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Studies on the function of amino acids and amines in the central nervous system of insects (*Schistocerca americana gregaria* and *Periplaneta americana*).

Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy

by

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July, 1982.

In the name of

ALLAH

Most Gracious Most Merciful

Thanking

HIM

with a full heart and devoted tongue

TO MY PARENTS

Acknowledgements

I wish to express my thanks to everyone who assisted me during the course of work, especially I am grateful to:

Dr. R.H.C. Strang for his able guidance, limitless encouragement and constructive criticism;

Professor R.M.S. Smellie for making the facilities of the department available;

Dr. A.F.H. Anderson for helpful criticism and advice;

Mr. Andrew Wilson for expert technical assistance;

Mr. Jaleel A. Miyan and Dr. Martin Burns for assistance and guidance in electrophysiological studies;

Dr. B.N. Zaba for critically reading the text and suggestions for its presentations;

Miss. L. Owen and E. Williams for careful typing of manuscript;

My wife Rehana Jabbar for consistent encouragement and unfailing help.

I acknowledge the financial support from Talbot Crosbie Bequest, Faculty of Medicine, University of Glasgow.

The following publications relate to the contents of this thesis.

1. A method for the quantitative study of the amines and amino acids of the insect nervous system.

   A. Jabbar and R.H.C. Strang.

   In "Insect Neurobiology and Pesticide Action". pp. 261-66 (1979). (M. Sherwood, Ed.).

   Society of Chemical Industry, London.

2. Synthesis of alanine in the nervous tissue of locust (Schistocerca americana gregraria).

   A. Jabbar and R.H.C. Strang.

   8th Meetg. Int. Soc. Neurochem. Abstr. p. 187 (1981), Nottingham, U.K.

3. Physiological stress and insecticide poisoning both cause the accumulation of an amino-compound in the haemolymph of insects.

   A. Jabbar and R.H.C. Strang.

   8th Meetg. Int. Soc. Neurochem. Abstr. p. 214 (1981), Nottingham, U.K.

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Abbreviations

Abbreviations used in this thesis are those recommended in the Biochemical Journal Instructions (1978) with the following additions.

A,	Adrenaline
ACh,	Acetylcholine
AlAT,	Alanine aminotransferase
AOAA,	Aminooxyacetic acid
AspAT,	Aspartate aminotransferase
AT,	Amino transferases
Ch,	Choline
CNS,	Central nervous system
DA,	Dopamine
Dans-Cl,	1,1-dimethylamino-naphthalene-5-sulphonyl-chloride
DAO,	diamine oxidase
DDT,	1,1,1-trichloro,2,2-bis(p-chlorophenyl) ethane
DFP,	di-isopropyl phosphorafluoridate
DMSO,	dimethylsulfoxide
DOPA,	dihydroxyphenylalanine
DOPAC,	dihydroxy phenylacetic acid
DTNB,	dithio-bis-nitrobenzoate
Gaba,	γ-aminobutyric acid
GAD,	Glutamic acid decarboxylase
γ-BHC,	γ-Benzylhexachloride
GDH,	Glutamate dehydrogenase
α-GP,	α-glycerophosphate (Glycerol-3-phosphate)
GPDH,	α-glycerophosphate dehydrogenase

5-HIAA,	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
HTP,	Hydroxytryptophan
HVA,	Homovanilic acid
IAA	Isoamylamine
o-IMPC,	o-isoproxyphenyl-N-methyl carbamate
INT,	2-p-iodophenyl-3-p-nitrophenyl-5-phenyl monotetrazolium chloride
α-Kg,	α-ketoglutarate
LDH,	Lactate dehydrogenase
MAO,	Monoamine oxidase
NA,	Noradrenaline
NADA,	N-acetyldopamine
NAOA,	N-acetyloctopamine
NATA,	N-acetyltyramine
NM(NMN)	Normetanephrine
OA(OCT),	Octopamine
OAA,	Oxaloacetic acid
PDH,	Proline dehydrogenase
PEP-CK,	Phosphoenolpyruvate-carboxykinase
PMS,	Phenazine methosulfate
POPOP,	1,4-di-[2-(5-phenyloxazolyl)]-benzene
PPO,	2,5-diphenyloxazole
PUT,	Putrescine
SLS,	Sodium laural sulfate
TA,	Tyramine
TCA,	Tricarboxylic acid cycle

TEPP, Tetraethyl pyrophosphate

TP, Tryptamine

VNC, Ventral nerve cord

SUMMARY

The main aim of the present investigations was to study amino compounds in the locust (Schistocerca americana gregaria) central nervous system (CNS). The results may be summarized as follows:

1. A method of separating amino compounds and their derivatives was developed. It involved electrophoresis with subsequent thin layer chromatography of dansyl derivatives and was sensitive enough to estimate as low as 1p mole of an amino compound in less than 1mg tissue and 50-60nl of haemolymph.

2. Of the monoamines of interest, dopamine, octopamine, tyramine, N-acetyloctopamine, N-acetyltyramine and 5-hydroxytryptamine were detectable in the thoracic ganglia. N-acetylamines were found to be the major end product of tyrosine metabolism in the CNS.

3. Among the amino acids; alanine, arginine, aspartic acid, cysteine/cystine, glutamic acid, glycine, histidine, iso-leucine, leucine, lysine, phenylalanine, proline, taurine, tryptophan, tyrosine and valine were all present in the locust CNS and haemolymph. γ-Aminobutyric acid (Gaba) could only be detected in CNS.

4. The incubation of the isolated thoracic ganglia in saline devoid of mono-saccharides resulted in substantial decrease (generally 5-50%) in the concentration of most of the amino acids. The exception to this decline was a 2-3 fold increase in alanine.

5. During the incubation of the isolated thoracic ganglia, glutamate, aspartate and Gaba were mostly retained in the tissue, whereas the rest of the amino acids readily diffused out. The efflux was mainly during the first 5-15 minutes and was greater at 32°C than 21°C but non-significantly different from that at 37°C.

6. In the locust CNS alanine was synthesized only by the transamination reaction between pyruvate and glutamate. The glutamate itself was generated from a number of amino acids, mainly proline, glutamine and aspartate.

7. Proline was found to be the best substrate for the locust CNS (whole tissue and mitochondrial preparations) among the substrates studied, namely glucose, glutamine, glutamate, α-glycerophosphate (α-GP), pyruvate, and succinate.

8. The locust CNS showed detectable activities of the following enzymes: aspartate aminotransferase, alanine aminotransferase, malate dehydrogenase, proline dehydrogenase, malic enzyme (NAD^+), glutamate dehydrogenase and oxaloacetate decarboxylase. The enzymes alanine dehydrogenase, malic enzyme ($NADP^+$), pyruvate carboxylase and phosphoenolpyruvate carboxykinase were all undetectable in this tissue.

9. The increase in α-GP, malate and succinate and disappearance of fumarate over one hour of anaerobic incubation of thoracic ganglia suggested that the ganglia were well equipped with various possible pathways for the limited regeneration of NAD^+ during anaerobiosis.

