed, in the case of flight muscle the specific activity was still increasing on the seventh day (Fig. 3.16).

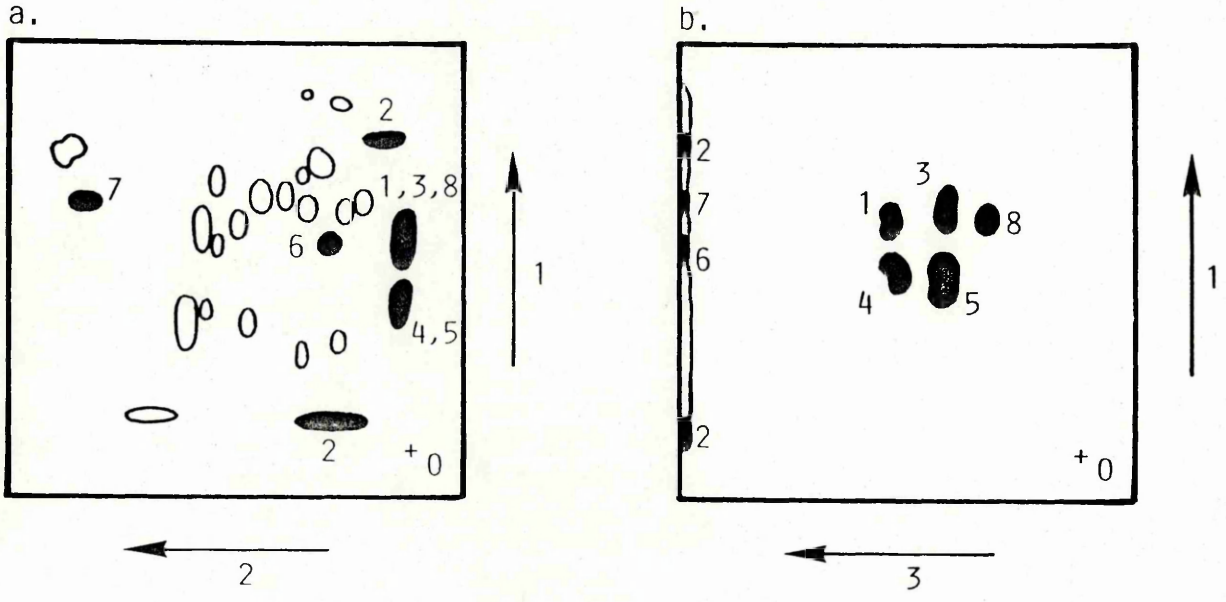


Figure 3.13 Separation of taurine and its potential precursors using two-dimensional chromatography on polyamide layers. In the first dimension the solvent was 3 % formic acid, while in the second dimension (2) toluene: acetic acid (9:1 v:v) or (3) ethyl acetate: 2-propanol: 88 % ammonia (4:10:3 v:v:v). Solvent 3 moved almost all of the amino containing material with the solvent front as shown in b. The absence of possible interfering substances was established by comparison with resolved extracts from S. gregaria made previously in the laboratory (Jabbar, 1982), some of which are indicated by the open spots on the chromatograms. 1 = Cysteic acid, 2 = Cystine, 3 = Cysteine sulphinic acid, 4 = taurine, 5 = hypotaurine, 6 = cysteine, 7 = cysteamine, 8 = Dansyl-hydroxide.

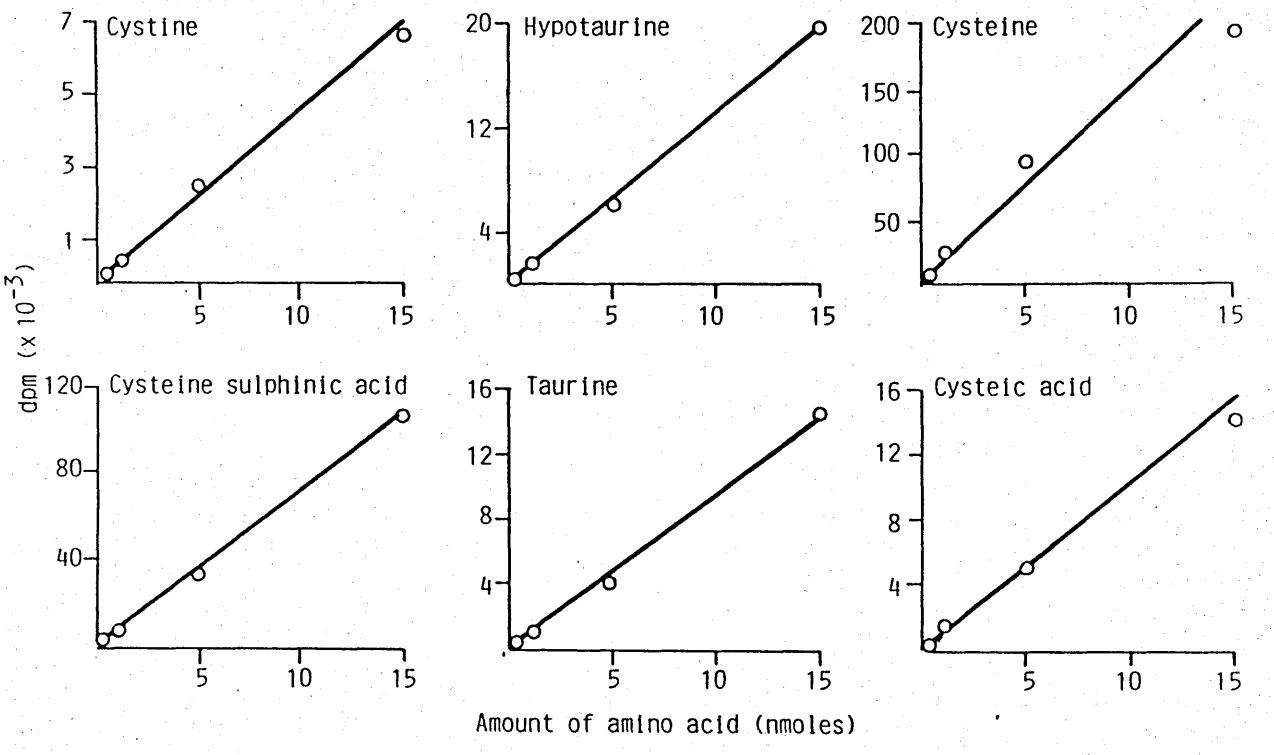


Figure 3.14 Standard curves for reaction of ^{3}H-dansyl chloride with known quantities of taurine and its possible precursors. After resolution on polyamide TLC plates, spots were visualised by u.v. light, marked, and cut out for quantitative estimation by scintillation counting.

Table 3.8 Concentration of taurine and its biosynthetic precursors in different locust tissues

	μmoles amino acid/g or ml wet weight	
	1 day old adult	25 days old adult
Haemolymph		
Cysteic acid	6.8 ± 2.7	8.1 ± 3.2
Cystine	5.1 ± 1.0	6.1 ± 0.5
Cysteine sulphinic acid	2.1 ± 0.6	3.0 ± 0.8
Taurine	0.8 ± 0.2	1.7 ± 1.0
Hypotaurine	0.3 ± 0.1	0.2 ± 0.1
Cysteine	3.6 ± 1.1	3.9 ± 1.1
Muscle		
Cysteic acid	6.3 ± 2.4	7.1 ± 2.5
Cystine	4.0 ± 0.8	3.1 ± 0.8
Cysteine sulphinic acid	11.2 ± 2.9	17.6 ± 4.5
Taurine	12.9 ± 3.5	21.6 ± 6.6
Hypotaurine	0.9 ± 0.1	0.7 ± 0.2
Cysteine	8.1 ± 2.2	11.5 ± 1.6
Thoracic ganglia		
Cysteic acid	4.2 ± 0.5	5.9 ± 1.6
Cysteine	0.8 ± 0.1	2.2 ± 0.3
Cysteine sulphinic acid	1.3 ± 0.5	0.8 ± 0.1
Taurine	3.7 ± 1.1	4.1 ± 0.4
Hypotaurine	0.6 ± 0.1	1.9 ± 0.4
Cysteine	2.8 ± 0.3	3.1 ± 0.6
Fat body		
Cysteic acid	1.1 ± 0.2	3.8 ± 0.8
Cystine	2.1 ± 0.6	1.3 ± 0.4
Cysteine sulphinic acid	1.9 ± 0.4	2.8 ± 0.3
Taurine	1.3 ± 0.4	2.6 ± 0.8
Hypotaurine	0.6 ± 0.2	0.2 ± 0.1
Cysteine	4.7 ± 1.2	6.1 ± 2.3

Table 3.8 continued on following page.

Table 3.8 (continued)

	μmoles amino acid/g or ml wet weight	
	1 day old adult	25 days old adult
Eye		
Cysteic acid	3.8 ± 0.5	4.1 ± 0.8
Cystine	0.7 ± 0.2	1.2 ± 0.3
Cysteine sulphinic acid	0.9 ± 0.3	2.4 ± 0.9
Taurine	6.8 ± 1.0	8.0 ± 0.9
Hypotaurine	0.1 ± 0.05	0.3 ± 0.1
Cysteine	3.9 ± 0.4	4.1 ± 0.7

Amino acid concentrations were estimated by ^{3}H-dansylation followed by resolution on polyamide TLC plates. The values obtained were then quantified by comparison with known quantities of standrds. Results are the mean ± S.D. of four locusts in each case.

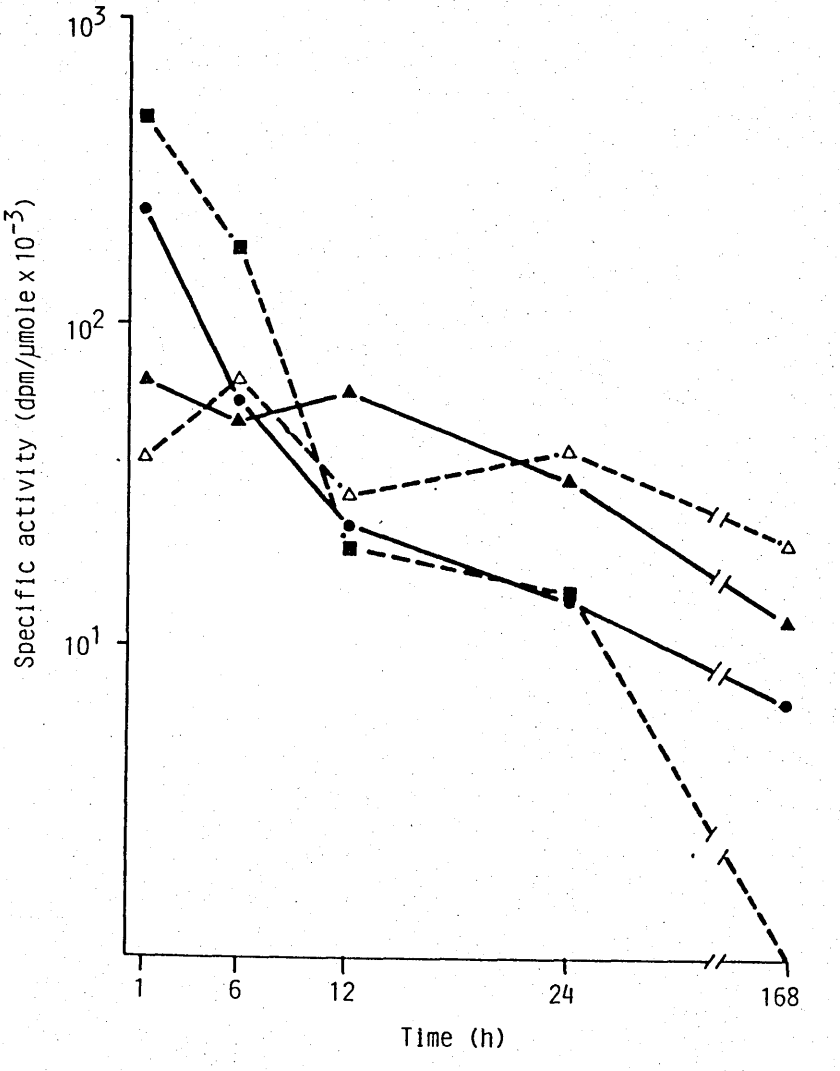


Figure 3.15 Incorporation of label from ^{35}S-cysteine into taurine and its precursors in the haemolymph of locusts at different times after injection of 20µCi of ^{35}S-cysteine into the haemolymph. Haemolymph was extracted and then resolved by TLC in one dimension, after which bands were scraped and estimated by scintillation counting. Cysteine (■---■), Cystine (●——●), Cysteine sulphinic acid (△--△), taurine (▲——▲). Results are averages from 3 locusts.

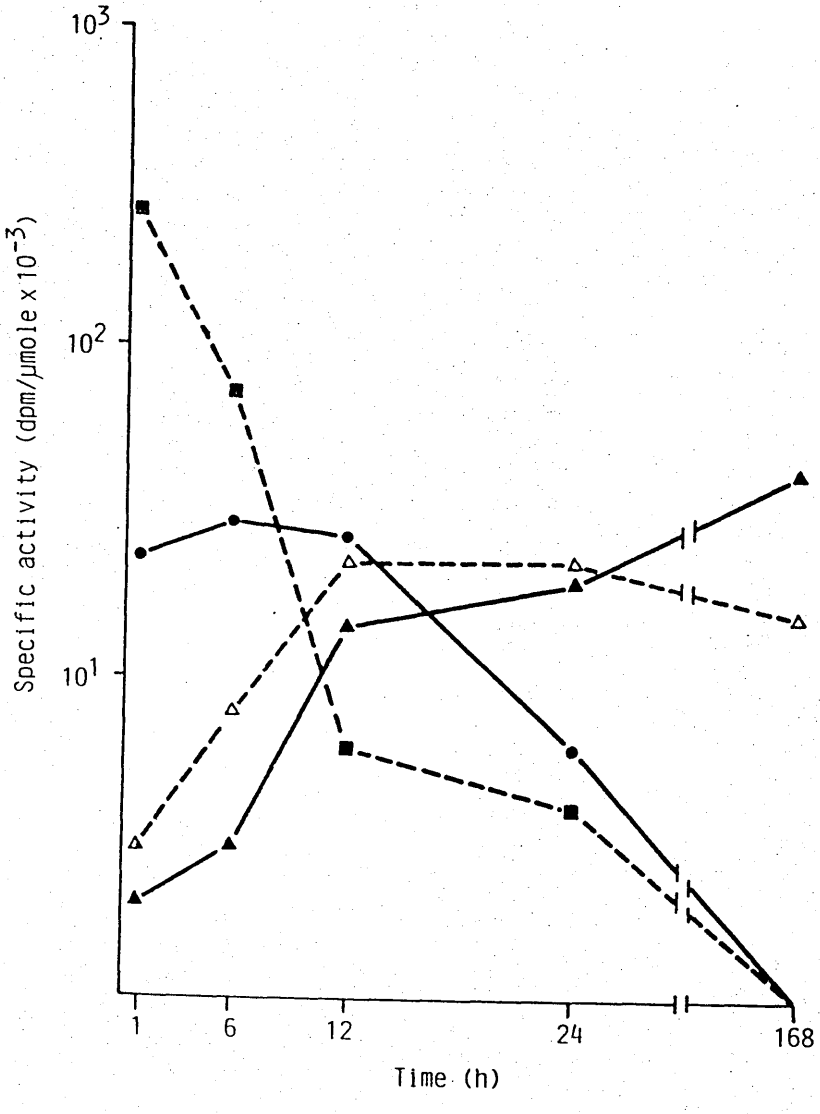


Figure 3.16 Incorporation of label from ^{35}S-cysteine into taurine and its precursors in flight muscle of locusts at different times after injection of 20μCi of ^{35}S-cysteine into the haemolymph. Flight muscle was extracted and the extract resolved by TLC in one dimension, after which bands were scraped and estimated by scintillation counting. Cysteine (■--■), cystine (●—●), cysteine sulphinic acid (△--△), taurine (▲—▲). Results are averages from three locusts.

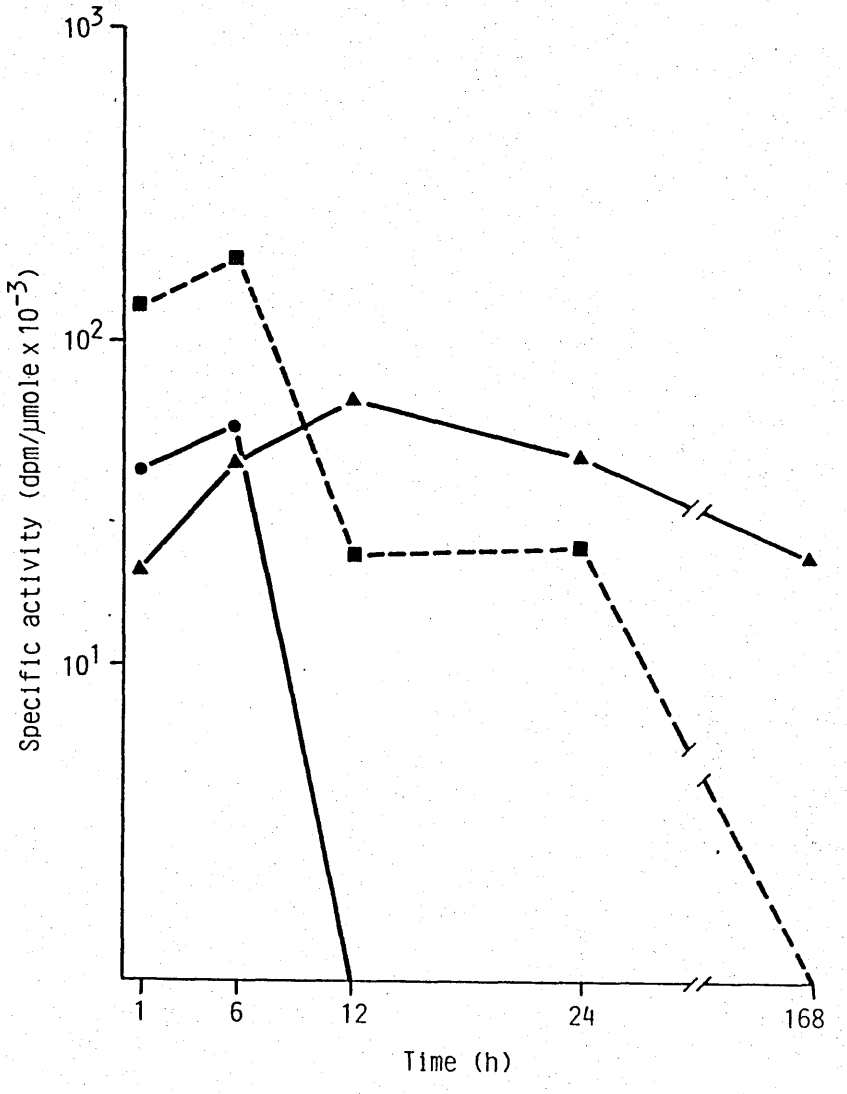


Figure 3.17 Incorporation of label from ^{35}S-cysteine into taurine and its precursors in thoracic ganglia of locusts at different times after injection of 20μCi of ^{35}S-cysteine into the haemolymph. Thoracic ganglia were extracted and the extract resolved by TLC in one dimension, after which bands were scraped for estimation by scintillation counting. Cysteine (■---■), cystine (●——●), taurine (▲——▲). Results are averages from three locusts.

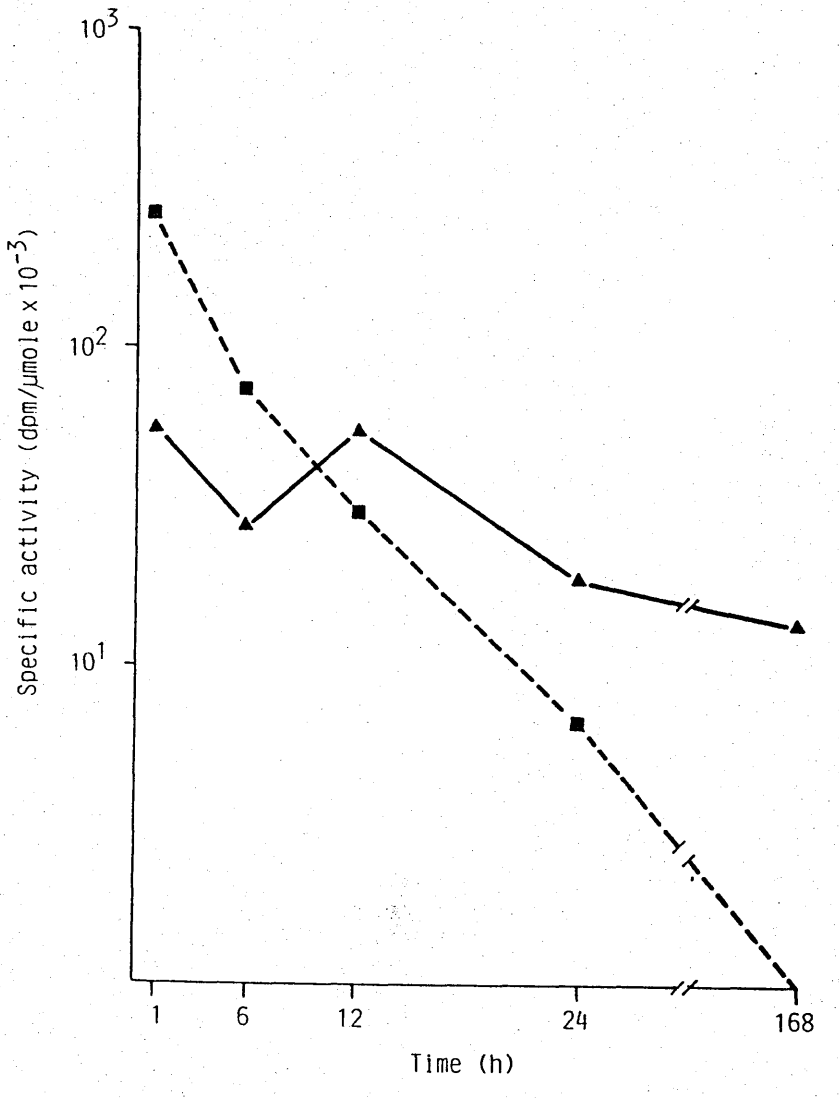


Figure 3.18 Incorporation of label from ^{35}S-cysteine into taurine and its precursors in fat body of locusts at different times after injection of 20µCi of ^{35}S-cysteine into the haemolymph. Fat body was extracted and the extract resolved by TLC in one dimension, after which bands were scraped for estimation by scintillation counting. Cysteine (■---■), taurine (▲—▲). Results are averages from three locusts.