10. The studies on the effect of chemical and physiological stress revealed that the enforced walking of locusts and cockroaches and injection of DDT, γ-BHC, dieldrin, malathion, methyl parathion, fenvalerate, chlordimeform and nicotine showed some sort of paralytic effect, accompanied by the accumulation of a neuroactive substance (taurine) in the haemolymph. No accumulation of taurine could be found in the nervous tissue.

11. The incubation of isolated ganglia of locust with these insecticides failed to show the presence of taurine either in the tissue or the medium; nor did the electrical stimulation cause the release of such a compound.

12. Quantitative analysis of the haemolymph from γ-BHC treated locusts showed increase in the concentration of alanine, arginine, leucine, and threonine but the most marked was the 2-3 fold increase in taurine. Proline concentration declined during these treatments.

13. Abnormal bursts of hyperactivity were observed in the abdominal nerve cord dissected out of cockroaches previously treated with γ-BHC. Similarly the haemolymph from insecticide-treated locusts increased the rate of spontaneous firing in the isolated VNC of cockroach.

14. Application of the amino acids arginine, threonine, leucine, isoamylamine to isolated untreated nerve cord of cockroaches caused excitation, wherease taurine reversibly inhibited the spontaneous activity.

15. Finally, putrescine (a diamine) was found in the locust CNS and haemolymph in substantial concentrations. The incubation of the isolated thoracic ganglia of locust with putrescine and injection

of [^{14}C]-putrescine into live insects did not show any significant rise in the concentration of Gaba for which it is a potential precursor.

SECTION ONE

INTRODUCTION

1.1 Advantages of the invertebrate nervous system

Perplexed at the complexity of the mammalian nervous system, neurobiologists have increasingly turned to invertebrates in their studies on the links between the biochemistry and physiology of the nervous tissue and their ultimate function: that of determining behaviour.

Reduced to their most basic, the advantages of invertebrate nervous tissue lie in two factors; "size" and "simplicity". Size, in this context refers not to the amount of tissue available in one animal, but to the size of individual cells, which is often great enough not only to allow an unambiguous identification but to yield sufficient material for biochemical measurements. The most outstanding example of this is the giant axon of the squid, without which we would have little clear idea of the origin of the resting and action potentials of nerve cells in general (Hodgkin and Katz, 1949; Katz, 1952).

The nervous systems of invertebrates have been utilized in a wide range of physiological and biochemical studies. Examples include electrophysiological recordings from the nervous tissue either showing excitation or inhibition of nerve impulses in response to the respiratory movements of locusts (Miller, 1966); cleaning of antennae by cockroach (Luco and Arnada, 1964); the optokinetic memory of locust (Horridge, 1966); flight in locust (Wilson, 1961, 1964, 1968; Wilson and Wyman, 1965); moth wing movement (Kammer, 1967), singing in cricket (Bentley and Kutsch, 1966) and Grasshopper (Elsner, 1968), etc. and results indicate that all these and many more actions are amenable to analysis. Similarly, the

biochemical and pharmacological investigations have led to the suggestion that glutamate instead of acetylcholine is the neuromuscular transmitter in invertebrates (Kerkut et al., 1965; Usherwood and Machili, 1966). A neurohumoral role for octopamine has been observed in insects (Candy, 1981) as well as being the transmitter involved in the luminescence of firefly (Robertson and Carlson, 1976).

The smallest unit of the insect nervous system employed in this work was the individual ganglion. It is in relation to the individual ganglion that advantage was taken of the "simplicity" of this invertebrate tissue. The "simplicity" may be said to have two important aspects in the reported work. One concerns the decentralization of function in invertebrate nervous systems, in which the brain has not assumed the hegemony of function which is found in the vertebrates. This means that many activities may have their cellular origins entirely within a ventral or thoracic ganglion, without the participation of the cerebral ganglion.

Backed by electrophysiological evidence, Roeder et al. (1960) were able to show the major role of the abdominal ganglia of insects in sexual movements and egg laying. Similarly Roeder (1963) has shown that the praying mantis is very inactive after removal of cerebral ganglia, but hyperactive after removal of suboesophaegeal ganglion, showing simply a modulatory role for these ganglia. The same is true for the cockroach. Locusts occasionally walk, jump or fly spontaneously after the removal of cerebral ganglia (Hoyle, 1970).

Horridge (1962), working with the nerve cord of headless locusts and cockroaches, and Eisenstein and Cohen (1965) working on the prothoracic ganglion of cockroach, showed that a decapitated insect could learn to keep its legs raised if given appropriate electric shocks. Following this up Hoyle (1965) has demonstrated an electrophysiological correlate to this "learning" situation in a headless locust. He showed that if the leg was stimulated every time the frequency of discharge to the anterior coxal aductor muscle (which lifts the leg) fell below a preset level, the discharge rate of this neurone could be tripled and remained high for many hours. In insects receiving stimulii unrelated to the low frequency of discharge in the neurone, there was no increase in frequency of firing. All this points to the fact that a number of functions are controlled by the parts of the nervous system other than cerebral ganglia. In other words the mammalian concept of the "brain" with its almost complete centralization of control of most activity, may have little relevance for the invertebrates. In these simpler animals each ganglion has a much more independent existence and each may (crudely) be said to constitute a "brain". From this it might be hoped that the biochemistry and physiology of the part may reflect those of the whole in a way not possible in the mammals. It would of course be unwise to take it for granted that these so-called minibrains are decentralized in almost every respect.

The other facet of simplicity of the insect CNS is the practical one that the neuroanatomy of locust and cockroach is such that the individual ganglia are well fitted to function <u>in vitro</u>.

Advantage can be taken of the fact that insects lack the vascular system of more advanced animals so that metabolites are directly absorbed or transported into the periphery of the ganglia from the haemolymph. This is easily reproduced *in vitro* by incubating the tissue in artificial media. Moreover, the insect ganglia show spontaneous nervous activity *in vitro* for a considerable length of time (Roeder and Roeder, 1939; Finlayson and Osborne, 1970, 1977; Clement and Strang, 1978). This can easily be monitored, and shown to be influenced by various factors (Flattum & Sternburg, 1970; Hue *et al.*, 1978).

The major drawback encountered with such a system is the diminished supply of O_2 to the ganglia due to rupture of tracheal system which normally permeates all the nervous tissue (Burrows, 1980). This may be partly offset by an increased concentration of oxygen in the bathing medium. Clement and Strang (1978) claimed that an adequate amount of O_2 reached all parts of incubated ganglia, on the basis of low lactate production. As will be discussed later in this thesis, however, lactate is probably a poor guide to anaerobiosis in locust nervous tissue; and ganglia *in vitro* are likely to be partially anaerobic.

To conclude, no tissue removed to artificial surroundings can be expected to reflect exactly the conditions *in vivo*. Nevertheless, in the case of the ganglia of insect CNS the previous reports cited give hope that results obtained *in vitro* will have some relevance to the living insect.

1.2 Organization of insect nervous system

Although there is a wide range of anatomical diversity throughout the class "Insecta", the nervous system can be broadly divided into the cerebral ganglia and ventral nerve cord (VNC), (Lane, 1974). The VNC consists of a series of ganglia linked by a paired interganglionic connectives. Fusion between these ganglia may occur during development, as it occurs with cerebral ganglia. This can be either during embryonic growth, or at different times after hatching (Pipa, 1973). The degree of fusion of the ganglia in the nerve cord is highly variable, with two extremes represented by, at one end, the cockroach where three distinct thoracic ganglia (commonly termed as pro-meso- and metathoracic) and six abdominal ganglia are found (Fig. 1), and at the opposite end, the housefly, which displays fusion of all the ganglia of the VNC into one large thoracic ganglion from which nerves emanate to all the other parts of the body. Other species may be intermediate in the degree of fusion (Bullock and Horridge, 1965).

The ganglia of the nerve cord are composed of a cortex of neuronal perikarya or cell bodies, ensheathed by glial cells, surrounding a central neuropile (Fig. 2), while the connectives consist only of axonal processes invested by glial cells (Lane, 1974).

Fig. (3) shows the diagrammatic representation of locust CNS. In the work reported here the main tissues used were the thoracic ganglia of locust and abdominal nerve cord of cockroach.

1.3 Electrophysiology of the insect ventral nerve cord (VNC)

Neurophysiological studies are, in most cases, based on the observation and recordings of the electrical impulses transmitted along nerve fibres. The introduction by Gasser and Erlanger (1922)

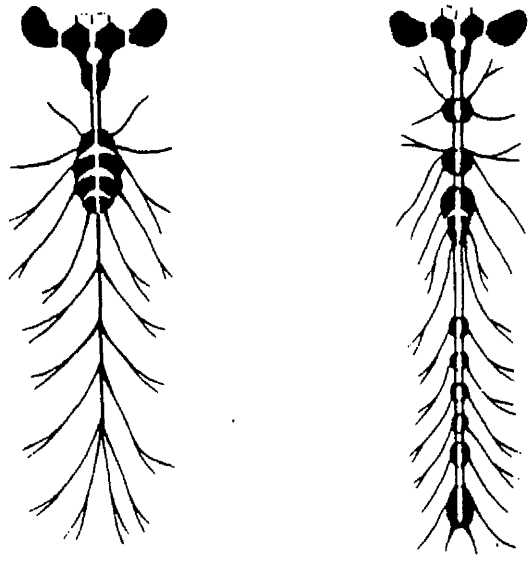


Fig.1 Diagram indicating differences in the degree of fusion of the ventral ganglia in the housefly *Musca*, on the left, compared with the cockroach, *Blatta*, on the right. *Musca* has only one large thoracic ganglion while *Blatta* has three separate thoracic ganglia as well as six abdominal ganglia. (After Bullock and Horridge 1965.)

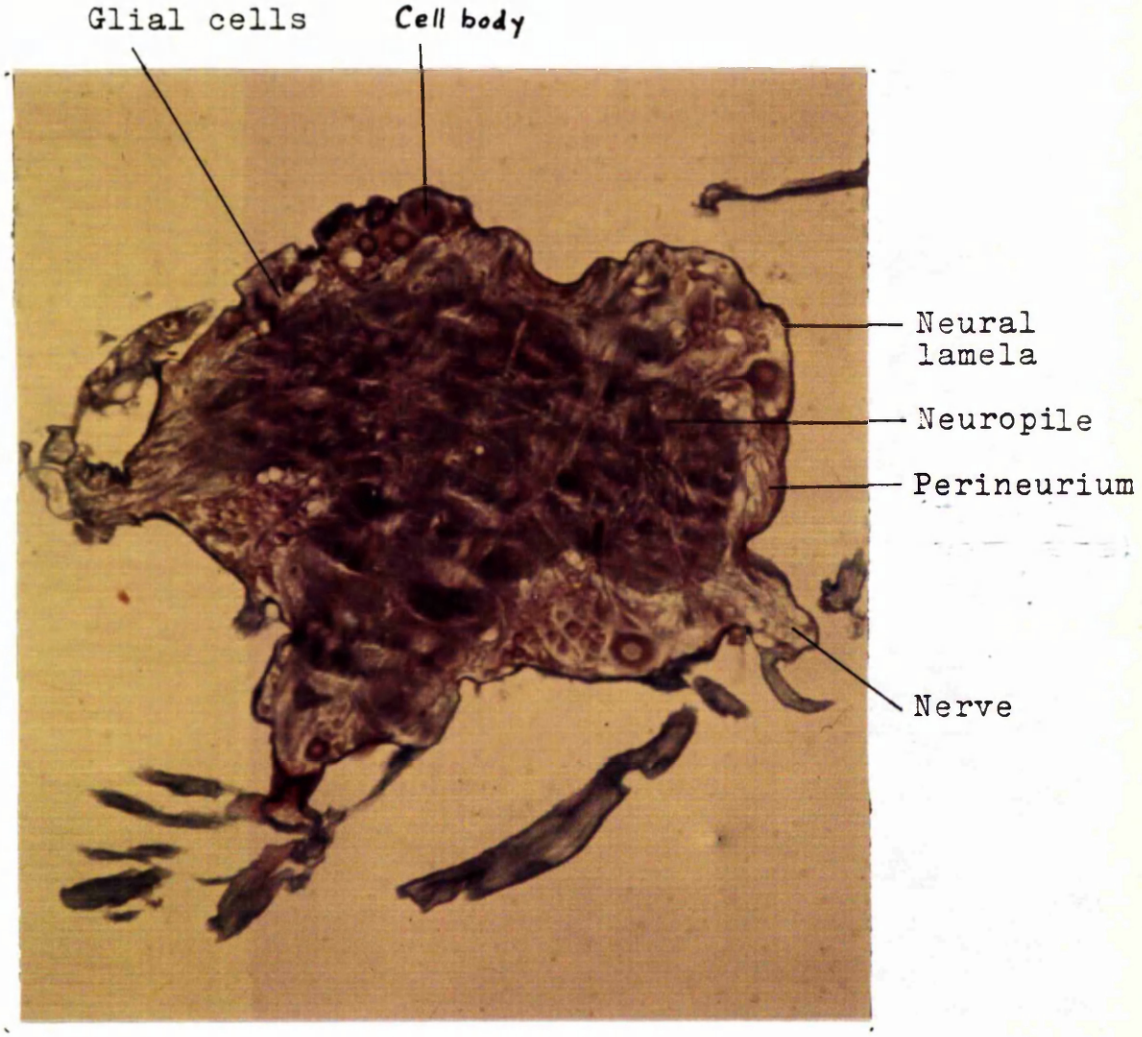
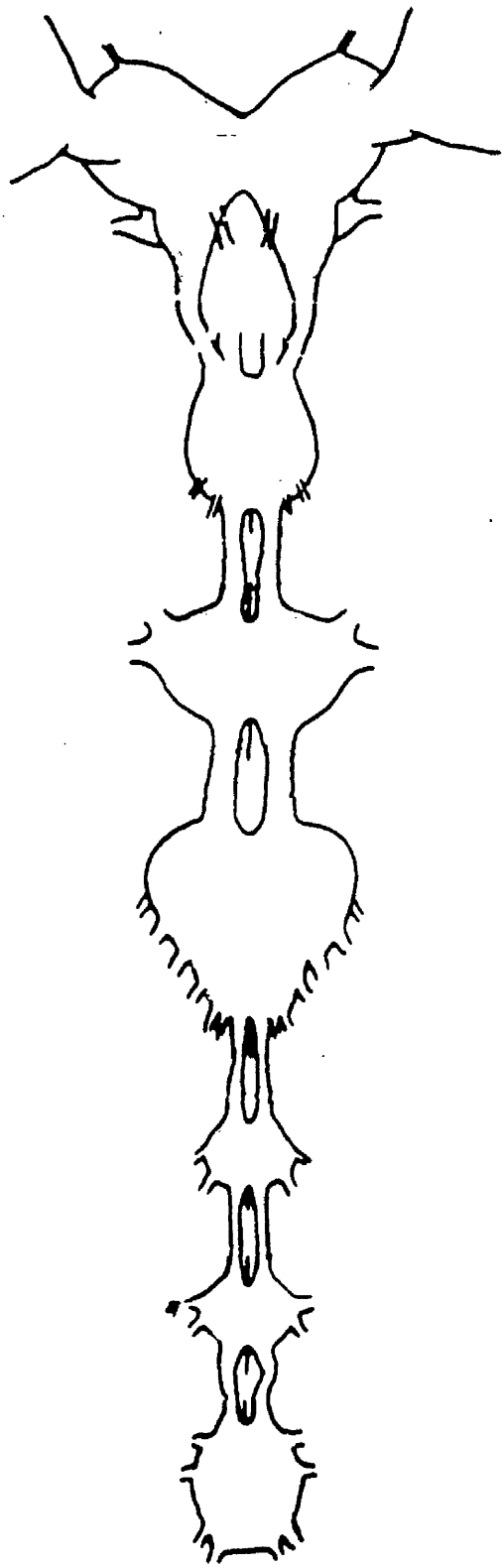