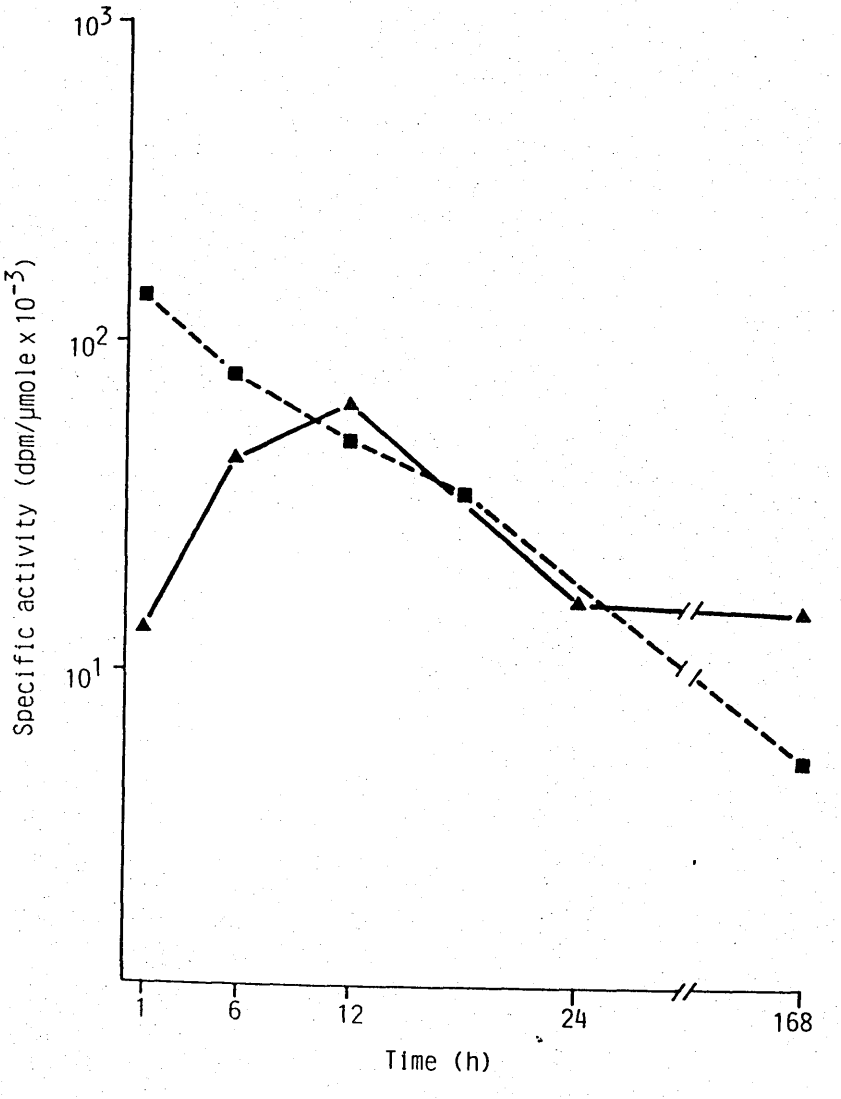


Figure 3.19 Incorporation of label from ^{35}S-cysteine into taurine and its precursors in eyes of locusts at different times after injection of 20µCi of ^{35}S-cysteine into the haemolymph. The eyes were extracted and the extracts resolved by TLC in one dimension after which bands were scraped for estimation by scintillation counting. Cysteine (■---■), taurine (▲—▲). Results are averages from three locusts.

3.15 Effects of age, stress and flying on incorporation of ^{35}S into taurine and its precursors

Table 3.9 shows the effects of age, stress and flying on the incorporation of label from ^{35}S-cysteine into taurine and its precursors. Compared with the adult at 25 days of age incorporation of label in 1 day old adults was very much greater, which is consistant with the large increase in taurine concentration in flight muscle after final ecdysis (Fig. 3.3). Picrotoxin-induced stress was also found to increase incorporation of label from ^{35}S-cysteine, as was 2 hr continuous flying, although in the latter case the effect was comparatively small (Table 3.9).

Studies using locust synaptosomal preparation

3.16 Uptake of ^{3}H-taurine and ^{3}H-GABA into synaptosomes

Figure 3.20 shows the uptake of either ^{3}H-taurine or ^{3}H-GABA into locust synaptosomes. GABA uptake into the synaptosomes was rapid, reaching a plateau after 5 min. In contrast to GABA, taurine was not accumulated into the synaptosomal preparation in any detectable way (Fig. 3.20). No uptake was detected in the presence of 1mM unlabelled taurine either and the result obtained under these conditions was identical to that shown in Figure 3.20.

3.17 Effect of taurine and nipecotic acid on ^{3}H-GABA uptake into synaptosomes

Both taurine (Fig. 3.21) and nipecotic acid (Fig. 3.22) caused a concentration-dependent decrease in ^{3}H-GABA uptake into locust synaptosomes. Nipecotic acid was much more effective in

Table 3.9 Effects of age, stress and flying on ^{35}S incorporation from ^{35}S-cysteine into taurine and its precoursors

	Specific activity of amino acid (dpm/μ mole				
	Haemolymph	Flight muscle	Thoracic ganglia	Fat body	Eye
25 day old adult					
Cystine	59.1 ± 7.2	31.5 ± 5.4	50.8 ± 11.9	0	0
Cysteine sulphinic acid	70.4 ± 16.6	8.1 ± 2.2	0	0	0
Taurine	59.5 ± 12.4	3.4 ± 0.7	39.3 ± 9.1	27.1 ± 7.2	19.6 ± 3.3
Cysteine	190.8 ± 21.8	81.9 ± 16.7	135.6 ± 19.8	80.3 ± 12.2	79.4 ± 9.7
1 day old adult					
Cystine	24.9 ± 4.2	32.6 ± 5.1	0	52.2 ± 9.9	0
Cysteine sulphinic acid	314.2 ± 38.1	113.4 ± 19.3	54.6 ± 7.7	182.5 ± 43.8	47.0 ± 5.8
Taurine	237.4 ± 32.0	97.5 ± 13.8	178.1 ± 26.0	731.6 ± 91.4	103.7 ± 16.5
Cysteine	50.3 ± 17.0	16.2 ± 4.1	35.9 ± 10.1	44.8 ± 6.7	31.9 ± 2.9
25 day adult (picrotoxin)					
Cystine	22.4 ± 5.7	9.0 ± 3.1	0	0	0
Cysteine sulphinic acid	316.9 ± 67.2	26.7 ± 5.7	11.9 ± 2.0	174.2 ± 27.0	19.6 ± 3.4
Taurine	121.3 ± 20.0	20.8 ± 4.6	106.9 ± 15.6	366.5 ± 56.9	102.1 ± 17.2
Cysteine	26.3 ± 5.2	16.8 ± 3.2	41.1 ± 8.3	18.1 ± 3.0	64.4 ± 10.3
25 day adult (flown)					
Cystine	28.7 ± 6.3	31.5 ± 7.0	0	0	0
Cysteine sulphinic acid	70.2 ± 13.1	8.9 ± 1.3	3.7 ± 0.6	18.2 ± 5.8	2.4 ± 0.7
Taurine	100.6 ± 19.0	9.4 ± 2.1	107.3 ± 23.4	62.7 ± 8.9	34.3 ± 4.8
Cysteine	95.2 ± 26.9	14.7 ± 5.2	30.5 ± 4.0	39.4 ± 11.1	42.7 ± 9.1

Samples were taken 6 hr after ^{35}S-cysteine injection. In the case of flown insects, locusts were flown for 2 hr, 4hr after ^{35}S-cysteine injection. Results are mean ± s.e.m. of three locusts in each group.

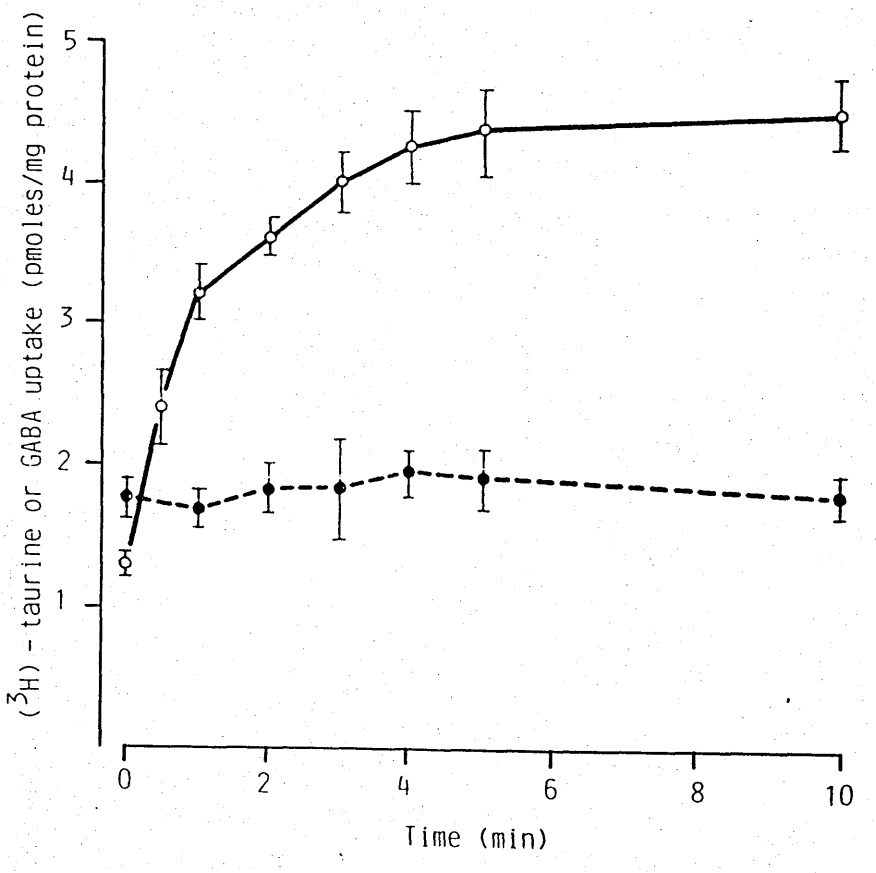


Figure 3.20 Uptake of ^{3}H-taurine (●----●) and ^{3}H-GABA (o——o) into locust synaptosomes. Synaptosomes were incubated with 2μCi of ^{3}H-amino acid in insect saline which in the case of GABA contained 50μM AOAA. Incubations were stopped by filtration and blank filter counts were subtracted from the results. Results are mean $\pm$ s.e.m. of three samples each.

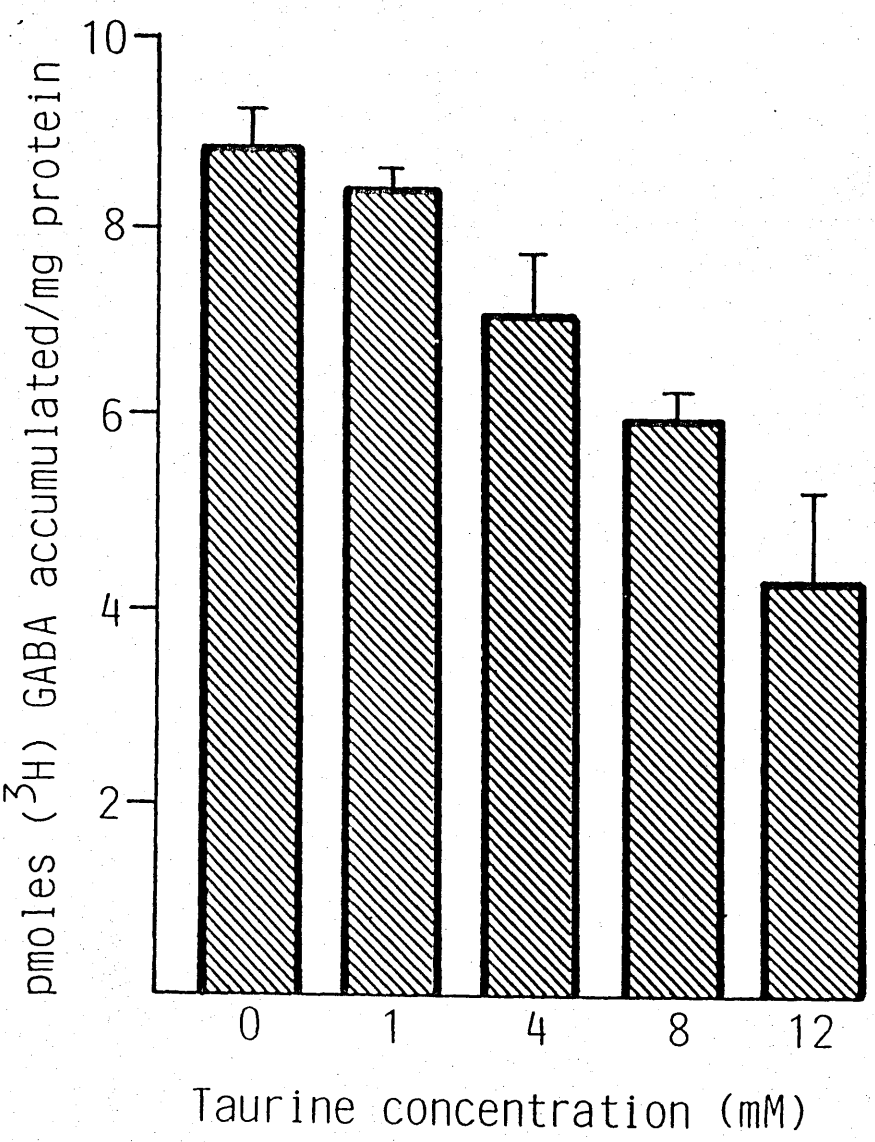


Figure 3.21 Effect of increasing taurine concentration on uptake of ^{3}H-GABA into locust synaptosomes. Synaptosomes were incubated for 3 min as described previously (see legend to Fig 3.20). Results are mean $\pm$ s.e.m. of three samples each.

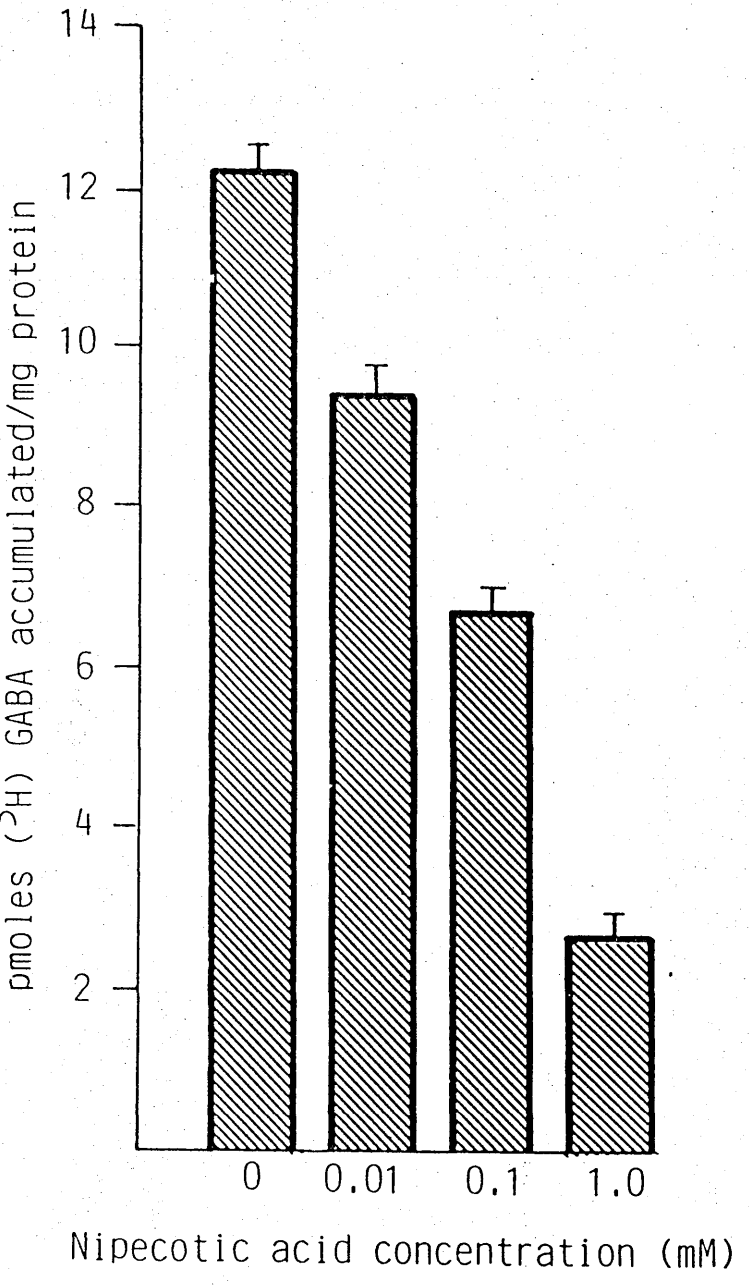


Figure 3.22 Effect of increasing nipecotic acid concentration on uptake of ^{3}H-GABA into locust synaptosomes. Synaptosomes were incubated for 3 min as described previously (see legend to Fig. 3.20). Results are mean $\pm$ s.e.m. of three samples each.

this respect however, decreasing GABA uptake by about 50 % at 0.1mM, which is approximately one hundred times less than the concentration of taurine required to produce the same effect.

3.18 Effect of drugs and temperature on $^{45}Ca^{++}$ uptake by synaptosomes

Resting synaptosomes accumulated $^{45}Ca^{++}$ and this reached a plateau between three and five minutes (Fig. 3.23). As shown in Figure 3.24a, when taurine was added to the incubation medium for resting synaptosomes it caused a concentration dependent inhibition of $^{45}Ca^{++}$ accumulation, although this only achieved statistical significance at the highest taurine concentration used.

When synaptosomes were incubated in the presence of either veratridine or 100mM K^+ the amount of $^{45}Ca^{++}$ accumulated was much greater than in the resting state (Fig 3.24b,c). In both veratridine and K^+-depolarised synaptosomes taurine caused a concentration dependent decrease in $^{45}Ca^{++}$ accumulation, which was reduced to the level seen in resting synaptosomes at the highest taurine concentration (Fig 3.24b,c).

Table 3.10 shows the effects of various procedures on $^{45}Ca^{++}$ accumulation in resting and veratridine-depolarised synaptosomes. In the resting state low temperature ($0^{o}C$) greatly reduced $^{45}Ca^{++}$ accumulation, indicating that this process is not merely due to diffusion. When depolarised synaptosomes were incubated in the presence of either 10mM GABA or leucine, neither was found to affect significantly $^{45}Ca^{++}$. This suggests specificity of the taurine effect, which at the same concentration reduced ^{45}Ca accumulation by about 50 % in veratridine-depolarised synaptosomes. Tetrodo-

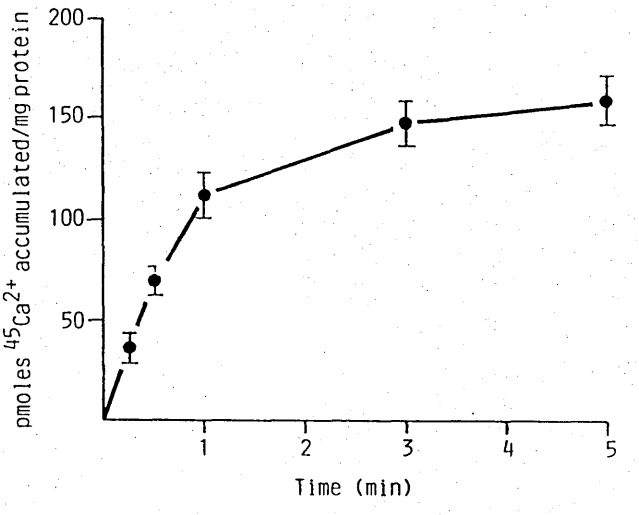


Figure 3.23 Accumulation of $^{45}Ca^{++}$ by resting locust synaptosomes. Synaptosomes were incubated in insect saline containing 2μCi of $^{45}Ca^{++}$ at 30^{o}C for the times indicated. Incubations were stopped by filtration and blank filter counts were subtracted from the results. Results are the mean $\pm$ s.e.m. of threesamples each.

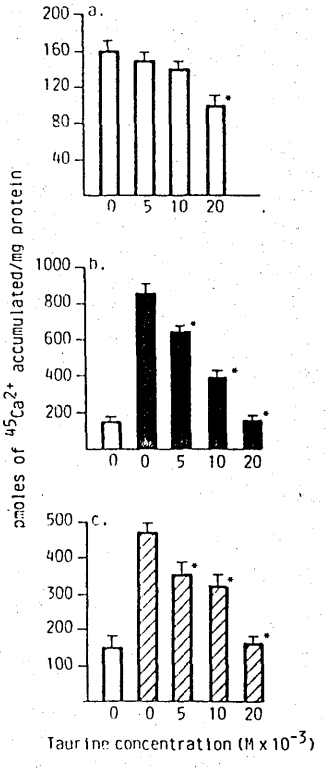


Figure 3.24 Effect of taurine on $^{45}Ca^{++}$ accumulation by locust synaptosomes. Synaptosomes were either resting (open bars) or depolarised by 100μM veratridine (solid bars) or 100mM KCl (hatched bars). The synaptosomes were incubated for 3 min as described previously (see legend to Fig. 3.23). Results are the mean $\pm$ s.e.m. of three samples. The results indicated (*) are significantly different from the control ($P < 0.01$).

Table 3.10 Effect of low temperature and drugs on $^{45}Ca^{++}$ accumulation in locust synaptosomes.

Treatment	$^{45}Ca^{++}$ accumulation (pmoles/ mg of protein)
Resting	
30°C	192 ± 13
0°C	47 ± 7*
Depolarised	
Veratridine (100μM)	741 ± 33
+ GABA (10mM)	714 ± 33
+ Leucine (10mM)	655 ± 63
+ Tetrodotoxin (1μM)	338 ± 22*
+ Verapamil (10μM)	376 ± 37*

Synaptosomes were incubated for 3 min as described previously (see legend to Fig. 3.23). The results indicated (*) are significantly different from the control ($P < 0.01$). Results are the mean ± s.e.m. of three samples.

toxin, a sodium channel antagonist, markedly reduced $^{45}Ca^{++}$ uptake, as might be expected since depolarisation induced by veratridine is considered to result from an opening of sodium channels (Villegas et al., 1976), which would in turn cause the opening of voltage-dependent calcium channels. Verapamil, a calcium channel antagonist, was observed to reduce $^{45}Ca^{++}$ accumulation, with a potency similar to that found in mammalian synaptosomes (Turner and Goldin, 1985), indicating that $^{45}Ca^{++}$ is entering the depolarised synaptosomes through calcium channels.

3.19 Release of ^{3}H-ACh and ^{3}H-GABA from locust synaptosomes

Since it has been observed that locust synaptosomes form ^{3}H-ACh from ^{3}H-choline, and the former is released on depolarisation (Breer and Knipper, 1984), the material released from the ^{3}H-choline loaded synaptosomes will be referred to as ACh. It should be emphasised, however, that the radioactivity collected would be likely to contain a high proportion of ^{3}H-choline due to the rapid metabolism of released ACh.

In the absence of depolarising agents a spontaneous efflux of ACh and GABA was observed, and this was similar for the two neurotransmitters (Fig. 3.25). When depolarised by either veratridine or 100mM KCl release of ACh was observed (Fig. 3.26), but in the case of GABA no apparent response to veratridine stimulation was seen and the effect of 100mM KCl was slight (Fig. 3.27).

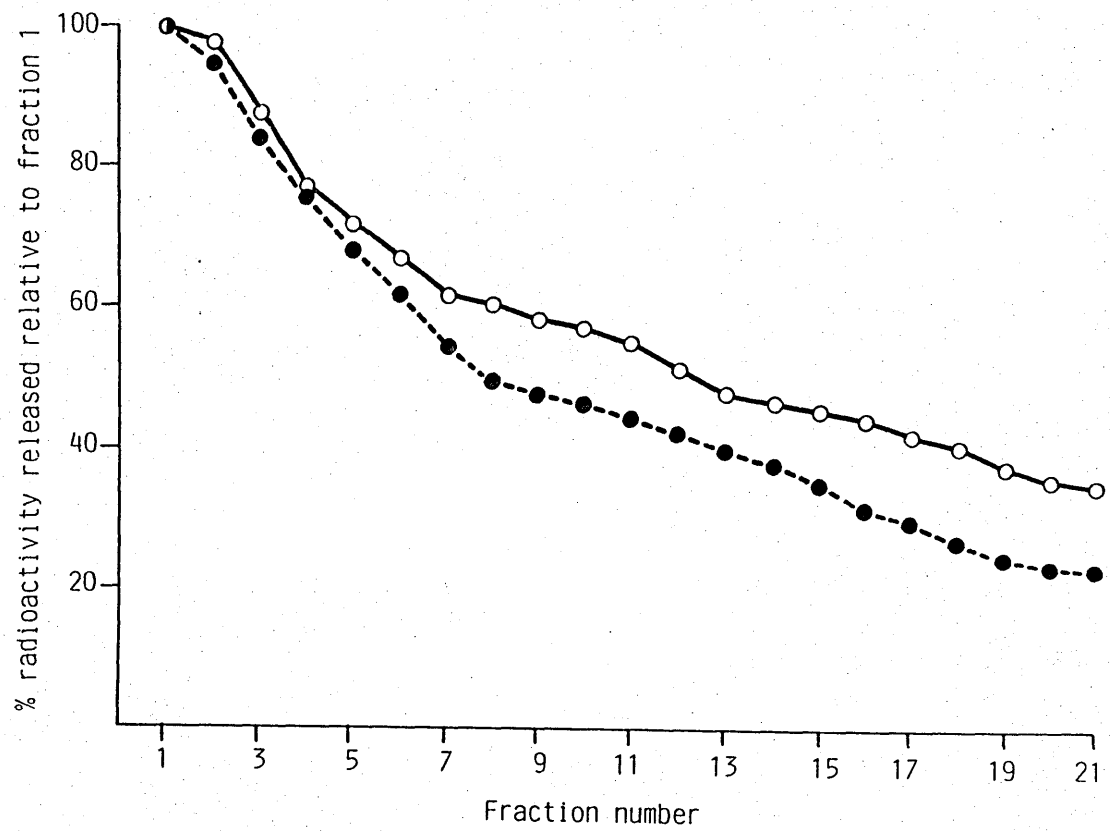


Figure 3.25 Spontaneous efflux of either ^{3}H-ACh (o——o) or ^{3}H-GABA (●---●) from locust synaptosomes. Fraction 1 corresponded to 1945 ± 193 cpm for GABA, and 4619 ± 309 cpm for ACh. Results are the averages of triplicate samples of which the s.e.m. was less than 12 % of the mean for each point.

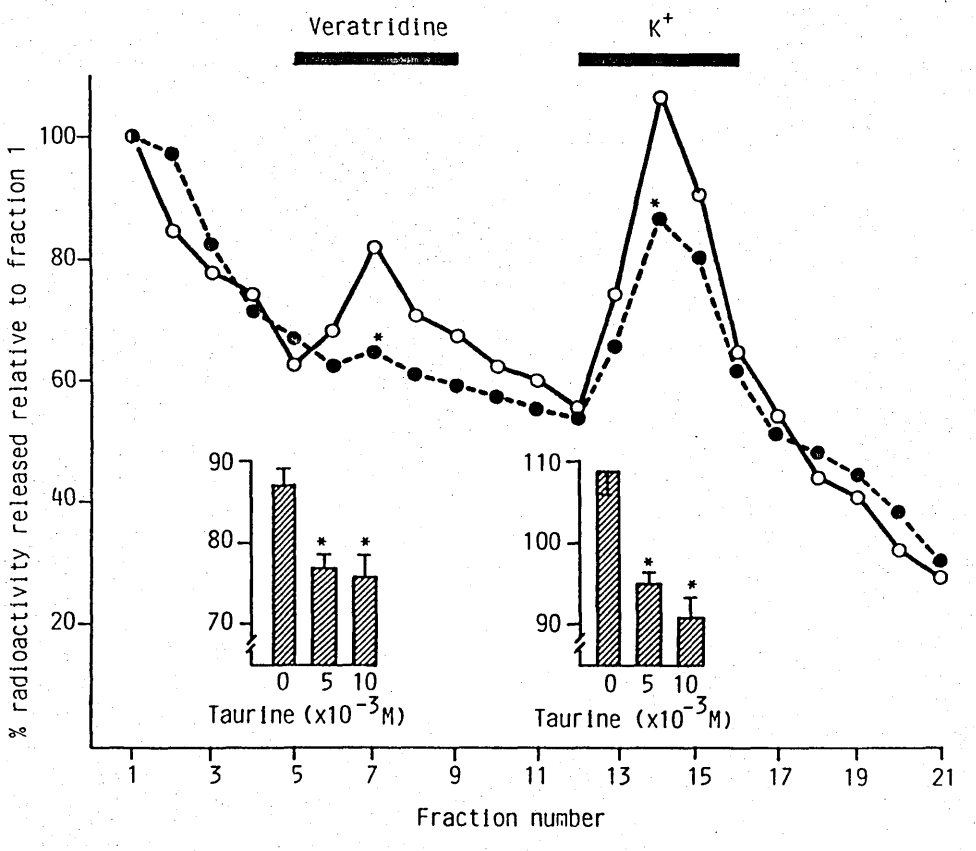


Figure 3.26 Effect of taurine (20mM) on veratridine (100μM) or K^+ (100mM) induced release of ^{3}H-ACh from locust synaptosomes. Taurine absent (o——o), taurine present (●----●). Histogram inserts correspond to data obtained for fractions 7 and 14 using different taurine concentrations as shown. The scale on the y-axis (vertical) of the insert corresponds to that of the y-axis of the main graph. Fraction 1 corresponds to 4817 $\pm$ 476 cpm. Results are the averages of triplicate samples of which the s.e.m. was less than 10 % of the mean for each point. * $P < 0.05$ compared with the control.

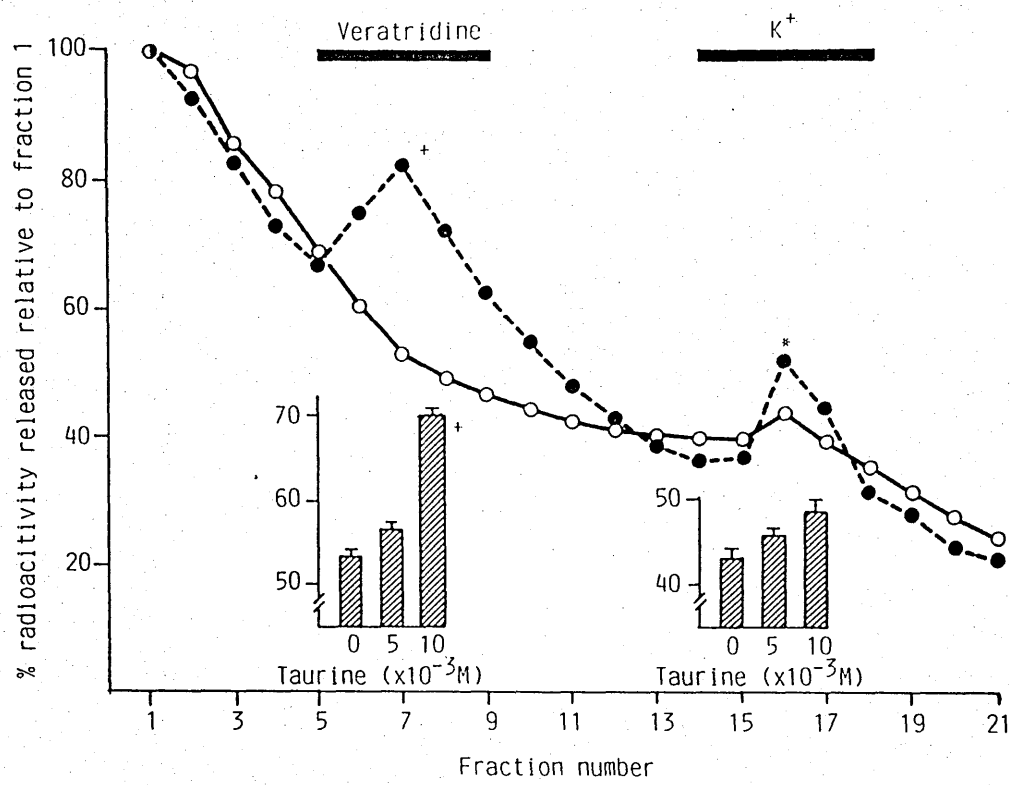


Figure 3.27 Effect of taurine (20mM) on veratridine (100μM) or K^+ (100mM) induced release of ^{3}H-GABA from locust synaptosomes. Taurine absent (o——o), taurine present (●- - -●). Histogram inserts correspond to data obtained from fractions 7 and 16 using different taurine concentrations. The scale of the y-axis (vertical) of the inserts coresponds to that of the y-axis of the main graph. Fraction 1 corresponds to 1891 $\pm$ 79 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 10 % of the mean. * $P < 0.05$, + $P < 0.01$, compared with the control.

3.20 Effect of taurine on release of ^{3}H-ACh and ^{3}H-GABA

As shown in Figure 3.26 taurine caused a concentration-dependent decrease in ^{3}H-ACh release from locust synaptosomes in response to both types of depolarisation. In marked contrast to its effect on ACh release, taurine caused an apparent concentration-dependent increase in ^{3}H-GABA release (Fig. 3.27). The effect of taurine on veratridine-induced GABA release was relatively much greater than the effect on high K^{+}-induced release (Fig. 3.27).

3.21 Comparison of the effect of nipecotic acid and taurine on ^{3}H-GABA release

Since it seemed possible that the effect of taurine on GABA release might be due to prevention of GABA reuptake (see Fig. 3.21) the effect of nipecotic acid on GABA release was compared with that of taurine. As had been observed previously, no release of GABA was detectable after veratridine depolarisation and the response to high K^{+} was slight (Fig. 3.28). In the absence of depolarising agents taurine increased GABA efflux, and this was enhanced when synaptosomes were depolarised (Fig. 3.28). Like taurine, nipecotic acid alone increased GABA efflux and this was similarly further increased by depolarising agents (Fig. 3.29). It can be seen that nipecotic acid is much more effective in increasing GABA efflux than taurine, which is consistent with its comparative potency in reducing GABA uptake into synaptosomes (see Figs. 3.21 and 3.22). Since GABA reuptake appeared to be so avid as to obscure depolarisation-induced release of the amino acid it was decided to include 1mM nipecotic acid in experiments to characterise GABA release.

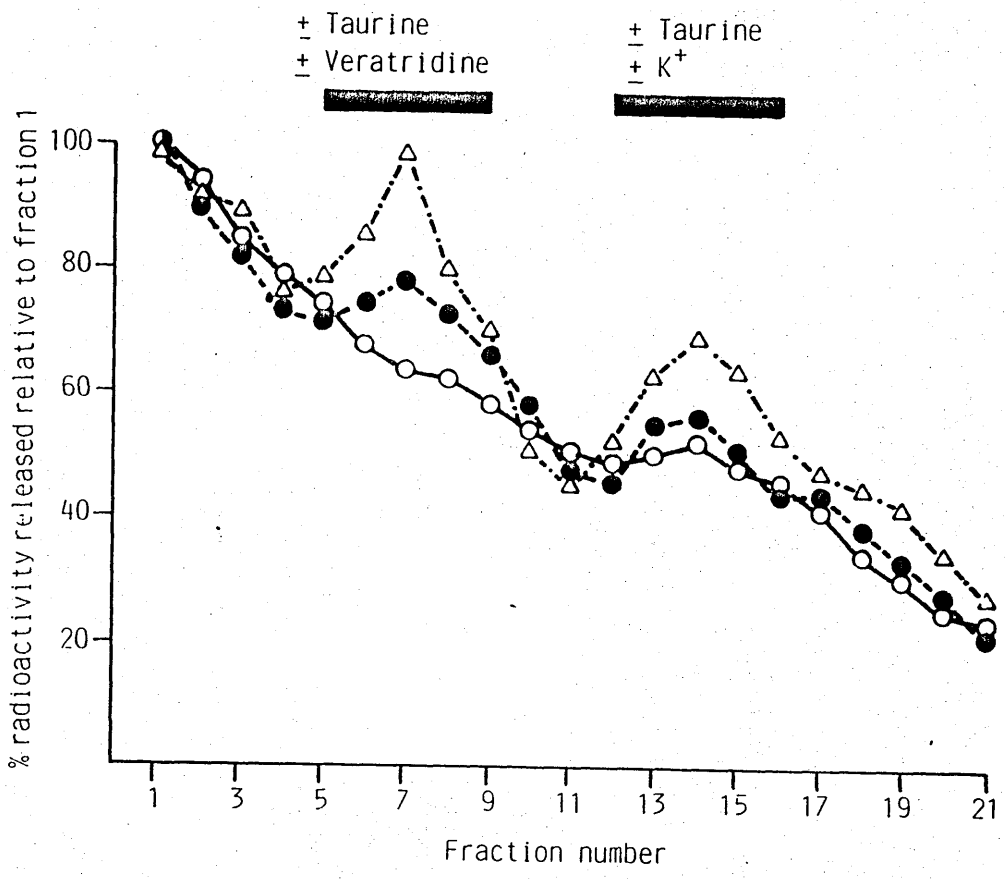


Figure 3.28 Effect of taurine (20mM) on ^{3}H-GABA release from locust synaptosomes. Taurine alone (●----●), veratridine and K^+ in the absence of taurine (o——o), veratridine and K^+ in the presence of taurine (△-·-△). Fraction 1 corresponded to 2098 ± 312 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 15 % of the mean for each point.

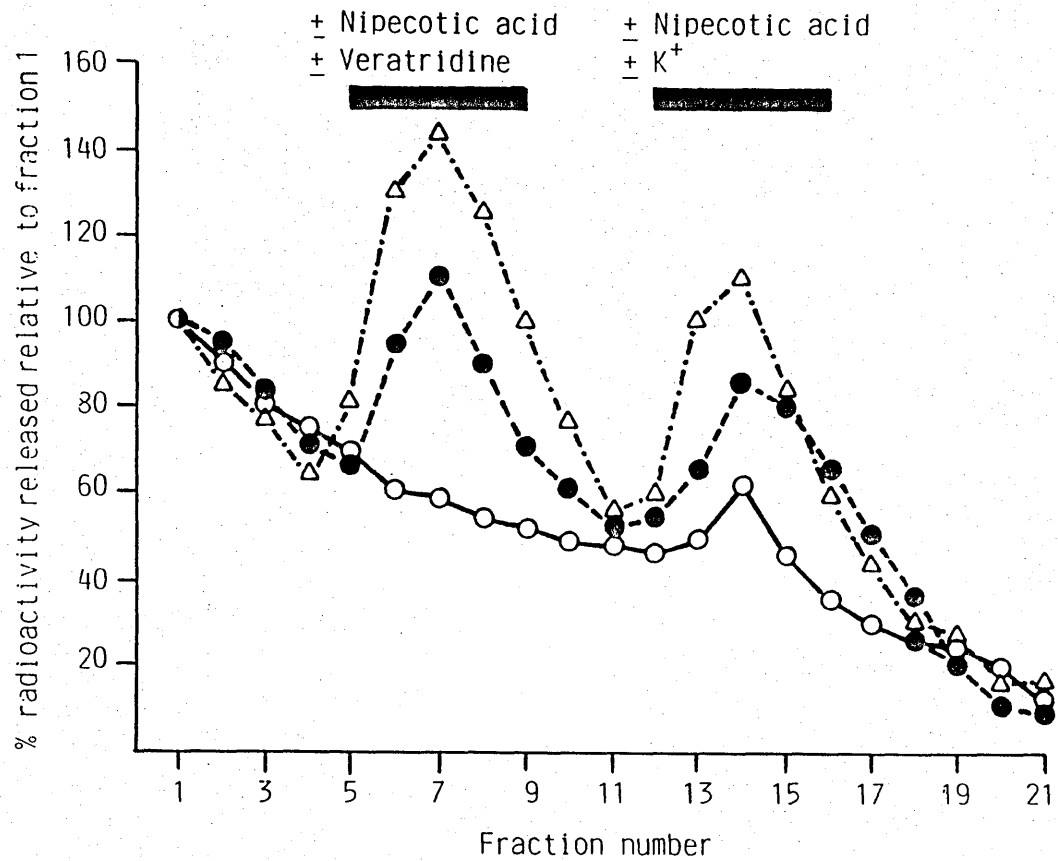


Figure 3.29 Effect of nipecotic acid (1mM) on ^{3}H-GABA release from locust synaptosomes. Nipecotic acid alone (●---●), veratridine and K^+ in the absence of nipecotic acid (o——o), veratridine and K^+ in the presence of nipecotic acid (△---△). Fraction 1 corresponded to 1823 ± 77 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 10 % of the mean for each point.

3.22 Calcium dependency of ^{3}H-ACh and ^{3}H-GABA release

Since an influx of calcium into the synaptic terminal is considered to be a requirement for neurotransmitter release (Miledi, 1973; Llinas and Heuser, 1977) this was studied with respect to the present system. When no calcium was present in the incubation medium (which contained 1mM EGTA) release of ACh was considerably reduced but was not completely abolished (Fig. 3.30). In the absence of calcium, but using nipecotic acid to reveal GABA release by preventing its reuptake, GABA efflux was reduced to a level which was observed in the presence of nipecotic acid alone (Fig. 3.31 and see Fig. 3.29). This indicates that the neurotransmitter release induced by veratridine and high K^{+} was Ca^{++}-dependent.