Fig 2. Transverse section of the metathoracic ganglion of locust, Schistocerca americana gregaria.

(Courtesy of R.H.C.Strang)

Central Nervous System of *Schistocerca gregaria*



Cerebral ganglia

Suboesophageal ganglion

Mesothoracic ganglion

Metathoracic ganglionic mass

4th abdominal ganglion

6th abdominal ganglion

Terminal abdominal ganglion

Figure 3

of the cathode ray oscilloscope made it possible to accurately monitor electrical events in excitable tissues. The basic electrophysiology of insect central nervous system is similar to that of other invertebrates and vertebrates (Wiersma, 1967). The studies to advance understanding of the neural mechanism of actions have taken advantage of the electrical properties of the neuronal (excitable tissue) membranes. In this respect the classic studies with the squid giant axon (Hodgkin and Katz, 1949) have proved to be a milestone. Differences exist in the ionic composition of intracellular and extracellular fluids, Na^+ concentration being higher outside and K^+ concentration being higher inside (Yamasaki and Narahashi, 1958, 1959; Treherne and Moreton, 1970; Pichon et al., 1972). The membrane is permeable to K^+ but only sparingly so to the other ions including Na^+, Cl^- and Ca^{++}, and the resting membrane potential (E_{RP}), (inside negative with respect to outside), is close to the equilibrium for K^+ (E_K) defined by the Nernst equation

$$E_{RP} \quad E_K = \frac{RT}{F} \ln \frac{K_o}{K_i}$$

where, R, T and F are respectively the gas constant, the absolute temperature and the Faraday constant (Pichon, 1974). Any agent which would disturb this balance by changing the permeability of membrane to these ions would result in an altered potential which when conducted to the axonal membrane to the nerve ending (synapse) causes the release of chemical transmitter which generates similar phenomenon in the post-synaptic membrane. This potential is called an action potential (AP) and would occur when the membrane potential

becomes less negative (depolarized) beyond a certain level (threshold) by a stimulating outward current across the membrane. At this point the sodium permeability of the membrane undergoes a large and fast generative increase. Thus the membrane becomes suddenly permeable to Na^+, and the membrane potential approaches the sodium equilibrium potential (E_{Na}) given by the equation

$$E_{AP} \quad E_{Na} = \frac{RT}{F} \ln \frac{Na_o}{Na_i}$$

where, E_{AP} is the membrane potential at the peak of the action potential, and Na_o and Na_i as the external and internal sodium concentration respectively (Treherne and Maddrell, 1967a, b; Weidler and Diecke, 1969; Treherne, 1972; Pichon et al., 1972). Since the external sodium concentration far exceeds the internal concentration, the membrane potential is reversed in polarity during the action potential.

The increased Na^+ permeability ceases almost immediately and the K^+ starts increasing beyond its resting level so that the membrane becomes almost exclusively permeable to K^+ bringing back the membrane potential towards the resting level. Thus the falling phase of the action potential is produced. As a result of these permeability changes Na^+ enters and K^+ leaves during the AP. Metabolic energy in the form of ATP plays an important role after excitation, pumping out the extra Na^+ that has entered and bringing in K^+ in exchange. This mechanism, called Na^+/K^+ Pump, is stimulated by an increase in internal Na^+ concentration. It also works at rest, keeping the proper concentration gradiants of Na^+ and K^+.

The electrical activity of the neurones is monitored at two different levels i.e. in single neurones or axons by implanting microelectrodes into the nerves or by recording the gross activity in a number of nerve fibres by the extracellularly placed suction or hook electrodes. The microelectrodes were first introduced by Ling and Gerard (1949) and have remained one of the most important tools for the neurobiologists ever since. The most widely used microelectrodes is the glass capillary filled with an electrolyte, e.g. potassium acetate, sulphate or chloride for intracellular recording, and a sodium salt for extracellular recording. Such electrodes can be used for focal external recordings or can penetrate inside the cell down to 2μm without damaging the membrane. This way recording from cell bodies, which has been carried out successfully for several years in molluscs and crustaceans, has also been achieved in insects (Hoyle, 1970; Hoyle and Burrows, 1973a, b). On the other hand the onset of Action Potentials (APs) in the nerve cord can be recorded by cutting the nerves and sucking into the glass or plastic electrode filled with an electrolyte or simply by placing the nerves on the silver wire hooks. This gives the sum of the APs generated in the nervous tissue and is commonly used to study the effects of various agents on nervous activity inside the living insects or particularly in the isolated nerve cords. Recordingsof the synaptic potentials have been made by Yamasaki and Narahashi (1960) at the level of sixth abdominal ganglia of P. americana with external electrodes placed as near as possible to the site of electrogenesis. Following the stimulation of the cerebral ganglion, they observed a potential

exhibiting the characteristics of an excitatory post synaptic potential in the abdominal ganglion. Hoyle and Burrow (1970) recorded intracellularly the excitatory post-synaptic potentials and inhibitory post-synaptic potentials in the identified neurones of locust, S. gregaria. Weiant (1958) recorded the nervous activity from the nerve no 5 of the locust metathoracic ganglia which remained otherwise intact in the insect. Then he severed the connectives between meso- and metathoracic ganglia and observed an increase in spiking and suggested that some inhibitory factors from cerebral ganglia were suppressing the activity of the thoracic ganglia.