3.23 Effect of tetrodotoxin on ^{3}H-ACh and ^{3}H-GABA release

To confirm that veratridine-induced release of neurotransmitter was based on activation of sodium channels, the effect of TTX on release was studied. TTX was observed to abolish veratridine induced release of both ACh and GABA, but in neither case was high K^{+}-induced release affected (Figs 3.32 and 3.33).

Studies using isolated flight muscle and nervous tissue mitochondria

3.24 Enzymic characterisation of the flight muscle mitochondria

Figure 3.34 shows the enzymic characterisation of the different fractions throughout the process of mitochondrial preparation (see Fig. 2.4). The most important point is that the final mitochondrial

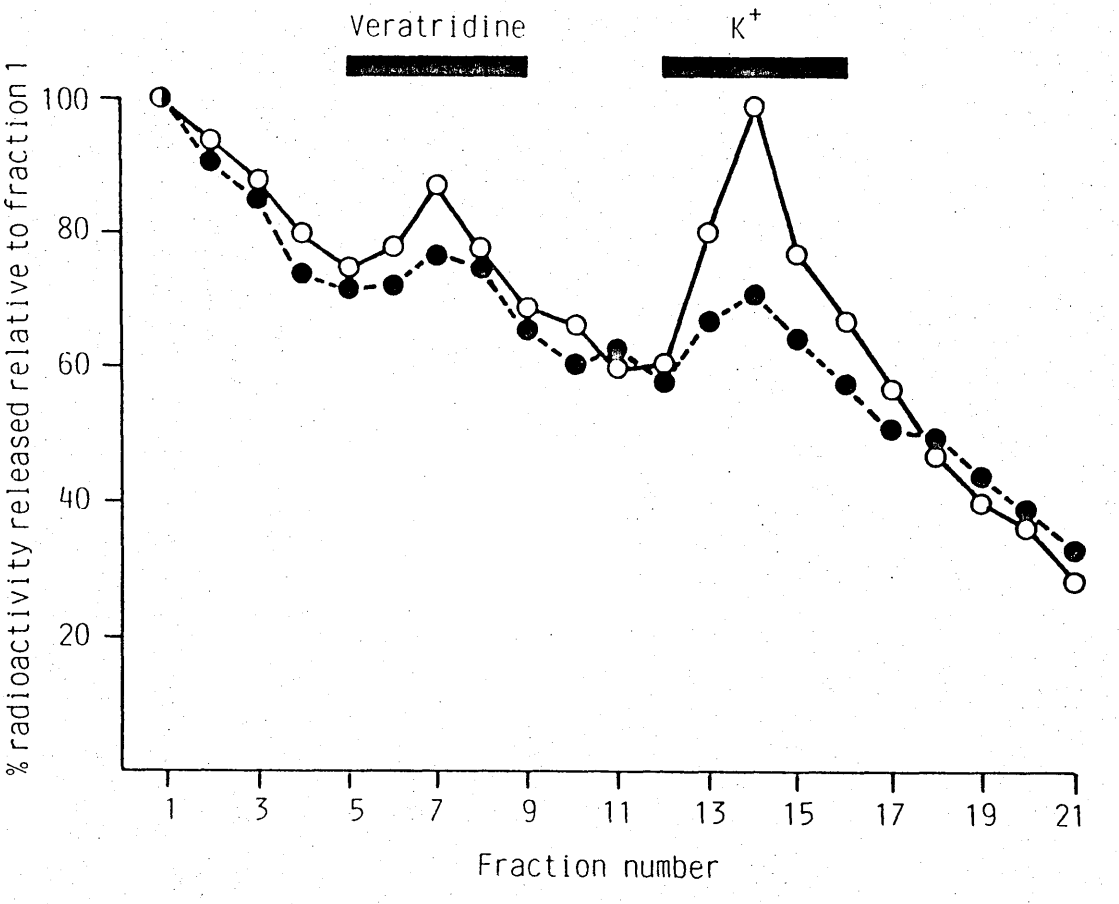


Figure 3.30 Calcium dependency of ^{3}H-ACh release from depolarised locust synaptosomes. Synaptosomes were superfused with insect saline in which calcium was either present (o——o), or absent (●----●), and in the latter case 1mM EGTA was present in the saline. Fraction 1 corresponded to 4353 $\pm$ 367 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 10 % of the mean for each point.

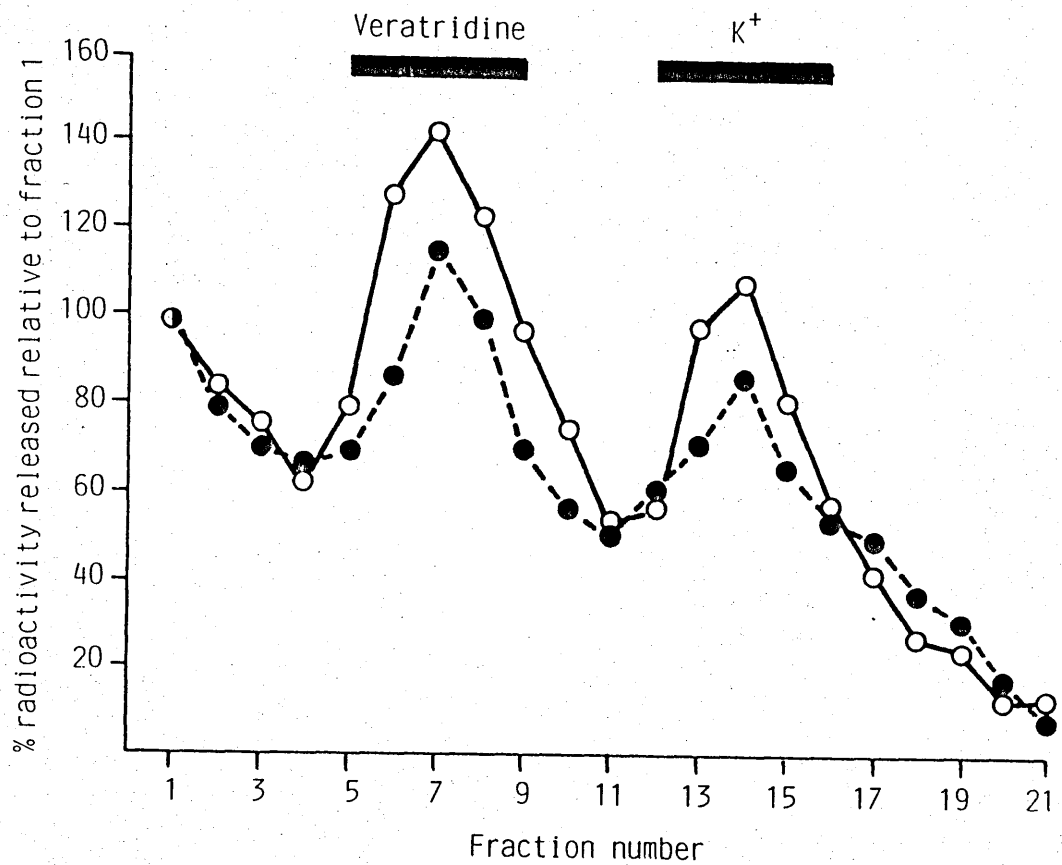


Figure 3.31 Calcium dependency of ^{3}H-GABA release from depolarised locust synaptosomes. Synaptosomes were superfused with insect saline in which calcium was either present (o——o), or absent (●----●), and in the latter case 1mM EGTA was present in the saline. 1mM nipecotic acid was present in the saline during the periods of veratridine (100μM) and high K^{+} (100mM) stimulation (solid bars) in order to reveal GABA release. Fraction 1 corresponded to 1955 $\pm$ 184 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 10 % of the mean for each point.

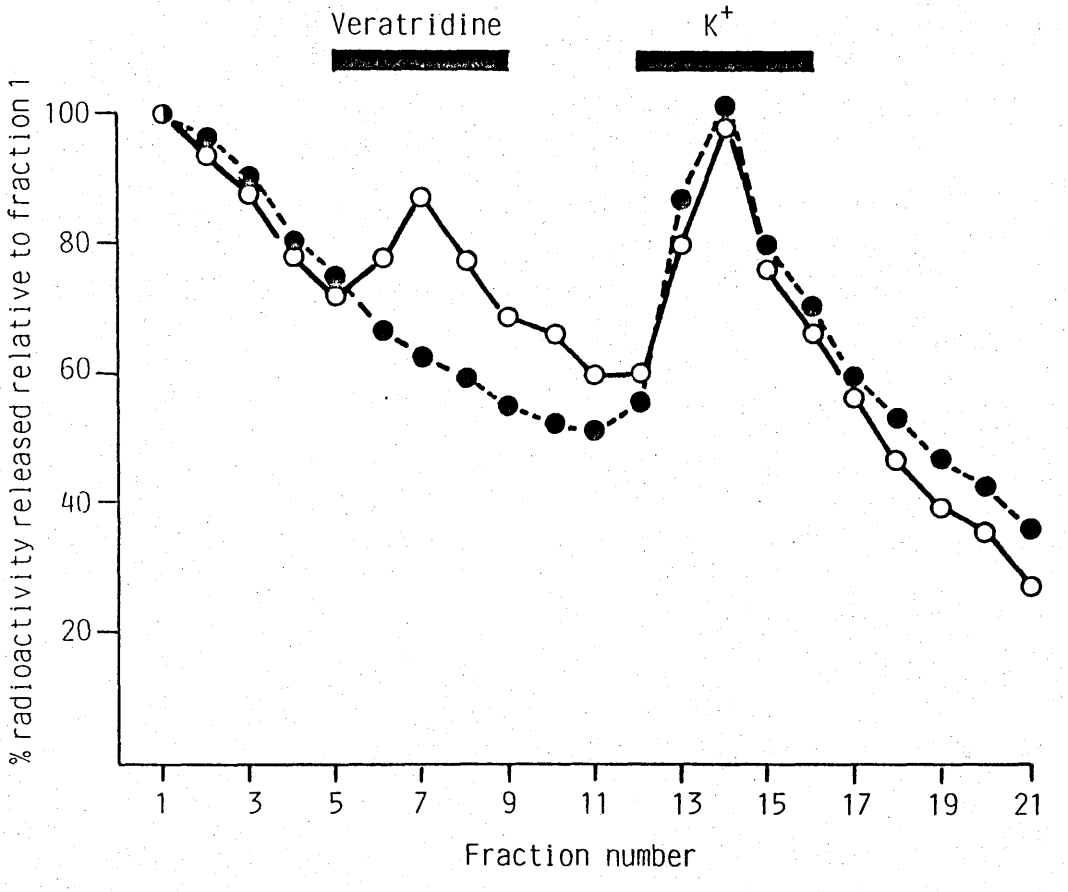


Figure 3.32 Effect of tetrodotoxin (1μM) On ^{3}H-ACh release from depolarised locust synaptosomes. TTX present (●----●), TTX absent (o——o). TTX was only present in the saline during the periods of veratridine (100μM) or high K^{+} (100mM) stimulation (solid bars). Fraction 1 corresponded to 4108 $\pm$ 104 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 10 % of the mean value for each point.

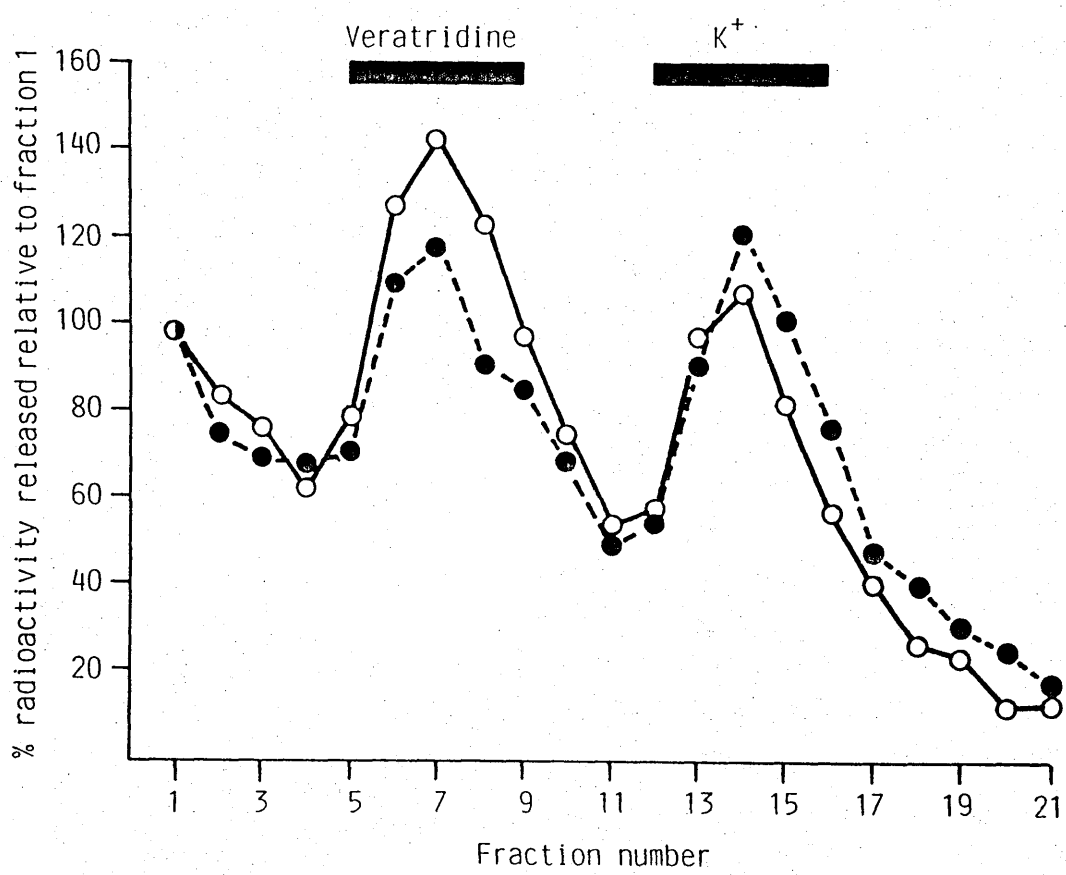


Figure 3.33 Effect of tetrodotoxin (1μM) on ^{3}H-GABA release from depolarised locust synaptosomes. TTX present (●----●), TTX absent (o——o). TTX was only prsent in the saline during the periods of veratridine (100μM) or high K^{+} (100mM) stimulation (solid bars). 1mM nipecotic acid was also present in the saline during these periods in order to reveal ^{3}H-GABA release. Fraction 1 corresponds to 1831 $\pm$ 59 cpm. Results are the average of triplicate samples of which the s.e.m. was less than 15 % of the mean value for each point.

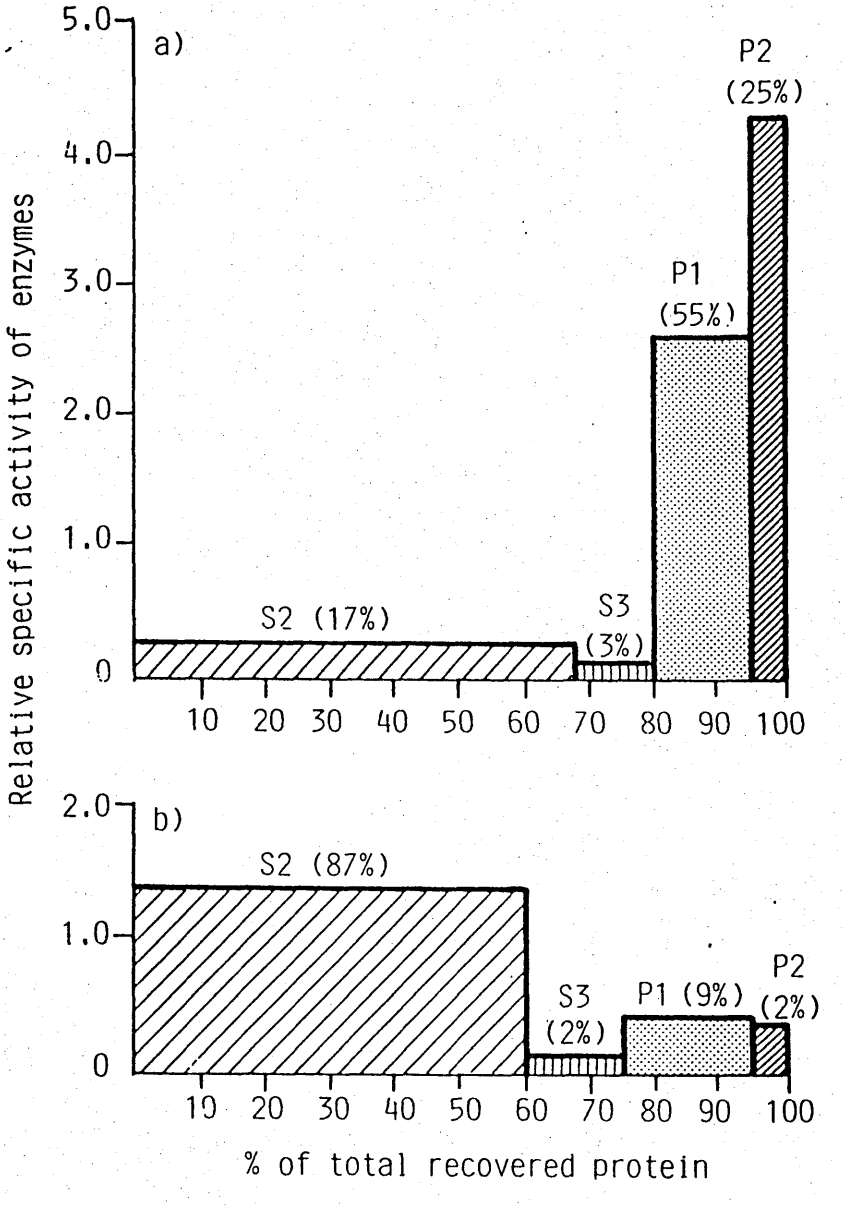


Figure 3.34 The Figure shows the specific activities of glutamate dehydrogenase and NAD^+-linked ∝-glycerophosphate dehydrogenase relative to S_1 (see Fig. 2.4). The activities were estimated after treatment of the fractions with 0.1 % Triton X-100 to release any occluded activity. Figures in brackets show the % of the total recovered enzyme activity in each fraction.

pellet contained a very low level of α-glycerophosphate dehydrogenase activity, indicating little cytoplasmic contamination, but was highly enriched in the mitochondrial enzyme glutamate dehydrogenase. Since some 95 % of glutamate dehydrogenase activity was occluded, and was released only after detergent treatment, it is clear that the mitochondria were both pure and intact.

3.25 Uptake of $^{45}Ca^{++}$ into locust mitochondria and the effect of taurine

Mitochondria isolated from both flight muscle and nervous tissue accumulated $^{45}Ca^{++}$ rapidly for 30 seconds after they had been introduced to the incubation medium, and thereafter more slowly, during the 10 min incubation (Fig 3.35). Accumulation of $^{45}Ca^{++}$ in mitochondria was found to be strongly influenced by, but not absolutely dependent upon the concentration of phosphate in the medium (Fig. 3.36), and on the basis of this 10mM phosphate was used thereafter. When taurine was added to the incubation medium no effect on $^{45}Ca^{++}$ accumulation was observed (Fig 3.37). As shown in Table 3.11 ruthenium red and incubation at $0^{o}C$ both greatly reduced (by at least 70 %) $^{45}Ca^{++}$ uptake into mitochondria from both tissues.

3.26 Efflux of $^{45}Ca^{++}$ from mitochondria and the effect of taurine

Since taurine had no detectable effect on $^{45}Ca^{++}$ uptake into mitochondria, it was decided to study any possible effect the amino acid might have on calcium efflux. Efflux of calcium from muscle and nervous tissue mitochondria requires a sodium gradient (Nicholls and Akerman, 1982). In the presence of 10mM Na^{+} a slight efflux of $^{45}Ca^{++}$ was observed, but this failed to reach statistical significance (Fig. 3.37). However, in the presence of ruthenium red, which

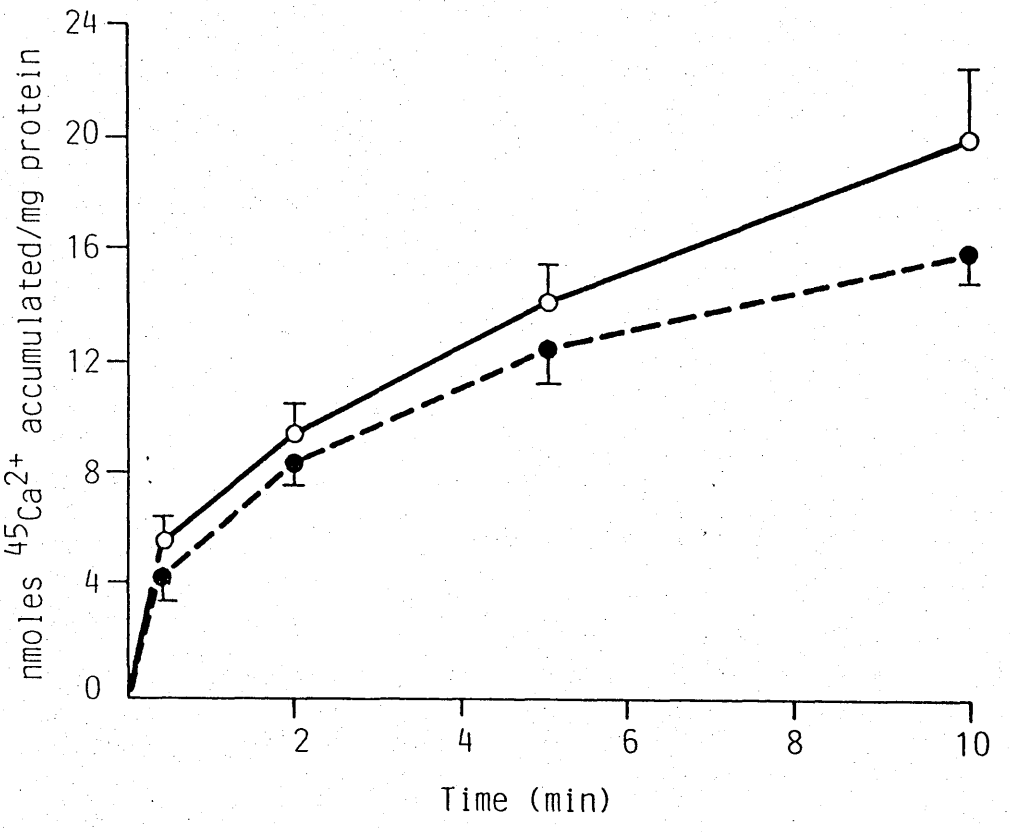


Figure 3.35 Time course of $^{45}Ca^{++}$ uptake into locust mitochondria. Mitochondria were obtained from flight muscle (●----●) or nervous tissue (o——o), and were incubated with 2μCi of $^{45}Ca^{++}$ at 30°C. Incubation was stopped by filtration and blank filter counts were subtracted from the results. Results are the mean $\pm$ s.e.m. from three samples.

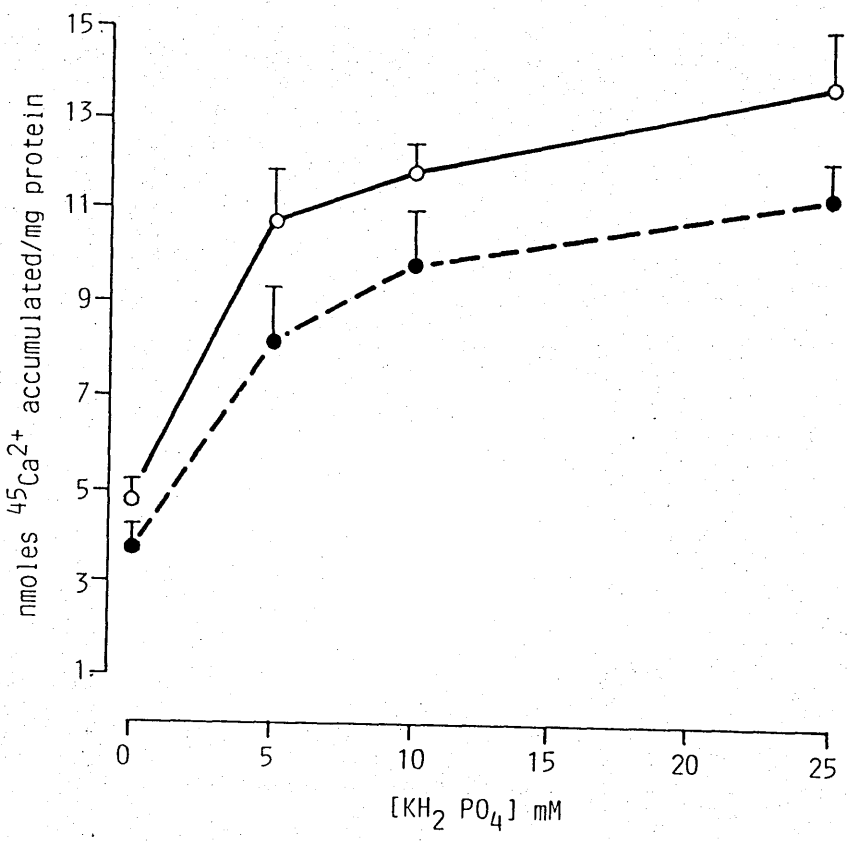


Figure 3.36 Effect of increasing phosphate concentration on uptake of $^{45}Ca^{++}$ into locust mitochondria. Mitochondria were obtained from flight muscle (●----●) or nervous tissue (o——o), and were incubated for 2 min as described previously (see legend to Fig. 3.35). Results are the mean $\pm$ s.e.m. from three samples.

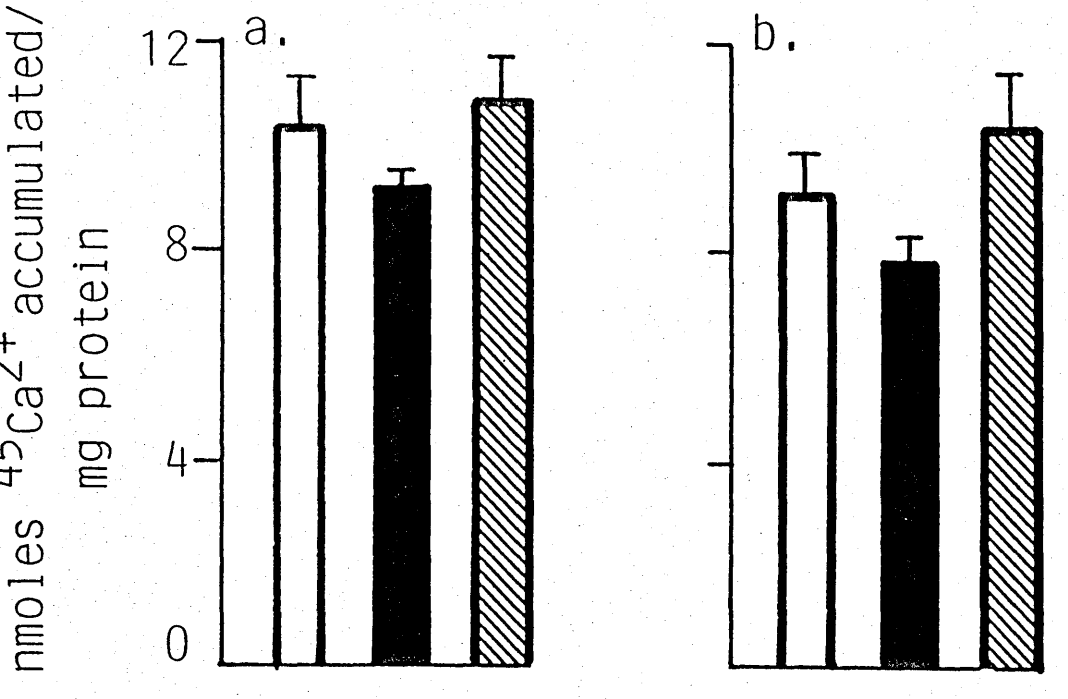


Figure 3.37 Effect of taurine (20mM) or Na^+ (10mM) on accumulation of $^{45}Ca^{++}$ by locust mitochondria from nervous tissue (a) or flight muscle (b). Incubation medium contained either no additions (open bars), 10mM Na^+ (filled bars) or 20mM taurine (hatched bars). Mitochondria were incubated for 2 min as described previously (see legend to Fig. 3.35). Results are the mean $\pm$ s.e.m. of three samples.

Table 3.11 Effect of cold ($0^{o}C$) or ruthenium red ($5\mu M$) on $^{45}Ca^{++}$ uptake into locust mitochondria

Treatment	$^{45}Ca^{++}$ uptake (nmoles/mg protein)	
	Nervous tissue mitochondria	Flight muscle mitochondria
Control	10.3 ± 1.1	8.7 ± 0.9
Ruthenium red	$1.3 \pm 0.3^{*}$	$1.1 \pm 0.2^{*}$
Cold ($0^{o}C$)	$2.7 \pm 0.7^{*}$	$1.6 \pm 0.4^{*}$

Mitochondria were incubated for 2 min as described previously (see legend to Fig. 3.35). Results are the mean $\pm$ s.e.m. of three samples. * indicates significant difference from control ($P < 0.001$).

inhibits calcium uptake into mitochondria (see Table 3.11), a clear efflux of $^{45}Ca^{++}$ was observed (Fig. 3.38). It can be seen from Figure 3.38 that even in the presence of ruthenium red, if Na^{+} is absent no efflux of $^{45}Ca^{++}$ is observed. In the data shown in Figure 3.39 both ruthenium red and EGTA were used to prevent further $^{45}Ca^{++}$ uptake into mitochondria, and in both cases a significant efflux of $^{45}Ca^{++}$ was seen. In the presence of Na^{+}, when taurine was added to the incubation medium, the efflux of $^{45}Ca^{++}$ was abolished (Fig. 3.39), but once again it can be seen that taurine had no effect on $^{45}Ca^{++}$ uptake alone. The effect of taurine was similar in both muscle and nervous tissue mitochondria, whether ruthenium red or EGTA was used.

Studies on ^{3}H-taurine binding to locust membranes

3.27 Association of ^{3}H-taurine with a whole locust head membrane preparation

Table 3.12 summarises the results obtained in assessing taurine binding to a membrane preparation obtained from whole locust heads. ^{3}H-taurine bound to the membrane preparation to the extent of 1.10 pmoles/mg of protein, but in the presence of 1mM unlabelled taurine this was significantly reduced, indicating competition for a specific site. If the preparation was not dialysed no taurine binding was observed, suggesting that endogenous taurine (or some other interfering substance) was still present in the preparation. ^{3}H-taurine binding was found to be sensitive to both low temperature (0^{o}C) and the absence of sodium ions (Table 3.12). Since sensiti-

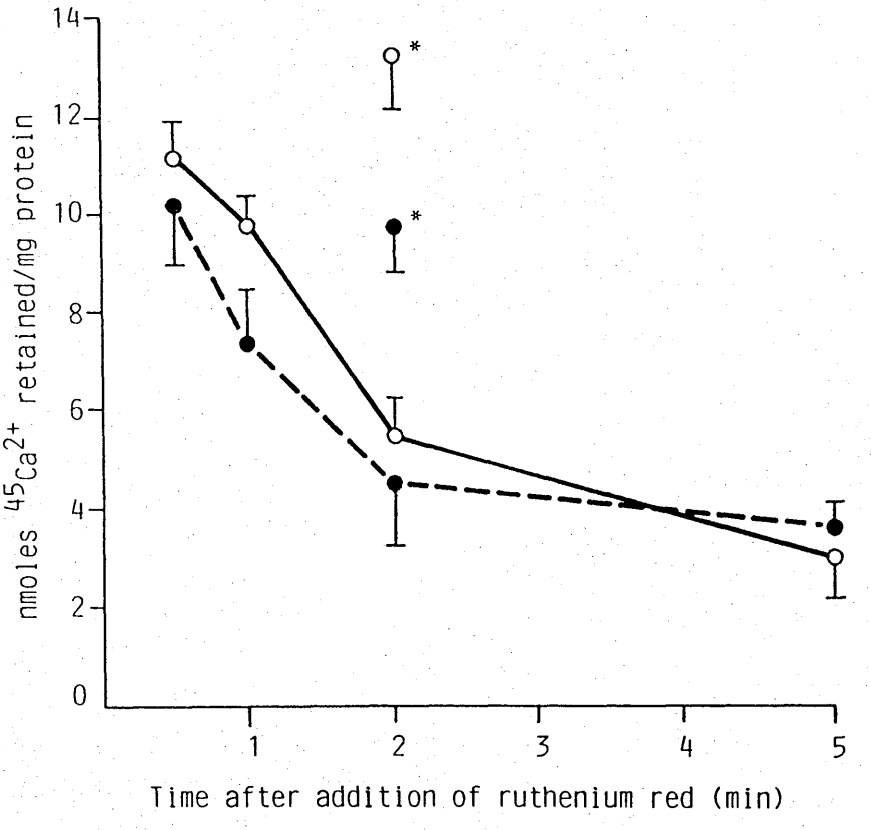


Figure 3.38 Time course of Na^+-dependent $^{45}Ca^{++}$ efflux from locust mitochondria. Mitochondria were obtained from flight muscle (●----●) or nervous tissue (o——o) and were incubated for 2 min prior to addition of ruthenium red. Incubations were performed and stopped as previously described (see legend to Fig 3.35). The two points marked by asterisks indicate the lack of efflux in the absence of 10mM Na^+ (data derived from Fig. 3.39). Results are the mean ± s.e.m. of three samples.

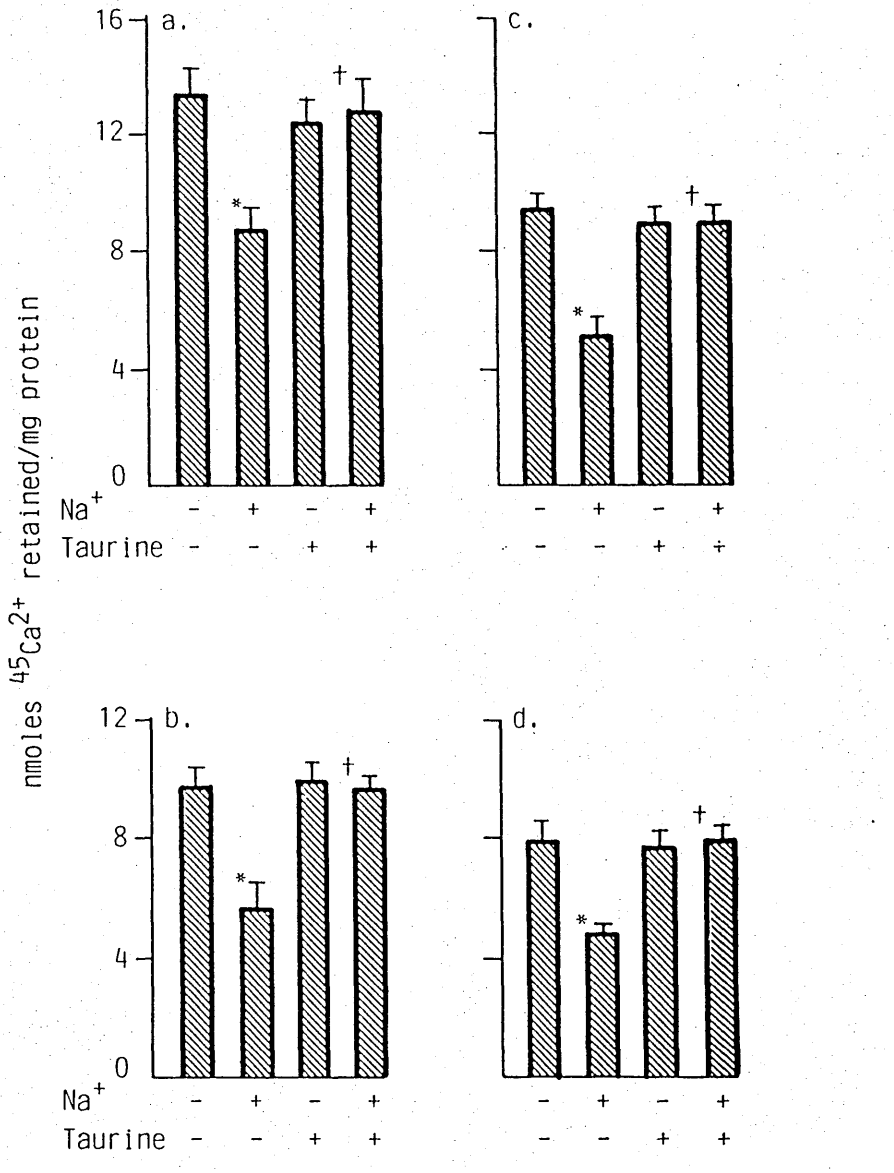


Figure 3.39 Effect of taurine on Na^+-dependent efflux of $^{45}Ca^{++}$ from locust mitochondria. Mitochondria were obtained from flight muscle (b,d) or nervous tissue (a,c) and were incubated as described (see legend to Fig. 3.35) for 2 min prior to addition of either ruthenium red (a,b) or EGTA (c,d) to terminate $^{45}Ca^{++}$ uptake. The reaction was stopped by filtration 2 min later. Na^+ (10mM) and/or taurine (20mM) were present or absent as indicated below each histogram. Significant differences ($P < 0.05$) are shown * or + where results are compared to the appropriate controls: * - compared to Na^+, taurine absent; + - compared to taurine absent. n = 3.

Table 3.12 ^{3}H-taurine binding to a locust membrane preparation

Sample description	^{3}H-taurine binding (pmoles/mg of protein)
Assay stopped by centrifugation	
No additions to medium	1.10 ± 0.04
+ 1mM unlabelled taurine	0.68 ± 0.03*
Undialysed preparation	0.68 ± 0.04*
Na^{+} relaced by choline	0.74 ± 0.04*
Assayed at 0°C	0.71 ± 0.02*
Assay stopped by filtration	
Filter washed with H_2O	0.74 ± 0.04
Filter washed with H_2O + 1mM unlabelled taurine in medium	0.57 ± 0.03*
Filter washed with saline	0.75 ± 0.02
Filter washed with saline + 1mM unlabelled taurine in medium	0.60 ± 0.01*
Filter counts in absence of membranes	0.29 ± 0.01

Results are the mean ± s.e.m. of five samples in each group.

* indicates statistically significant differences ($P < 0.02$) compared with samples with no additions to the medium, as appropriate to each procedure for terminating the assay.

vity to these conditions is characteristic of many uptake systems, an experiment was performed to consider the possibilty that taurine might be accumulated within vesicles formed by the membrane preparation. In this case the incubation was terminated by filtration followed by washing with either saline or distilled water. If taurine was being accumulated within vesicles it would be expected that these would be lysed by distilled water, but not saline, and that under the former conditions apparent binding would be lower. As shown in Table 3.12 there was no observable difference in taurine binding under both conditions, and in both cases this was significantly greater than if 1mM unlabelled taurine was present (Table 3.12). In the absence of the membrane preparation there was quite a high degree of ^{3}H-taurine binding to the filter alone, but this showed no difference between the two washing conditions and the results (Table 3.12) are therefore the average of all filter counts.

Electrophysiological studies with taurine and GABA

There is much data to suggest that the effects of taurine on neurons is very similar to that of GABA, and often the pharmacology of these two amino acid shows considerable similarities. The electrophysiological studies have therefore concentrated on a comparison of effects of taurine and GABA on the locust neuronal cell body (somatal) preparation, and the pharmacology of the receptors for both compounds.

3.28 Effect of taurine and GABA on membrane potential in locust somata

Both taurine and GABA produced similar hyperpolarising effects on the membrane potential in resting neurons using acetate filled

micro-electrodes. When the membrane potential was varied by passage of polarising D.C. through the recording pipette it was found that taurine and GABA had very similar reversal potentials, in the region of -75 mV (Fig. 3.40).

3.29 Effect of taurine and GABA on resistance changes in locust somata

Figures 3.41 and 3.42 show the effect of taurine and GABA respectively on the resistance across the somatal membrane during repetative application of current pulses. Both substances caused a dose-dependent decrease in membrane resistance and this was accompanied by a hyperpolarisation. Comparison of the two figures reveals very great differences in the iontophoretic ejection currents to produce an equivalent effect on membrane resistance. In order to assess whether this difference was due to a difference in potency between GABA and taurine, or differences in iontophoretic mobility of the two compounds, the latter was estimated by including ^{3}H-amino acid in the pipette. Over a 5 min period it was calculated, using the specific activities of taurine or GABA in the pipette, that 0.07μmoles of taurine (ejection current 400nA) was ejected, while over the same period 0.06μmoles of GABA (40nA ejection current) was ejected. Thus under the iontophoretic conditions used it appears that taurine has about 10 % of the iontophoretic mobility of GABA. It can be seen from Figures 3.41 and 3.42 that even accounting for such a difference in iontophoretic mobility GABA appears to be considerably more potent than taurine.

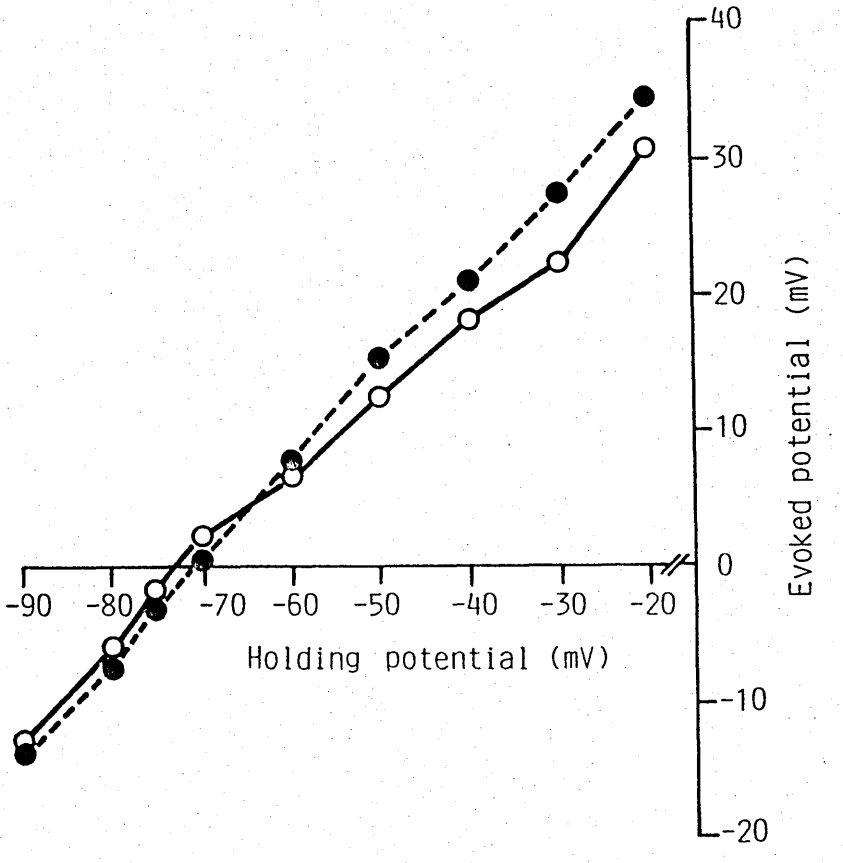


Figure 3.40 The evoked-potential/ holding-potential relationship seen in locust somata in response to iontophoretic application of either taurine (o——o) or GABA (●----●). The point of intersection of the two curves with the abscissa represents the reversal potential for the amino acid-induced current, which for both was about -75 mV.