Similarly, isolated preparations have been employed in the study of the effect of pharmacological agents on the activity of CNS of cockroach (Roeder and Roeder, 1939; Finlayson and Osborne, 1970, 1977). The nervous activity in these isolated cordswhich is independent of the motor input or any external stimulus is termed as the spontaneous nervous activity. Gahery and Boistel (1965) used isolated preparations including cercus and cercal nerves of cockroach and found that Gaba had an inhibitory effect and 3-hydroxytyramine showed an excitation in spontaneous activity. Many insecticides e.g. organochlorides (Narahashi, 1976) and neurotoxins e.g. Nicotine (Flattum and Sternburg, 1970) and Tetrodotoxin (Pichon et al., 1972) have shown to affect the nervous activity by perturbing the ionic balance by changing the permeability of the membrane to the ions (Na^+, K^+ and Cl^-).

Due to the potential changes in membrane (during AP) certain chemical compounds are released at synapses and, these substances released from the synapse and causing potential changes in post-synaptic membrane are characterized as excitatory or inhibitory transmitters accordingly if they increase or decrease the spiking in the cell. In this context acetylcholine and glutamate have been shown to be the central and peripheral excitatory transmitters in invertebrates (Cottrell and Leverack, 1968; Florey, 1967; Florey and Michelson, 1973; Usherwood and Machili, 1968; Dowson and Usherwood, 1973). Gaba, DA and recently reported taurine have an inhibitory effect on the activity in the nerve cord of insects e.g. locust and cockroach (Usherwood, 1973; Lea and Usherwood, 1973; McGeer et al., 1961; McLennan and Hagan, 1963; Pelhate et al., 1978; Hue et al., 1978).

This excitation or inhibition of the nervous activity has the ultimate control of functions of various organs of animals in normal and abnormal (i.e. stressful) conditions. As will be shown later factors accumulating in the haemolymph of insects paralyzed under stress are neuroactive and their prime action may be their neurotoxicity; the present studies would be extended to include the electrophysiology of the VNC of insects. It will be a good guide and tool to get an insight into the physiological state of the insect and possibly the mode of action of agents like insecticides in the stressful conditions.

1.4 Amino compounds and the insect nervous system

As its title implies, this thesis is concerned with the importance of amino compounds, i.e. amino acids and amines, for the functioning of the nervous system of the locust and cockroach. The experimental

work which will be reported falls into two distinct catagories; that which was concerned with amino compounds associated with the nervous system in the normal course of the insects' life, and that concerned with the effect of extremely stressful conditions, particularly insecticide poisoning. It has long been thought that under these conditions amino compounds may play some part in the aeteology of the poisoning (Sternburg and Kearns, 1952; Colhoun, 1960; Hawkins and Sternburg, 1964).

Although in the experimental section the work with the insecticides is reported first (as it was done first), it seems better in the introduction to consider the normal state before the abnormal.

1.4.1 Amino compounds in the insect haemolymph

A good deal of the work which will be presented was concerned with the uptake into or release from the nervous system, of various amines and amino acids. Hence the concentration of these compounds in the haemolymph is of primary importance in their relation to the nervous tissue. A sharp contrast between the mammals and insects is the very high concentration of many amino acids in the haemolymph of the latter compared to the blood stream of the former (Table 1). The value of amino acid nitrogen in the insect blood is 5-50 times higher than the normal for mammalian plasma. Vertebrates have about 0.05g per cent free amino acid in the blood, in insects the amount rangesfrom 0.29-2.43g per cent (Wyatt, 1961). Moreover, striking differences are found in the pattern of amino acid content in the same insect at different stages of its life cycle. Because of these variations, few

statements of a general character regarding levels of individual amino acids can be made.

Of the common amino acids of proteins, all except tryptophan, methionine and cysteine or cystine may occur in high concentration in one insect or another. The highest concentrations and the greatest range of variations are recorded for the amino acids, proline and tyrosine (Gilmour, 1961). This variation is perhaps due to proline being an important constituent of insect cuticle and tyrosine as precursor of phenolic compounds responsible for tanning and hardening of that cuticle. In addition, more and more data is accumulating pointing to proline as an energy source in insect flight muscle (Bursell, 1981). Proline has been shown to be synthesized from alanine in fat body and made available via haemolymph to various tissues in colorado beetle (Weeda et al., 1980). Although the prime source for various amino acids in the haemolymph is the diet, the use of conventional methods of growing the larvae on chemically defined diet together with the deletion procedure, indicates that the ten amino acids (arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, iso-leucine, valine) which are known to be necessary for the growth of mammals, have been proved to be also essential for insects (Lipke and Fraenkel, 1956; House, 1962). This has indirectly been confirmed by following the incorporation of ^{14}C from labelled glucose into the non-essential amino acids in the larvae of Phormia regina, Agrotis orthogonia, Ctenicera destructor (Kasting and McGinnis, 1958, 1960, 1962; Kasting et al., 1962) as well as Neodiprion pratti (Schaeffer, 1964). The possible importance of these

high concentrations of amino acids will be discussed in subsequent sections.

1.4.2 Amino compounds in insect CNS

The profile of the free amino acids and amines in the nervous tissue of mammals and insects is given in Tables 1 and 2 respectively. Despite the variable distribution, the common feature of these substances is their overall higher concentrations, both in nervous tissue and the haemolymph of insects compared to vertebrates. Essentially, all the amino acids and amines found in higher vertebrates are also present in the insects. Using the microtechnique of dansylation Osborne and Neuhoff (1974) detected as many as 20 different amino compounds in the haemolymph, muscle and nervous tissue of cockroach. In all the tissues analyzed proline was the most abundant amino acid, followed by glycine and alanine. Glutamic acid and aspartic acid occur in the nervous tissue at a higher concentration than in the haemolymph where they are hardly detectable. Gaba is present in the nervous tissue and is not found in haemolymph. Various functions ascribed to the high concentrations of these compounds include; 1) maintenance of ion-balance, 2) protein synthesis, 3) neurotransmission and 4) acting as a possible source of energy. As the work presented in this thesis is concerned with the metabolism and neuroactivity of amino compounds, their known functions in these two respects will be reviewed below.

Table 1. Concentrations (μmol/g fresh weight; μmol/ml) of free amino acids in the nervous tissue and plasma/haemolymph of some mammals and insects

Amino Acids	RAT	CAT		HUMAN			LOCUST		COCKROACH	
	Brain	Brain	Plasma	Brain	Plasma	CSF	CNS	haemolymph	CNS	haemolymph
Alanine	0.57[a]	0.94[c]	0.77[c]	0.25[b]	0.41[d]	0.026[b]	6.94[e]	1.5[e]	3.33[i]	2.04[i]
Arginine	0.11[b]	0.08[c]	0.08[c]	0.10[b]	0.08[d]	0.018[b]	2.00[e]	0.89[e]		
Asparagine		0.11[c]	0.07[c]				0.56[e]	1.00[e]		
Aspartic acid	2.71[a]	2.23[c]	0.007[c]	0.96[b]	0.05[d]	0.0002[b]	5.70[e]	0.06[e]	1.16[i]	0.14[i]
Cysteine/cystine		0.08[c]	0.03[c]		0.002[d]			1.8[e]		
Gaba	2.00[a]	2.27[c]	0.001[c]	0.42[b]	0.04[d]	0.0[b]	2.10[e]	0.0[e]	1.54[i]	0.03[i]
Glutamic acid	9.42[a]	8.70[c]	0.12[c]	6.60[b]	0.64[d]	0.008[b]	8.40[e]	0.001[e]	2.76[i]	0.91[i]
Glycine	1.29[a]	1.35[c]	0.31[c]	0.4[b]	0.24d	0.007[b]	1.8[e]	6.3[e]	3.47[i]	8.11[i]
Isoleucine	0.02[b]	0.09[c]	0.06[c]	0.03[b]	0.10[d]	0.004[b]	0.001[e]	0.28[e]	0.13[i]	0.76[i]
Leucine	0.05[b]	0.24[c]	0.12[c]	0.07[b]	0.17[d]	0.011[b]	0.05[e]	0.61[e]	0.12[i]	0.47[i]
Lysine	0.21[b]	0.14[c]	0.19[c]	0.12[b]	0.19[d]	0.021[b]	6.20[e]	0.38[e]	0.17[i]	0.08[i]
Methionine	0.04[b]	0.10[c]	0.03[c]	0.03[b]	0.005[d]	0.003[b]			0.08[i]	0.23[i]
Phenylalanine	0.05[b]	0.07[c]	0.05[c]	0.05[b]	0.06[d]	0.008[b]	0.001[e]	0.54[e]	0.10[i]	0.93[i]
Proline	0.08[b]	0.14[c]	0.20[c]	0.10[b]	0.15[d]	-	11.10[e]	5.6[e]	10.90[i]	10.48[i]
Serine	1.07[a]	0.72[c]	0.20[c]	0.44[b]	0.10[d]	0.025[b]	1.40[e]	2.2[e]		
Taurine	5.36[a]	1.92[c]	0.06[c]	1.20[b]	0.03[d]	0.006[b]	12.74[f]	0.9[h]		0.4[j]
Threonine	0.66[b]	0.21[c]	0.12[c]	0.27[b]	0.11[d]	0.025[b]	22.40[g]			
Tyrosine	0.07[b]	0.07[c]	0.04[c]	0.06[b]	0.05[d]	0.008[b]	0.53[e]	1.2[e]	0.46[i]	3.34[i]
Valine	0.07[b]	0.18[c]	0.20[c]	0.13[b]	0.32[d]	0.016[b]	0.16[e]	0.78[e]	0.40[i]	1.05[i]

a = Ansell and Richter (1954); b = Siegal et al (1976); c = Tallen et al (1954); d = Steine and Moore (1954); e = Schlesinger et al (1977); f = Porcellati (1963); g = Osborne (1971); h = Irving et al (1979); i = Osborne and Neuhoff, 1974; j = Jacobson and Smith (1968).

Table 2. Concentrations (nmol/g fresh weight) of the biogenic amines in the nervous tissue of some mammals and insects

Amine	Human		Rat	Locust (*S. gregaria*)	Locust (*L. migratoria*)	Cockroach	
	Cortex	Hypothalmus	Brain	Cerebral ganglia	Cerebral ganglia Sub-oesophageal ganglia	Cerebral ganglia	Thoracic ganglia
Noradrenaline	0.15[a]	6.6[a]	2.51[b]	0.65[c]	1.41[e]		
Dopamine		0.51[a]	3.6[b]	5.67[c]	8.53[e]	6.97	1.17-3.19[f]
Octopamine				15.83[c]		27.69[g]	
5-hydroxytryptamine	0.06[a]	0.5[a]	2.52[b]	4.48-8.34[d]	13.28[e]	12.20[f]	8.23-11.63[f]

a = Carlsson et al (1980); b = Smith et al (1975), c = Robertson (1976);

d = Klemm and Axelsson (1973); e = Hiripi and S-Rozsa (1973);

f = Kusch (1975); g = Robertson and Steele (1974)

1.5 Metabolism and functions of amino acids

1.5.1 Metabolic functions of amino acids in invertebrates

a) Amino acids as fuels for energy metabolism

Though the exact functions of large quantities of proline found in insect CNS (Ray, 1964; Osborne and Neuhoff, 1974; Schlesinger et al., 1977) are unknown, in some insects e.g. tsetse fly (Bursell, 1963), blowfly (Sacktor and Childress, 1967) and Colorado beetle (de Kort et al., 1973; Weeda et al., 1980) the flight muscle is known to oxidize proline to form a number of metabolites including alanine and glutamic acid (Bursell, 1963, 1965, 1967; Sacktor and Childress, 1967; Meyer, 1980) as shown in Fig. 4. The resultant keto acids are metabolized through TCA cycle for the generation of ATP. The activities of enzymes responsible for proline metabolism have been determined in these tissues (Sacktor and Childress, 1967; Crabtree and Newsholme, 1970; Weeda et al., 1980) and suggest that proline functions as a rich source of energy in insect muscle. In one of the first reports about participation of proline in energy metabolism, Bursell (1963, 1965) observed that flight muscle of tsetse fly utilizes the intermediates of citric acid cycle but does not fully oxidize proline, which finally accumulates as alanine (see Fig. 4). In this pathway, a molecule of alanine is formed for each one of proline used. This pathway is dependent upon the enzyme L-alanine: oxoglutarate aminotransferase. An alternative mechanism involves the oxidative deamination of glutamate derived from proline, in which case the accumulation of ammonia is greater than that of alanine (Hansford and Johnson, 1975). The enzyme responsible for the initial metabolic step in the utilization of proline is proline-dehydrogenase, which in