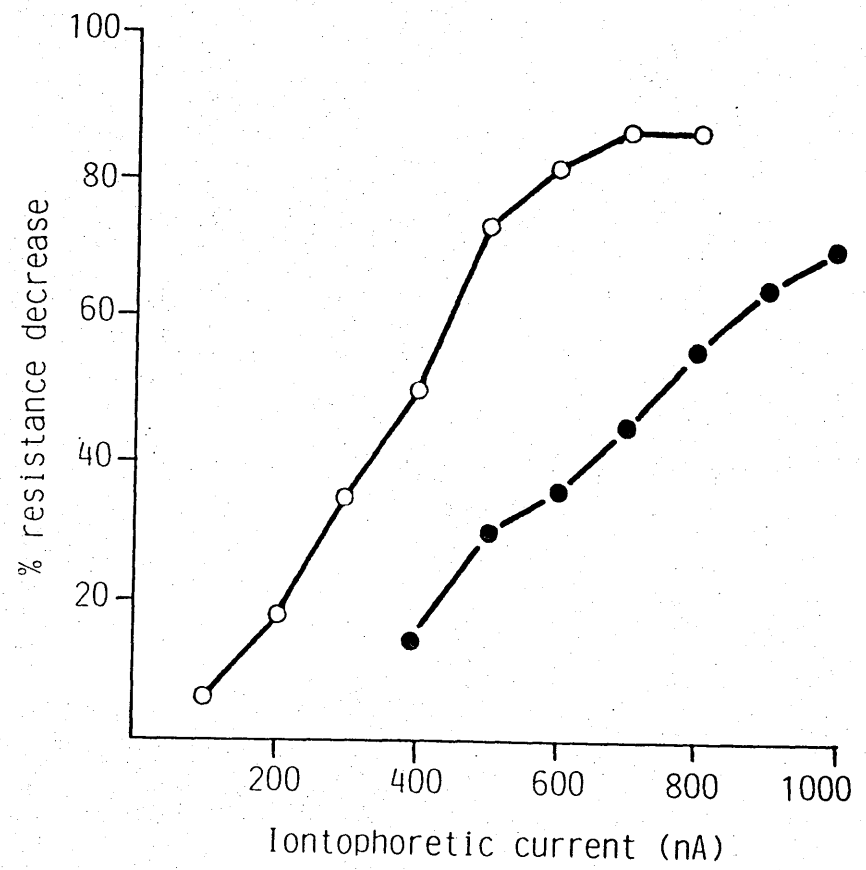


Figure 3.41 The effect of taurine on membrane resistance, as assessed by repetetive application of hyperpolarising current pulses into locust somata, in the absence (o——o) or presence (●——●) of TAG (100μM).

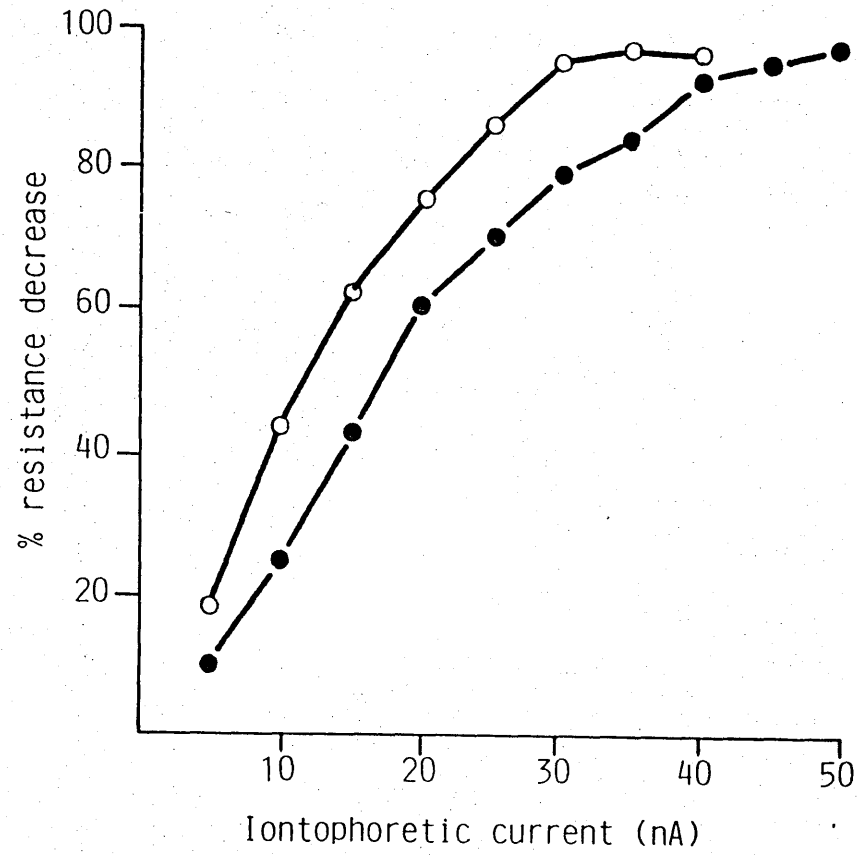


Figure 3.42 The effect of GABA on membrane resistance, as assessed by repetative application of hyperpolarising current pulses into locust somata, in the absence (o——o) or presence (●——●) of TAG (100μM).

3.30 Pharmacology of taurine and GABA in locust somata

Although the pharmacology of taurine has been poorly resolved due to a lack of specific agonists and blockers, 6-aminomethyl-3-methyl-4H-1,2,4-benzthiadiazine-1,1-dioxide hydrochloride (TAG) has been proposed as a specific taurine antagonist (Yarbrough et al., 1981). The effect of TAG on responses to taurine and GABA was therefore studied. Using a TAG concentration of 100μM, less than that used by Yarbrough et al. (1981), responses to taurine were indeed reduced, and this effect of TAG was not entirely overcome even at the highest dose of taurine which could be applied (Fig. 3.41), which might suggest a non-competative interaction. However, it can be seen that TAG was not entirely specific in its action since GABA responses were also reduced (Fig. 3.42), albeit to a lesser extent. This is also indicated in Figures 3.43 and 3.44. Picrotoxin, a known GABA antagonist, was found to progressively reduce then completely abolish the effects of both taurine and GABA on locust somata (Figs. 3.45 and 3.46). Finally the effect of flunitrazepam, a drug of the benzodiazepine class which are known to have modulatory effects on GABA responses (see Olsen, 1981), was studied. Figures 3.47 and 3.48 show that flunitrazepam augmented the responses to both taurine and GABA. The pharmacology of taurine and GABA on the locust somata preparation is therefore very similar.

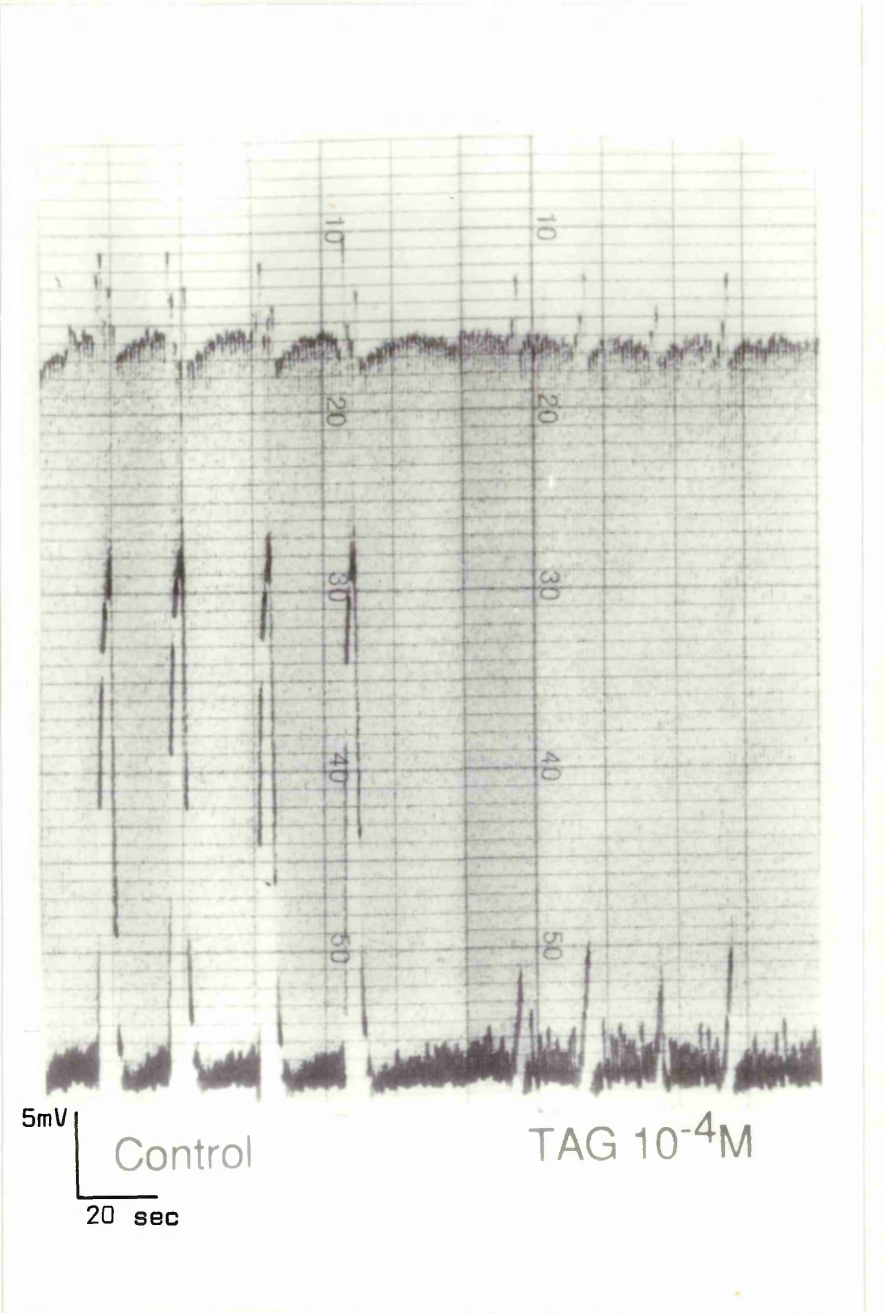


Figure 3.43 Effect of TAG on the reduction in membrane resistance induced by iontophoretic application of taurine (400nA, 3 sec), assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to taurine, TAG was bath applied to give a final concentration of 100μM. Taurine evoked conductance changes are largely abolished by the antagonist, but small hyperpolarisations are still evident. Upward deflections are hyperpolarisations. V_m was -42mV.

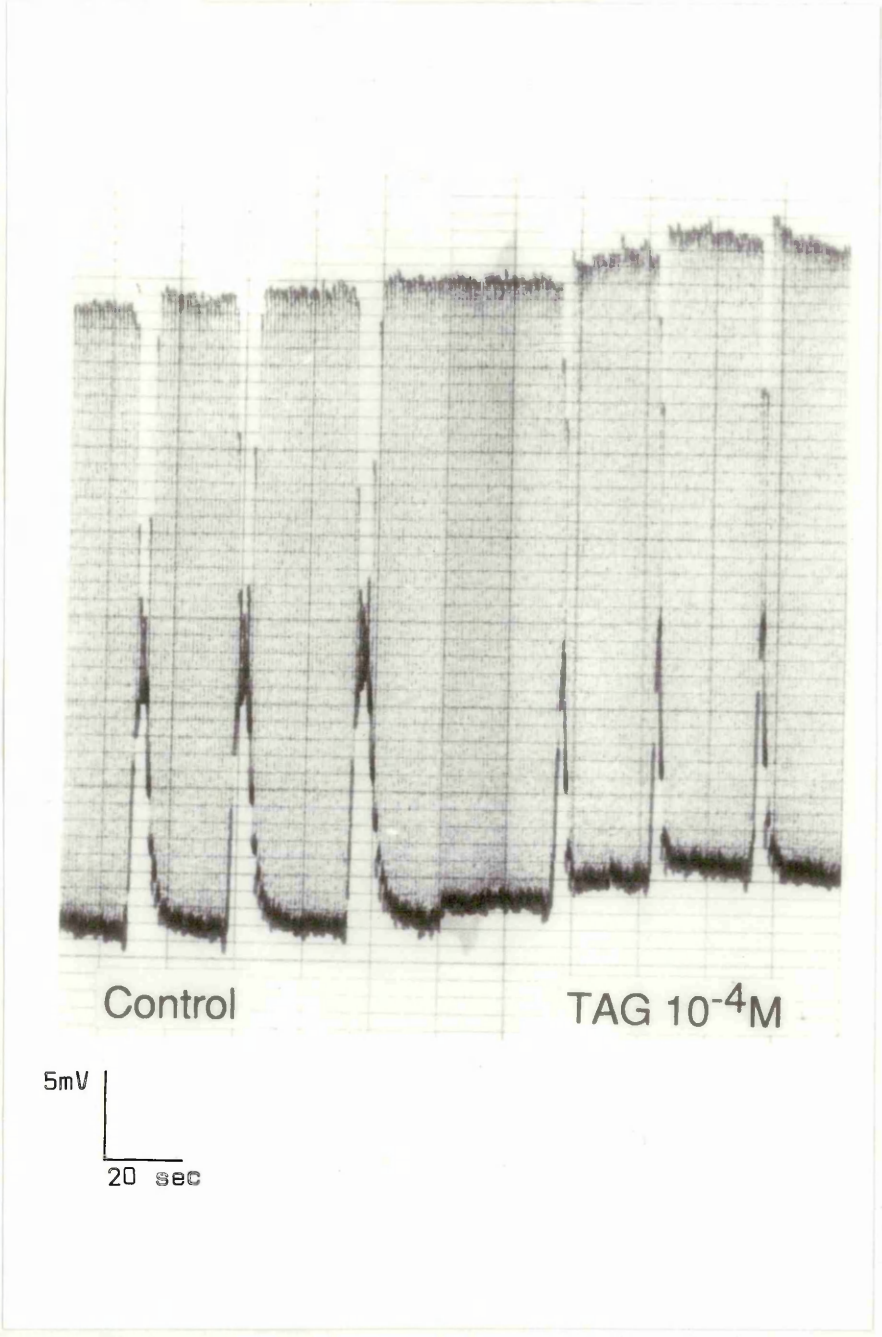


Figure 3.44 Effect of TAG on the reduction in membrane resistance induced by iontophoretic application of GABA (30nA, 3 sec) assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to GABA, TAG was bath applied to give a final concentration of 100μM. Upward deflections are hyperpolarisations. V_m was -41mV.

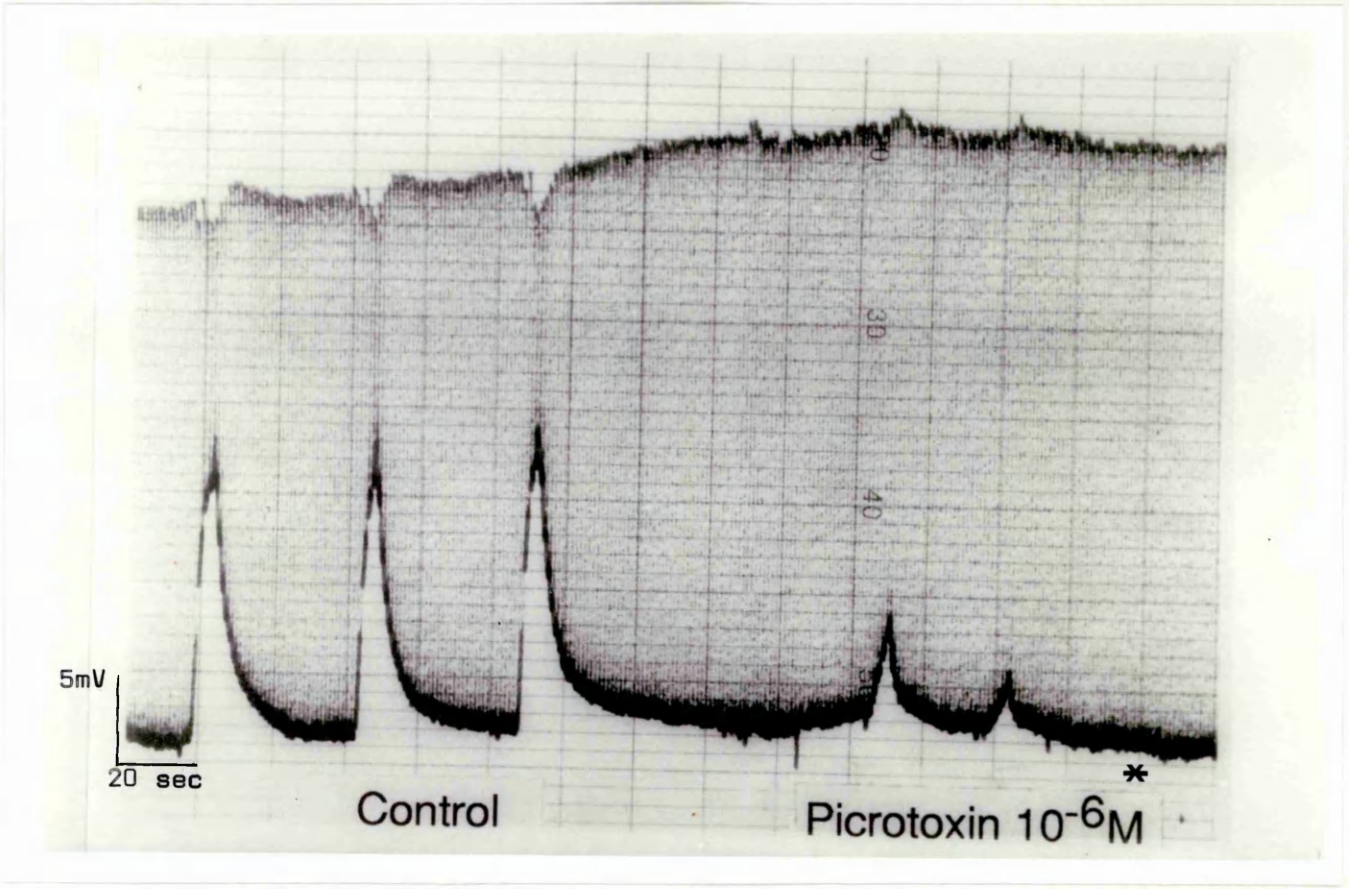


Figure 3.45 Effect of picrotoxin on the reduction in membrane resistance induced by iontophoretic application of taurine (400nA, 3 sec) assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to taurine, picrotoxin was bath applied to give a final concentration of 1μM. The asterisk (*) indicates a further application of taurine, but the response was totally blocked. Upward deflections are hyperpolarisations. V_m was -46mV.