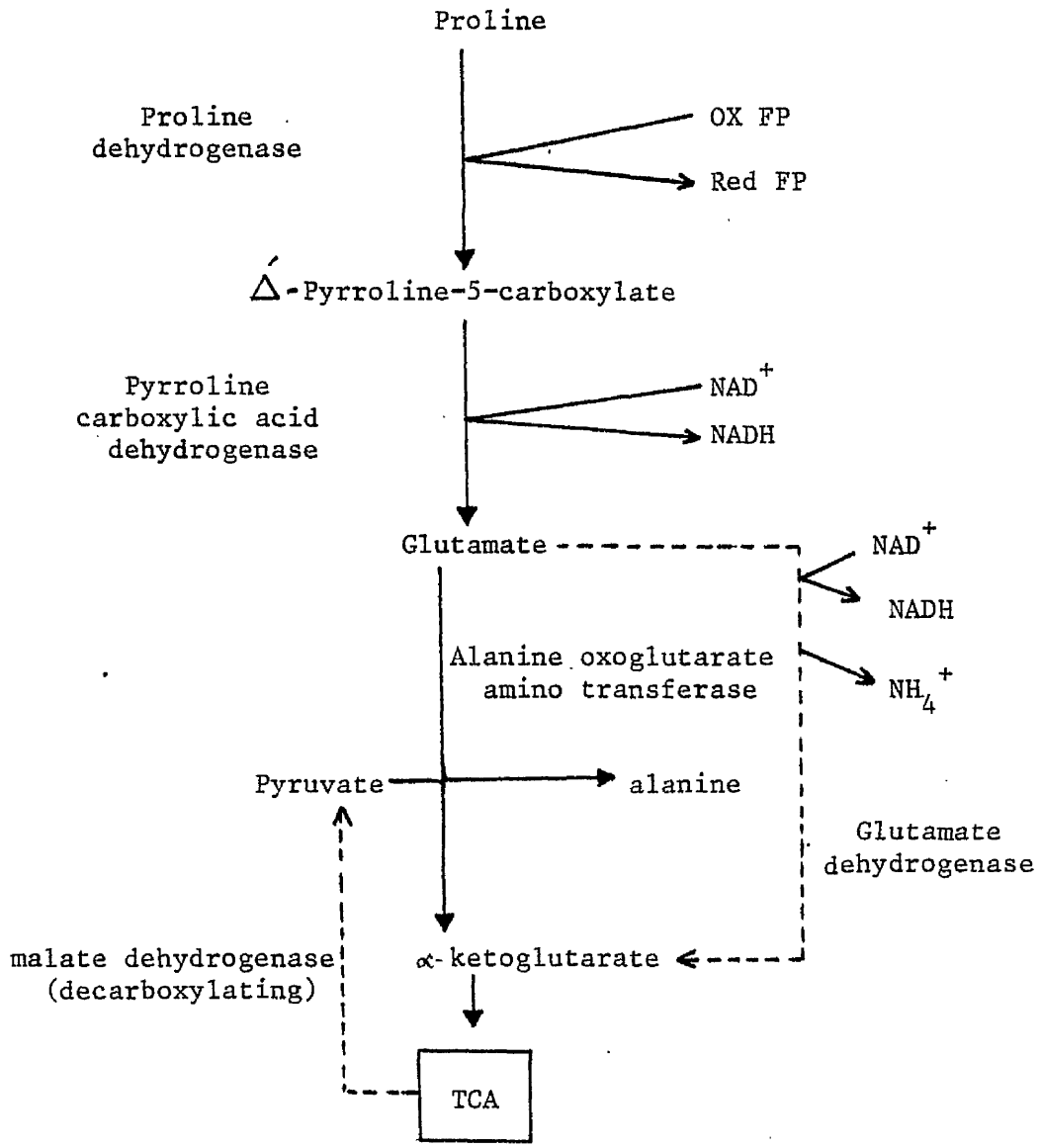


Fig. 4 - Proline metabolism in insect flight muscle. Continuous lines represent the major metabolic route whereas the dotted line show the minor catabolic pathway.

(After Kammer & Heinrich, 1978)

the thoracic ganglia of locust is present at a higher specific activity (5.2μmol/g/min., Strang, 1981) than flight muscle (1.7μmol/g/min., Crabtree and Newsholm, 1970) of the same insect. This is indicative of the fact that the ganglia are capable of oxidizing proline but the pathway subsequent to glutamate is unknown. Although there is little evidence for an energy role of these amino acids in flight muscle of cockroach and locust (Mayer and Candy, 1969), they could be potential fuels for CNS of locusts. The current evidence is indirect, but includes the utilization of [^{14}C]-glutamate (Bradford et al., 1969), and presence of proline dehydrogenase (Strang, 1981). In addition Clement (1979), found that ganglia in medium containing amino acids (with proline as a constituent) showed spontaneous activity for much longer than when glucose alone was the energy source. All these observations strongly suggest a possible metabolic function of this amino acid in the locust CNS.

While the information on the role of proline as a substrate in the insect nerve cord is lacking, some work has been reported on the metabolism of glutamate (one of the intermediates in proline metabolism in flight muscle) in insect nervous tissue. The already mentioned incorporation of label from [^{14}C]-glutamate into alanine (Bradford et al., 1969) in insect nerve cord is in agreement with the above pathway. Alanine is an amino acid which occurs in high concentrations in various insect tissues. In haemolymph its concentration is highly variable and may frequently depend on the physical condition of the insect. Alanine has been found to increase markedly under aerobic and anaerobic conditions in a wide

range of invertebrates (Stokes and Awapara, 1968). The most likely reason for it seems the rapid accumulation of pyruvate resulting from increased glycolysis, shown to occur in locust CNS *in vitro* (Clement and Strang, 1978). A fermentation mechanism to maintain the cytoplasmic redox potential resulting in net accumulation of alanine was suggested by Sacktor (1965) to be operative in insect flight muscle as shown below:

Pyruvate + glutamate ⟷ alanine + α-ketoglutarate

α-ketoglutarate + aspartate ⟷ glutamate + oxaloacetate

oxaloacetate + NADH ⟷ malate + NAD^+

Pyruvate + aspartate + NADH ⟷ Alanine + malate + NAD^+

Whether the accumulation of alanine in the locust nervous system under anaerobiosis is the result of this sort of mechanism is unexplored.