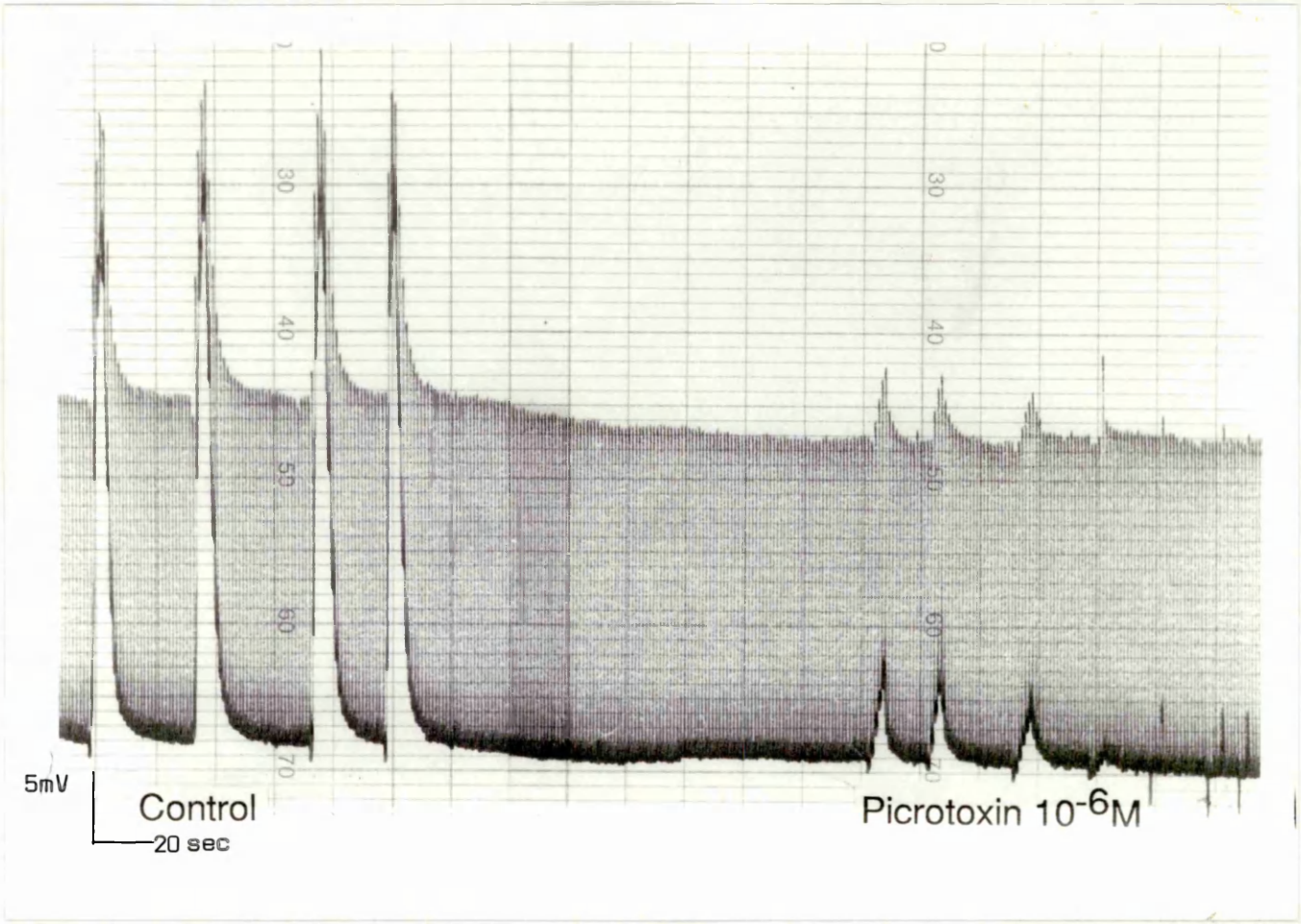


Figure 3.46 Effect of picrotoxin on the reduction in membrane resistance induced by iontophoretic application of GABA (30nA, 3 sec) assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to GABA, picrotoxin was bath applied to give a final concentration of 1μM. The progressive reduction and final abolition of GABA responses can be seen from the illustration. Upward deflections are hyperpolarisations. V_m was -32mV.

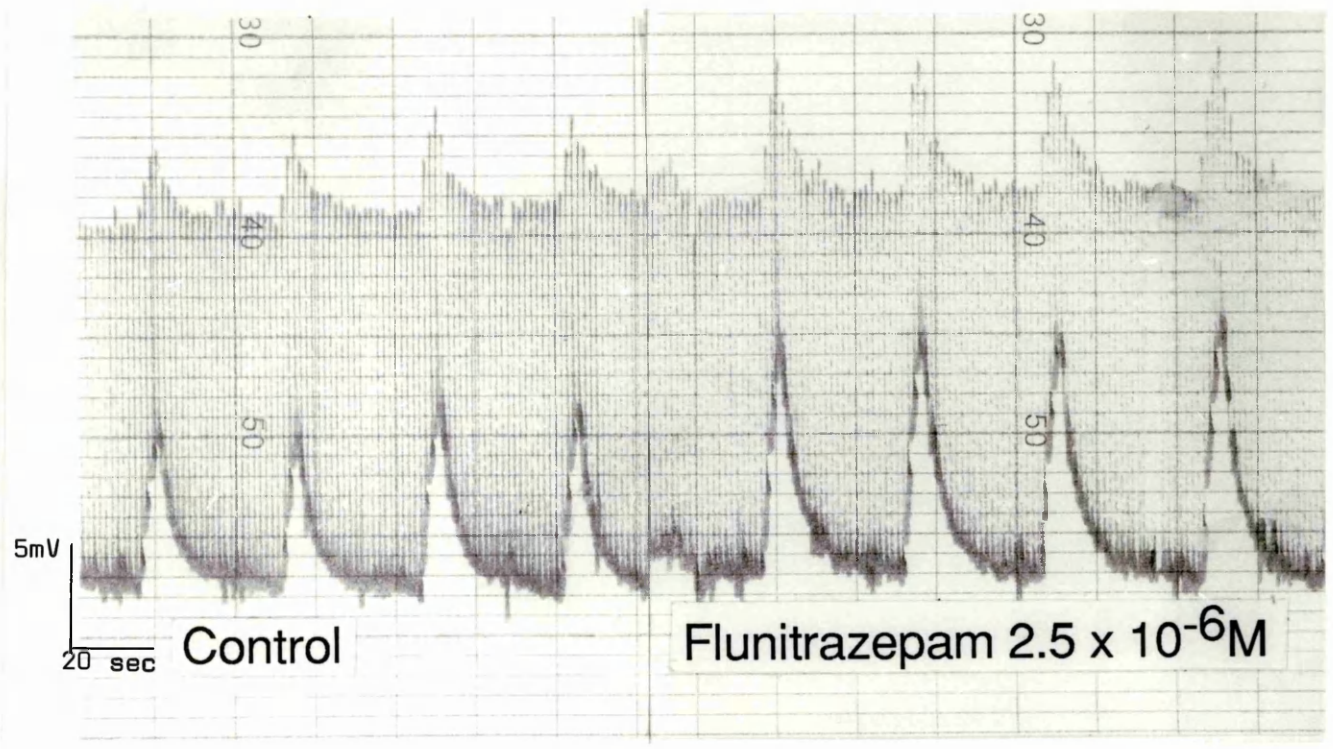


Figure 3.47 Effect of flunitrazepam on the reduction in membrane resistance induced by iontophoretic application of taurine (200nA, 3 sec) assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to taurine, flunitrazepam was bath applied to give a final concentration of 2.5μM, and an increase in the amplitude of the response was evident. Upward deflections are hyperpolarisations. V_m was -42mV.

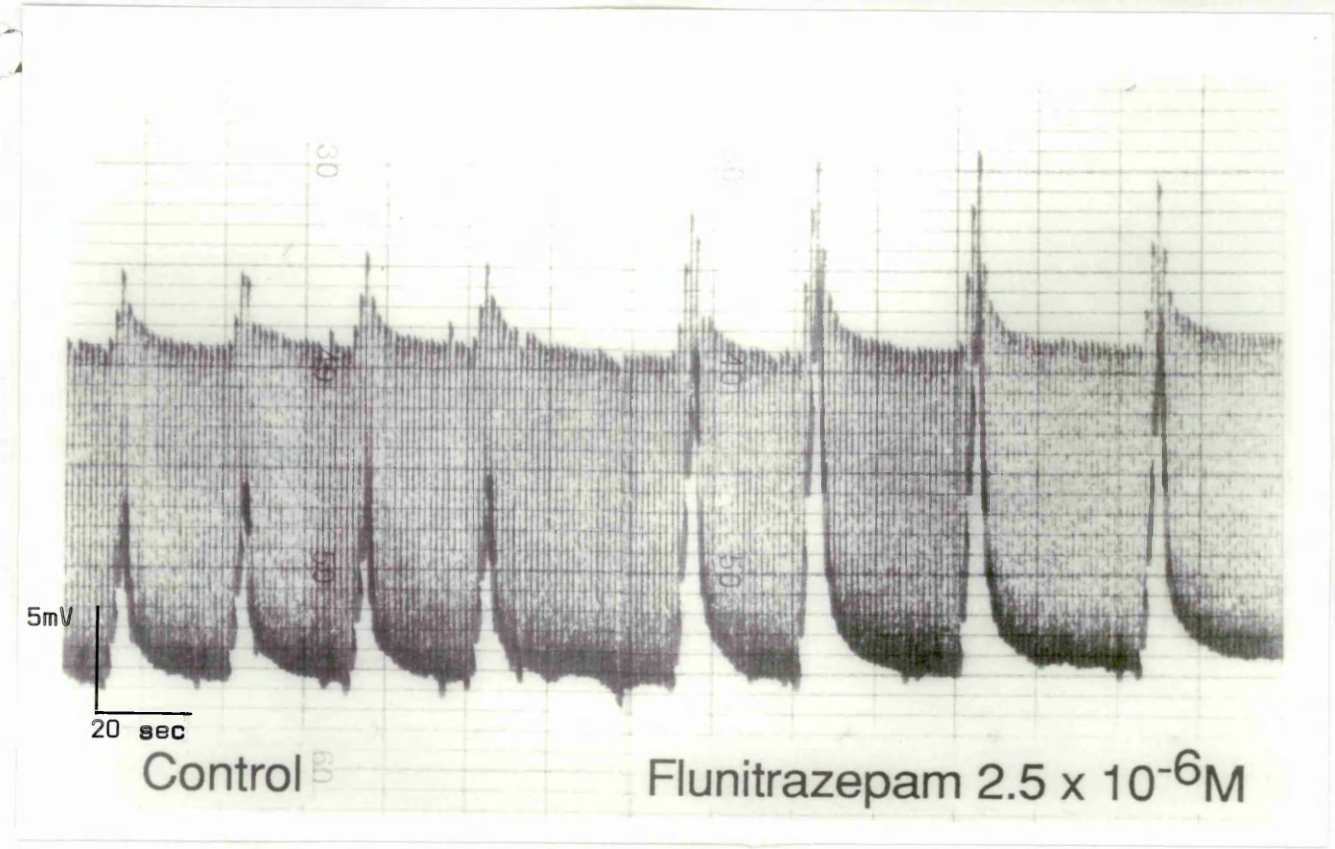


Figure 3.48 Effect of flunitrazepam on the reduction in membrane resistance induced by iontophoretic application of GABA (10nA, 3 sec) assessed by repetetive application of hyperpolarising current pulses into the locust somata. After obtaining control responses to GABA, flunitrazepam was bath applied to give a final concentration of 2.5μM, and an increase in the amplitude of the responses was evident. Upward deflections are hyperpolarisations. V_m was -44mV.

SECTION FOUR

DISCUSSION

The estimation of taurine concentration in various locust tissues confirms that the distribution of the amino acid in this insect shows many similarities to that in higher animals, considering the considerable differences in anatomy and physiology. The highest concentration is present in the flight muscle (about 25μmoles/g), and is relatively much higher than any comparable mammalian skeletal muscle (the highest reported is 14μmoles/g found in the rat, see Grosso and Bressler, 1976). A very high taurine concentration (54μmoles/g wet weight) in the flight muscle of L. migratoria has been reported (Van Marrewijk et al., 1980). The taurine concentrations found by Bodnaryk (1981b) in the head (2.8μmoles/g) and thorax (11.09μmoles/g) in M. configurata are very similar to those observed in S. gregaria. Bodnaryk's finding of little taurine in the moth pupae is consistant with its presence mainly in flight muscle. Weighing whole and dissected locusts has shown that the average weight of a fully grown adult female is about 2 g, of which some 40 % is due to the thorax. An examination of the concentration of taurine in whole thorax and flight muscle suggests that half the wet weight of the thorax is comprised by the flight muscles, which therefore contributes about 20 % or 400 mg of the average adult female. This means that 1 g of whole adult will contain about 6μmoles of taurine of which the 200 mg of flight muscle will contribute about 5μmoles or 83 % of the total. This is in good agreement with the statement of Bodnaryk (1981b) that the thorax of M. configurata contains 91 % of the total taurine.

The part of the nervous system which clearly shows a very high taurine concentration is the eye. This is similar to the situation in the mammalian retina which has a very high taurine concentration (Voaden et al., 1977). It is interesting that this should be the case consider-

ing the large phylogenic and structural differences between the insect and mammalian eye.

The association of taurine with fully developed flight muscle is confirmed by the results from other species of insect, selected for different degrees of flight muscle development. T. molitor is flightless, and the female of B. orientalis has only vestigial wings, while those of the male are quite well developed. Concentrations of taurine in the thoraces of all four insects examined showed a range of results consistant with the association of taurine with flight muscle. The highest concentration is found in the powerfully flying locust, whilst the flightless beetle has the least. The differences between the male and female cockroaches is also consistant with their wing development.

The results from the developing locust, however, show that taurine concentration in the flight muscle is not wholly related to increase in muscle mass, as the taurine concentration increase in the the young adult is greatest slightly after the maximal rate of muscle development, and is proportionately greater. The greatest biosynthetic activity of the taurine pathway takes place in the young adult and appears to be controlled by factors somewhat independent of flight muscle capacity. This high level of taurine biosynthesis in the young adult is supported by the studies on the metabolism of ^{35}S-cysteine in the locust.

During stress there is a marked re-distribution of taurine within the locust. Increase in taurine concentration occurs in the haemolymph, ganglia and fat body, while decreases occur in flight muscle and the eye, although whole body concentration is unchanged. Thus it appears

that taurine is released from from muscle during violent muscular activity. The release of taurine during exercise is not the result of the breakdown of muscle tissue as this would result in a large increase in the activity of arginine kinase in the haemolymph. In neither flying or picrotoxin treatment was there any increase in this very sensitive indicator, whereas in both cases there was a significant increase in taurine concentration. Flying did produce some increase in the volume of the haemolymph, which coupled with the small increase in the average activity of arginine kinase in the haemolymph, might indicate some tissue damage, which in turn might account for some of the release of taurine. There was, however, no such indication in the picrotoxin treated locusts in which the release of taurine was much more marked.

Although taurine biosynthesis appears to be stimulated by picrotoxin treament and flying it is unlikely that this could contribute significantly to the increased taurine concentration observed in tissues other than muscle and eye. No change in whole body taurine concentration occurs during stress and the present results indicate that taurine metabolism in the locust is very slow indeed, so a massive increase in taurine turnover during the time periods considered is highly improbable. This leaves a re-distribution of taurine as the only possible cause for the increase of taurine concentration in haemolymph and other tissues, and it is clear that the flight muscle must be the source of most of this taurine. Why such a re-distribution should occur is unclear. There are many changes which occur in the amino acid content of the haemolymph during stress, including considerable decreases in

the concentrations of some (Jabbar and Strang, 1985). Taurine might help to maintain the osmolarity of the haemolymph as is the case in marine invertebrates (Jacobsen and Smith, 1968), although there is no specific evidence for this.

The uptake of ^{14}C-taurine into excitable tissues in the locust showed many similarities with previous studies in mammals. Taurine tends to accumulate in brain and muscle and within these tissues has a long half-life (Awapara, 1957; Sturman, 1973). It is, however, usually the case that these tissues accumulate taurine relatively slowly. In the locust flight muscle the situation appears to be similar to that in mammaliam cardiac and skeletal muscle, a slow accumulation of taurine being observed, and this is followed by an equally slow decline. However, locust thoracic ganglia and eye accumulated taurine very rapidly, being maximal after 10 min, but once accumulated the ^{14}C-taurine was retained without any noticable decline after 14 days. Retinae, isolated from the frog, loaded with ^{35}S-taurine and maintained *in vitro*, showed no decline in taurine content six weeks after ^{35}S-taurine injection (Lake et al., 1977). *In vitro* studies on uptake and efflux using rat brain slices indicate a very low level of taurine efflux from brain tissues (Oja, 1971; Collins, 1974).

Taurine clearance from the haemolymph also proved to be similar to data obtained from mammals on taurine clearance from the blood after intravenous administration of the amino acid. There appear to be two taurine pools in the locust as indicated

by the two-component decline in ^{14}C-taurine level in the haemolymph: a small rapidly exchanging pool and a larger more slowly exchanging pool. Such data has been found in rats (Sturman, 1973) and monkies (Matsubara et al., 1985).

Taurine clearance from the haemolymph proved to be highly sensitive to low temperature, indicating that non-diffusional processes are involved. Taurine is transported into vertebrate retina (Lake et al., 1977) and brain (Holopainen et al., 1984) by energy requiring transport systems. Taurine transport into locust tissues appears to be mediated by a specific uptake mechanism since it is inhibited by strucural analogues of taurine such as GABA, β-alanine and hypotaurine. These compounds are known to inhibit taurine transport into preparations from vertebrates in vitro (Borg et al., 1979; Larsson et al., 1986). The taurine metabolite isethionic acid had no significant effect on taurine clearance from haemolymph, which indicates that an amino group is a requirement for the transport system.

Taurine biosynthesis in insects has received virtually no attention up to now, and indeed there appears to be only one paper specifically dealing with the subject (Bodnaryk, 1981b), although others considering the whole of sulphur metabolism in insects are more numerous (e.g. Cotty et al., 1958; Henry and Block, 1960, 1961). What data exists indicates that there are no dramatic differences in the pathways for taurine biosynthesis in insects and vertebrates.

The endogenous concentrations of taurine and its possible precursors observed in the present study, as estimated by ^{3}H-dansylated amino acids, reveal that in general the concentrations of

of these compounds are similar to those observed for other amino acids in insects (Hackman, 1956; Levenbook and Dianamarca, 1966; Evans and Crossley, 1974; Van Marrewijk et al., 1980; Jabbar, 1982). The concentration of taurine established by dansylation compares well with that obtained by the fluoremetric determination, (Tables 3.1 and 3.8) in different locust tissues. This confirms the validity of the estimates of taurines precursors by dansylation. Apart from taurine in flight muscle, the other compound which occurs at very high concentration, also in flight muscle, is cysteine sulphinic acid, a substance which in the prsent study appears to be a precursor in taurine biosynthesis. Cysteic acid, also a likely taurine precursor, was found at fairly high concentrations in all the tissues studied, particularly in the adult. On the other hand, hypotaurine, although detectable in all the tissues, was present at a comparatively low concentration.

Since it was not possible to determine ^{35}S incorporation into either cysteic acid or hypotaurine quantitatively the best that could be achieved with the available methods was to establish which tissues could actually form these compouds qualitatively. All of the tissues studied contained ^{35}S labelled precursors sufficient to yield a complete biosynthetic pathway for taurine (Figs. 3.9-3.12 and see Fig.]. Cysteic acid was detected in muscle, eye, ganglia and at trace amounts in fat body while hypotaurine was observed, at trace levels, in thoracic ganglia and eye. Cysteamine, which appears to be a taurine precursor in M. configurata (Bodnaryk, 1981a) and occurs in M. domestica (Cotty et al., 1958) but is absent in B. germanica (Henry and Block, 1961) was not detected in the

locust in the present study. Cysteamine was not found when estimates of endogenous concentrations of taurine precursors were made, nor was there any spot which would correspond with the known position of the NEM adduct of cysteamine after autoradiography, and also no radioactivity was found at the NEM-cysteamine band after one-dimensional TLC followed by scintillation counting. It therefore appears that taurine biosynthesis in the locust occurs via cysteine sulphinic acid and thereafter either cysteic acid or hypotaurine. Although neither of the latter two showed particularly heavy labelling, incorporation was nonetheless much greater into cysteic acid, and on the basis of the present results seems the more likely candidate of the two. It is of course possible that both are taurine precursors in the locust. In vitro studies of enzyme activities and metabolism of specific putative precursors would be a logical development of the present work.

After administration of ^{35}S-cysteine in vivo, all tissues studied were observed to have incorporated ^{35}S into taurine, even after only 1 hr. As would be expected, in all cases the initial specific activity of cysteine was greater than any other compound. In haemolymph where oxidising conditions prevail, cysteine and cystine were essentially in equilibrium throughout the duration of the experiment. In the more reducing conditions found intracellularly, cystine was either absent (fat body and eye) or was detected only when the specific activity of cysteine was very high (thoracic ganglia), with the exception of flight muscle where cysteine and cystine were roughly in equilibrium.

In the tissues examined, with the exception of haemolymph, taurine was the most persistant compound as demonstrated by its ability to retain a specific activity. In the case of flight muscle this was still increasing at the final time point observed. This is a clear reflection of the very slow turnover which is a characteristic of this amino acid, and confirms the results of the uptake studies using ^{14}C-taurine *in vivo* (Fig. 3.5).

When taurine biosynthesis was studied in 1 day old adult locusts, 6 hr after administration of 20μCi of ^{35}S-cysteine, and compared with the same procedures in 25 day old adults considerable differences were observed. It is clear that the biosynthetic pathway for taurine is very much more active in the 1 day old adult, as might be predeicted from the taurine levels found in the thoraces of developing locusts (Fig. 3.3). A further point of interest is that in the 1 day old adults cysteine sulphinic acid was detected *in vivo* in thoracic ganglia, fat body and eye which was not the case in unstressed 25 day old adults. On the other hand, tissues from adults incubated *in vitro* clearly indicated the presence of cysteine sulphinic acid. As well as this 25 day old adults treated with picrotoxin, and to a lesser extent after flight showed increased taurine biosynthesis and in these instances cysteine sulphinic acid was detected in thoracic ganglia, fat body and eye. It therefore appears that when ^{35}S-cysteine metabolism is very rapid a higher specific activity of cysteine sulphinic acid occurs indicating increased turnover of this amino acid, and activation of subsequent steps

in taurine biosynthesis. In mammals cysteine sulphinate decarboxylation to hypotaurine is considered to be the rate limiting step (De La Rosa and Stipanuk, 1985), while if cysteic acid is the immediate precursor of taurine, decarboxylation of cysteate is considered to be rate limiting (see Oja and Kontro, 1983). In mammals it is in fact generally considered that metabolism of cysteine to cysteine sulphinate and thence to cysteate is mediated by the same enzyme (L-cysteine: oxygen oxidoreductase, E.C. 1.13.11.20), and similarly decarboxylation of cysteine sulphinate and cysteate is also achieved by a single enzyme (L-cysteinesulphinate carboxylyase, E.C. 4.1.1.29), at least in brain and liver (see Oja and Kontro, 1983 for a comprehensive account of the subject).

The differences observed between the *in vivo* and *in vitro* studies on ^{35}S-cysteine metabolism by 25 day old adult locust tissues must obviously arise from the conditions found in the *in vitro* experiments. The only major difference was the appearence of ^{35}S-labelled cysteine sulphinate in all tissues *in vitro*. This might be due to the *in vitro* conditions favouring oxidation of ^{35}S-cysteine (the samples were continuously bubbled with 95 % O_2 / 5 % CO_2) compared with those in the intact animal. The appearence of ^{35}S-cysteine sulphinic acid may also be enhanced by the relatively very high dose of ^{35}S-cysteine used in the *in vitro* experiments. Thus in order to ensure that sufficient activity would be present for autoradiography 10μCi of ^{35}S-cysteine was incubated with not more than 10-50 mg of tissue *in vitro*, while for the *in vivo* studies 20μCi of ^{35}S-cysteine was administered to locusts weighing 1.5-2.5 g and was therefore proportionately much more

diluted.

Much of the results obtained in the present study in locusts has proved to be remarkably similar to findings obtained in mammals. A major difference, however, is the apparent inability of locust synaptosomes to accumulate ^{3}H-taurine. Although as might be expected 'mammalian data' essentially means data obtained only in rats, there are many studies in that species demonstrating both low and high affinity taurine transport into synaptosomes (Hruska et al., 1978; Kontro and Oja, 1978; Hanretta and Lombardini, 1986), and there is no reason to question the validity of such data. Moreover, taurine release from rat synaptosomes has been observed in a number of studies (Placeta et al., 1979; Hanretta and Lombardini, 1986). Such studies have been a major influence in establishing taurine as a putative neurotransmitter. It must therefore be considered that the lack of any apparent uptake into locust synaptosomes argues against taurine being a neurotransmitter in this insect. This is almost certainly not due to some functional abnormality in the locust synaptosomal transport systems since ^{3}H-GABA was readily accumulated, as was ^{3}H-choline. Furthermore, ^{3}H-taurine does not seem to accumulate in the nerve terminals of cockroach neurons or in roach glia grown in culture (Beadle et al., 1987). In mammals, the synaptic action of taurine is considered to be terminated, like GABA, by reuptake from the synapse into neurons and glia. Insect neuronal terminals and glia in culture readily accumulate GABA (Beadle and Lees, 1986; Beadle et al., 1987). This contrasts conspicuously with the findings with taurine and must cast further doubt on whether this amino acid is a neurotransmitter

in insects. As the present results show there is reasonable evidence to consider that taurine may be a neuromodulator in the locust, as it also appears to be in mammals. It may be therefore that a role for taurine as a neurotransmitter may have developed in higher animals. Considering the structural similarities of taurine and GABA this would not be difficult to envisage.

It is somewhat suprising that locust synaptosomes were unable to transport taurine, considering its structural similarity to GABA. Taurine proved to be fairly effective at inhibiting GABA transport into synaptosomes, although much less so than nipecotic acid, an established inhibitor of neuronal GABA transport in mammals (Krogsgaad-Larsen and Johnston, 1975). Inhibitors of GABA transport are often themselves transported (Johnston et al., 1975; Neal and Bowery, 1977) but this is evidently not the case with taurine in the locust.

The release of neurotransmitter from presynaptic nerve terminals is initiated by an increase in intraterminal calcium concentration (Miledi, 1973; Llinas and Heuser, 1977). Taurine reduces calcium uptake into resting (Remtulla et al., 1979; Pasantes-Morales and Gamboa, 1980) and K^+-depolarised rat brain synaptosomes (Yamamoto et al., 1981). Electrophysiological studies in guinea-pig cerebellar slices have shown that taurine can abolish calcium spikes in Purkinje neurons (Okamoto et al., 1983) and these authors therefore suggested that taurine may inhibit calcium entry into these cells.

Calcium uptake into nerve terminals occurs through specific channels which are opened by voltage changes across the plasma mem-

brane in response to an action potential. Calcium channels may also be activated by the association of a neurotransmitter with a specific receptor which regulates the state of the channel. Calcium accumulation in the resting state should only occur through random opening of calcium channels. Due to possible artefacts the situation found *in vitro* is potentially more complex. Calcium is extruded from the cell by a Na^+ : Ca^{++} exchange mechanism and a calcium-ATPase extrusion mechanism which is dependent on the proton gradient. Thus major ionic disturbances could profoundly modify calcium homeostasis, while the creation of 'inside-out' synaptosomes would lead to an accumulation of calcium by those processes which would normally extrude it from the cell. In the present work taurine reduced passive calcium influx a little, but was much more effective when a large calcium influx was induced by depolarisation. Since depolarisation causes a temporary reduction in the sodium gradient accross the plasma membrane, it could be argued that such an effect would increase net calcium accumulation by reducing Na^+ : Ca^{++} exchange. To rule out this possibility the effect of verapamil, a calcium channel blocker, was studied on veratridine induced calcium uptake. Verapamil reduced calcium uptake with a potency very similar to that observed in rat brain synaptosomes depolarised by K^+ (Turner and Goldin, 1985). This was encouraging since of the two depolarising agents used (K^+ and veratridine) veratridine would be far more likely to cause a calcium accumulation artefact since its depolarising action is based on activation of sodium channels (Villegas et al., 1976), while K^+ opens calcium channels because of a collapse in the membrane

potential. The effect of verapamil therefore indicated that calcium influx was occurring through actual calcium channels. This may also explain why taurine was more effective in blocking calcium uptake in depolarised rather than resting synaptosomes. Certain channel blocking drugs require the channel to be open in order to gain access to some moiety within the channel with which they interact. An example of this is picrotoxin, a GABA antagonist, which acts by obstructing the GABA regulated chloride channel of the GABA receptor complex (Olsen, 1981; Turner and Whittle, 1983). This raises the question of the *in vivo* relevance of taurine's action. In the present study taurine is being considered as an endogenous substance, not an applied pharmacological effector. The concentrations of taurine required to significantly reduce calcium influx in this and other studies (Remtulla et al., 1979; Pasantes-Morales and Gamboa, 1980) are very high compared with what are considered usual concentrations of other neuroactive substances. Most neuroactive substances are released into the extracellular space to act on other cells. Clearly an element of dilution occurs, and whether taurine could achieve such concentrations within microevironments in the extracellular space is unknown. However, the *in vivo* concentration of taurine in the nervous system in mammals (see Grosso and Bressler, 1976) and insects is high, particularly in areas where subcellular compartmentilisation occurs. Huxtable and Sebring (1986) have recently suggested that many of the diverse effects of taurine could be explained by its interaction with phospholipids in cell membranes. If this is the case then it may be that taurine could act intracellularly, which would suggest a very general action of this amino

acid. The effects of taurine on calcium binding to muscle intracellular membranes could be achieved by such an effect. It is also possible that, since ion channels must traverse the entire cell membrane, that taurine could interact with the calcium ionophre intracellularly. It is possible that by acting on phospholipids in the vicinity or within the calcium channels taurine might induce an open channel to revert back to the closed conformation or increase the energy required to open the channel, or might simply physically obstruct the channel.

The effect of taurine on depolarisation-induced calcium uptake suggested that this amino acid might modulate neurotransmitter release. Taurine was observed to reduce ACh release in a concentration-dependent manner, and over the range in which calcium uptake was reduced. In order to postulate a connection between these events, however, it was important to establish to what extent ACh release was calcium-dependent. Breer and Knipper (1984) found ACh release from L. migratoria synaptosomes to be wholly dependent on calcium in the saline, while in the present study ACh release was reduced, but not abolished, in the absence of calcium. A number of reports exist suggesting that taurine can reduce neurotansmitter release. Taurine decreases dopamine release from rat brain synaptosomes (Arzate et al., 1986), as wellas in vivo in free moving rats (O'Neil, 1986). Taurine has also been found to reduce ACh release from isolated rat cervical ganglia and rat cortical slices (Kuriyama et al., 1978), as well as light-evoked ACh release from isolated rabbit retinae (Cunningham and Neal, 1983). On the basis

of monitoring EPSP's Hue and Chanelet (1984) have suggested that taurine reduces ACh release in the sixth abdominal ganglion of P.americana. It appears that taurine shares a common spectrum of activity as a modulator of neurotransmitter release in both insects and mammals.

The effect of taurine on GABA release is at present contradictory. In guinea pig cerebellar slices taurine was observed to reduce K^+-evoked GABA release (Namima et al., 1983) and this was attributed to a possible action of taurine on presynaptic GABA autoreceptors. Leach (1979), however, had previously observed that taurine enhanced both veratridine and K^+-induced release of GABA from rat cortical slices, and as was observed in the present work with synaptosomes, the effect of taurine was relatively much greater on veratridine-induced release. Since nipecotic acid also increased GABA release it seems probable that this and the effect of taurine are the result of inhibition of GABA uptake and reuptake. In the present work both compounds were observed to cause concentration-dependent increases in GABA release from synaptosomes. Szerb (1982) found that nipecotic acid increased the efflux of GABA from rat cortical slices in high K^+ concentration depolarising conditions. Furthermore, electrical stimulation of rat brain slices failed to increase GABA efflux above resting level, but such an increase was observed in the presence of nipecotic acid (Szerb, 1982). Nipecotic acid may increase GABA efflux by mechanisms other than inhibiting GABA transport. The transport of nipecotic acid into the nerve terminal may result in increased

transport of GABA from storage sites into the extracellular space (Johnston et al., 1976), or may cause displacement of stored GABA by heteroexchange (Levi et al., 1978). However, since taurine is not transported by locust synaptosomes it is improbable that such mechanisms could be involved in its effect on GABA release. Since taurine can potentiate GABA release it is possible that some of the inhibitory effects of taurine reported might be due to this effect. The converse might also apply due to the competitive effects these amino acids have on each others uptake (Larsson et al., 1986).

Smith and Bygrave (1978) reported the dependence on external phosphate concentration of calcium transport into flight muscle mitochondria from the blowfly L. cuprina. In the absence of external phosphate, calcium transport was minimal, while phosphate concentrations above 2.5mM had an inhibitory effect on calcium uptake. Wohlrab (1974), however, observed that in the blowfly Sarcophaga bullata calcium transport was markedly higher at 20mM phosphate concentration than at 1mM. In the present work increasing phosphate concentration up to 25mM led to an increase in calcium transport by locust mitochondria. Taurine has been observed in the present work to reduce Na^+-dependent calcium efflux from locust flight muscle and thoracic ganglia mitochondria. Kuriyama et al. (1978) reported that taurine decreased calcium efflux from rat brain mitochondria. However, in these experiments Na^+ was present at an unphysiologically high concentration (150mM), and the data was insufficient to conclude an effect of taurine on Na^+-dependent calcium efflux. Dolara et al. (1973) suggested

that taurine increased calcium uptake into mitochondria from rat liver. No such effect was seen in the present studies, nor was any such effect of taurine observed by Kuriyama et al. (1978). It should be considered, however, that the concentration of calcium used in the work of Dolara et al. (1973) was in the millimolar range which is far greater than the intracellular free calcium concentration in vivo which is usually 1μM or less. In the present work, considering the concentration of the labelled calcium, the maximum impurities in the KH_2PO_4, and the calcium buffering effect of the NTA, the free calcium concentration would be about 500nM, which is very similar to the physiological intracellular concentration.

The current work suggests that taurine may play a role in modulating intracellular free calcium. The extent to which mitochondria may contribute to intracellular free calcium modulation in a functional sense is uncertain, particularly with respect to processes which require rapid changes in free calcium, since mitochondria are relatively slow. However, there is evidence that mitochondria could contribute to calcium regulation in cardiac muscle (Fry and Miller, 1985), and if this is so might well perform similar roles in nervous tissue where neurotransmission is to a great extent regulated by the intracellular free calcium concentration. This, along with the observed effect of taurine on calcium uptake into depolarised synaptosomes, indicates that taurine may act at more than one site to reduce intracellular free calcium, which in turn might tend to reduce neurotransmitter release in nervous tissue, and decrease contractility in muscle. Taurine is

known to increase calcium association with cardiac sarcolemma (Chovan et al., 1979; Chovan et al., 1980; Sebring and Huxtable, 1985) as well as skeletal muscle sarcoplasmic reticulum (Dolara et al., 1986; Huxtable and Bressler, 1973), and it would be very interesting to study such possible effects in insect muscle.

A criterion of most neuroactive substances is that they interact with specific receptors in order to mediate their effects. In the case of taurine, binding sites have been demonstrated in vertebrate nerve (Lahdesmaki et al., 1977), muscle (Kulakowski et al., 1980) and retinal preparations (Lombardini and Prien, 1983). In the present study a membrane preparation was made from whole locust head, which although crude in comparison with using a pure tissue, is not uncommon in insect studies where quantities of material are an important consideration (Cattell et al., 1980; Donellan et al., 1980; Mansour et al., 1980; Cohenand Casida, 1985). Binding sites for taurine were detected in the whole head membrane preparation, and it would be interesting to attempt to detect such sites in specific tissues such as muscle which can be obtained in reasonable quantity in the locust. The competative pharmacology of such binding sites should also be characterised.

Since in the present work, binding could only be found after washing and dialysis of the preparation, it is noteworthy that in vertebrate preparations difficulties have been encountered in removing endogenous taurine from membrane preparations (Lombardini and Prien, 1983; Marnela and Kontro, 1984). It appears that it may be characteristic of taurine that it is tightly bound within

the tissue, or perhaps is occluded by subcellular compartmentilisation.

There are a number of electrophysiological studies on taurine in a variety of preparations from both vertebrates and invertebrates. It has generally been found that taurine has an inhibitory effect on neural activity, similar to that of GABA. Both transmitters have a similar, though not necessarily identical, pharmacology in most preparations.

Taurine inhibits synaptic activity of the A6 abdominal ganglion in P.americana (Hue et al., 1978) and also inhibits spontaneous activity in the isolated nerve cord of the same insect (Jabbar and Strang, 1985). The inhibitory effects of taurine and GABA are in most cases due to increased chloride conductance with a consequent hyperpolarisation of the neuronal membrane (Nistri and Constanti, 1976; Hue et al., 1979; Homma, 1979). In the present studies taurine and GABA had a qualitatively similar effect on isolated locust somata. The reversal potential for both amino acids was very similar, at about -75mV, and such a reversal potential would be consistant with a chloride mediated effect. However, the efficacy of the two compounds did differ, with GABA being some 3-4 times more potent than taurine, even accounting for the lower iontophoretic mobility of taurine under the present experimental conditions. It should be stressed, however, that the true efficacy of the two amino acids would best be estimated by bath application. Giles and Usherwood (1985) using bath application, observed that GABA and taurine both

hyperpolarised isolated locust somata from S. gregaria. Under these conditions all cells tested were found to be responsive to GABA, but only two out of five responded to taurine. In the present study all cells tested were observed to respond to either taurine or GABA.

The pharmacology of taurine and GABA is often similar. Both amino acids are antagonised by picrotoxin in the cockroach, but in the same preparation only taurine appears to be sensitive to strychnine (Hue et al., 1979). In the lobster muscle fibre only GABA was found to be antagonised by picrotoxin (Nistri and Constanti, 1976). In frog spinal cord GABA was selectively blocked by picrotoxin, but not strychnine, with the reverse holding true for taurine (Sonnhof et al., 1975). In locust somata both GABA and taurine were antagonised by picrotoxin, with the antagonist concentration (1μM) being sufficient to abolish completely the responses to the amino acids. Lees et al. (1987) have found picrotoxin to be a potent antagonist of GABA in locust somata. These authors have also observed that the benzodiazepine flunitrazepam can augment responses to GABA in locust somata (Lees et al., 1987). The present findings confirm these observations in that GABA responses were augmented by flunitrazepam and underline the similarity between taurine and GABA in this preparation. Finally the effects of TAG, a proposed specific taurine antagonist (Girard et al., 1982; Yarbrough et al., 1981) was studied. TAG produced a clear antagonism to taurine in locust somata, but the effect of GABA was also reduced at the same TAG concentration (100μM), albeit to a lesser extent. Yarbrough et al. (1981) used 250-500 μM concentrations of TAG to study the effect of this drug on the

reduction in dorsal root potentials in the toad elicited by taurine and taurine analogues. TAG reduced the effects of taurine and β-alanine, but not GABA. In cat spinal cord TAG was observed to reduce taurine and to a lesser extent GABA responses (Curtis et al., 1982). Thus although TAG appears to be more effective in blocking the effects of taurine than GABA, it is clearly not very selective and the concentration difference for these effects is not great enough to make TAG a really useful pharmacological tool in taurine studies.

Considering the considerable similarities observed in the effects of taurine and GABA on S. gregaria somata, and the comparative pharmacology of the two amino acids, it seems likely that both are acting at a common site in this instance.

General conclusions

Although a number of studies considering taurine in insects exists up till now, there has been a lack of data obtained in a single insect species. Thus data concerning taurine in insects has been piecemeal and has not allowed an overall picture on the subject to emerge. The studies of Bodnaryk (1981a,b) have shown that taurine may be particularly associated with insect flight muscle, and that its biosynthetic pathway is similar to that in some vertebrate tissues. The present work has largely been on a single insect species, S. gregaria. The distribution of taurine in the locust does have similarities with that observed in vertebrates. It appears that in general taurine may be a significant constituent of the free amino acid pool in insect flight muscle, as is the case in vertebrate skeletal and cardiac muscle (see

Grosso and Bressler, 1976). This indicates that the function of taurine in muscle may be similar in both insects and vertebrates. Within the nervous system, the effects of taurine observed by both electrophysiology and biochemical methods are consistent with observations derived from mammalian studies. The major difference is the inability of locust synaptosomes to take up taurine. This would argue against taurine having a true neurotransmitter role in this species, and it would be of considerable interest to study this using synaptosomes derived from other insect species. In vertebrates the effects of taurine can be broadly grouped under two camps: those in which taurine is postulated as a neurotransmitter with GABA-like actions, and those in which taurine may act as a neuromodulator, probably affecting calcium homeostasis, and this is generally not mimicked by GABA. Taurine may well be a neuromodulator in locusts, but its role as a neurotransmitter is questionable. Certainly taurine has GABA-like effects on isolated somata, but this may be due to a relatively weak agonist action at a site which is modulated by GABA *in vivo*.

The failure to observe taurine uptake into synaptosomes could be due to the possibilty that only a very small population of the synaptosomes actually take up the amino acid, although there is no evidence for this. In mammalian synaptosomal preparations it is now possible to isolate specific subpopulations by a form of affinity chromatography. It would be interesting to attempt this with taurine in insects, but the methodological difficulties would probably be very considerable.

This work was originally stimulated by the observation that

taurine concentration is elevated in the haemolymph of stressed insects and occurs, even in the unstressed animal, at a concentration which has an inhibitory effect on nervous activity (Jabbar, 1982; Jabbar and Strang, 1985). There is only one tissue within the locust that could act as a pool of taurine to elevate haemolymph concentration to the extent observed; the flight muscle. Although it does seem that stress and enforced activity do stimulate taurine biosynthesis to some extent it does not seem that this would be sufficient to account for the large changes in taurine distribution observed.

Is taurine the much studied but elusive 'toxic factor' found in the haemolymph of poisoned insects? There have been so many compounds proposed, that it would be naive to catagorically suggest a single candidate. Indeed it is probably naive to imagine that a single toxin is responsible for the death of insecticide treated insects at all. Even using lethal doses (100μg) of Lindane it takes many hours to kill a locust, by which time its general metabolism will be grossly different from that in the normal animal. It approaches the impossible to state which of this myriad of biochemical disfunctions is the exact cause of death, and it seems unlikely that any single one is. Certainly taurine has effects *in vitro* that would strongly suggest that *in vivo* it should have an inhibitory effect on the nervous system, and this might become dileterious if the taurine concentration became excessively high. It must be considered that taurine is elevated in the haemolymph during the completely normal activity of flight, however, and in *L. migratoria* (Van Der Horst et al., 1980) this increase is much greater than that observed in *S. gregaria* in the present work. It

is inconceivable that any highly toxic substance would be released into the blood as a result of a normal physiological activity, and in any event taurine is not particularly toxic. Perhaps, however, taurine release may damp down the nervous system during prolonged flight or violent muscular activity and thereby decrease the tendency for flight and violent muscular activity.

ADDENDUM

After the completion of this thesis a publication was brought to my attention which is relevant to the present work. Breer and Heilgenberg (1985) studied various aspects of GABA neurochemistry in synaptofrom L. migratoria. These authors observed a rapid accumulation of GABA into synaptosomes, and this was released by 50 μM veratridine-depolarisation. The accumulation of GABA by L. migratoria synaptosomes was more rapid than that observed in the present work (see Fig. 3.20), reaching a plateau after 1 min (Breer and Heilgenberg, 1985). This difference may well be due to the Na^+ concentrations used in the two sets of experiments. Breer and Heilgenberg (1985) studied the relationship between Na^+ concentration and the rate of GABA uptake, which was maximal at 150mM Na^+, above which the rate was reduced. The authors therefore studied the time course of GABA uptake in the presence of 150mM Na^+, while in the present work the concentration was 214mM, which gives a much lower rate of transport (Breer and Heilgenberg, 1985). It was also found that GABA release by veratridine could be observed in the absence of an inhibitor of GABA transport, which was not the case in the present work (see Fig. 3.27). This may be due to the fact that Breer and Heilgenberg (1985) used a filtration assay to assess GABA release and under these conditions the (^3H) GABA would be washed away too rapidly to be reaccumulated, while this can occur during a superfusion assay.

SECTION FIVE

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