Though the information on metabolism of amino acids in relation to provision of energy under anaerobic conditions in insects is limited, some evidence comes from the investigation with the larvae of *Callitroga macellaria* (Meyer, 1977, 1978, 1980) and (by inference) from sea mussel *Mytillus edulis* (Zwann, 1977). In these invertebrates aspartate is transaminated with α-ketoglutarate to give oxaloacetate and glutamate, which on transamination with pyruvate yields alanine and α-ketoglutarate. Alanine has been found to increase both in aerobic and anaerobic conditions; showing a greater accumulation in anaerobic environment. In contrast to vertebrates where NAD^+ is produced by the action of lactate dehydrogenase with the formation of

lactate from pyruvate, in invertebrates NAD^+ is generated during the conversion of oxaloacetate to malate. As the mitochondria are impermeable to NAD^+ or NADH, the reducing power is probably transported in the form of malate. The malate is subsequently metabolized to fumarate and finally accumulates as succinate. The flux of these metabolites in the insect muscle tissue is probably insufficient to account for provision of all the oxidizing power required to maintain glycolysis. The other well studied shuttle mechanism implicated in this respect comprises the conversion of dihydroxyacetone phosphate (DHAP) to Glycerol-3-phosphate with the regeneration of NAD^+. Glycerol-3-phosphate can permeate through the mitochondrial membrane and is converted back to DHAP as illustrated in Fig. 5 (Mordue et al., 1980). The low concentrations of lactate (Clement and Strang, 1978) and large synthesis of alanine (Bradford et al., 1969; Ray, 1964) in locust thoracic ganglia suggest that a similar phenomenon might be occurring in this tissue but no conclusive experimental evidence is available to support this. An investigation into the concentrations of amino acids (aspartate, glutamate, alanine), intermediates of TCA cycle (malate, fumarate, succinate) and Glycerol-3-phosphate, both in aerobic and anaerobic conditions would be quite useful in throwing light on the mechanism of anaerobic energy metabolism in insect nervous tissue.

1.5.1.b Amino acids as precursor for other amino compounds

Two of the non-essential (in dietary terms) amino acids, serine and glutamate, are precursors of several important compounds in CNS. In vertebrate nervous tissue serine is converted to glycine, a putative neurotransmitter (Werman, 1972; Davidson, 1976; Aprison and Nadi, 1978; Levi et al., 1982). Similarly glycine can be

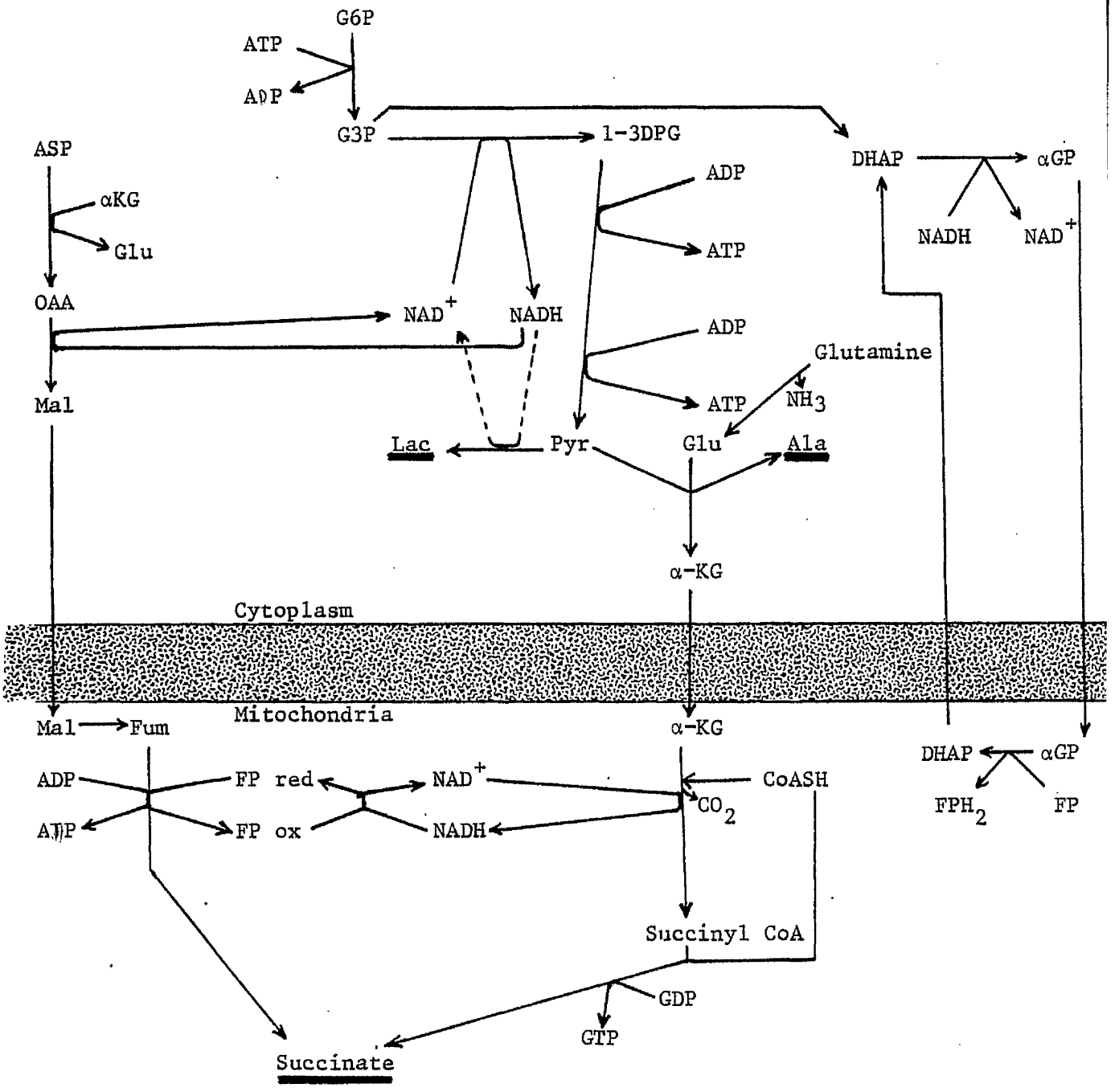


Figure 5 A metabolic map of α-GP shuttle and that accounting for the production of lactate, alanine and succinate as end products of carbohydrate and amino acid metabolism and regeneration of NAD^+.

G6P: Glucose-6-Phosphate; G3P: Glyceraldehyde-3-phosphate; 1-3DPG: 1,3 diphosphoglycerate; Pyr: pyruvate; Lac: lactate; Ala: Alanine α-KG: α-ketoglutarate; Glu: glutamate; Asp: aspartate; Mal: malate Fum: fumarate; OAA: oxaloacetic acid; FPox and FPred: flavoprotein oxidized and reduced; α-GP: α-glycerophosphate; and DHAP: Dihydroxyacetonphosphate. Adapted from Fremin et al., 1980.

converted back to serine, or it can act as a precursor of creatine, an important substance with high energy bond as creatine phosphate. The principal reactions of glycine are shown in Fig. 6. The glutamic acid and Gaba both of which have probable transmitter functions at the neuromuscular junctions in invertebrates are present in insect central ganglia (Ray, 1964; Evans, 1973; Osborne and Neuhoff, 1974; Jabbar and Strang, 1979). These amino acids also occur in honeybee brain in rather higher concentrations (Frontali, 1961, 1964). In nervous tissue from honeybee (Frontali, 1964), cockroach (Higgins et al., 1967) and locust (Bradford et al., 1969) labelled glutamate is in part converted to Gaba. Glutamate decarboxylase activities have been measured in some of these species. Their amount and properties resemble those of the mammalian brain enzymes (Frontali, 1964; Bradford et al., 1969). Major reactions of Gaba, glutamate and aspartate are shown in Fig. 7. Whether the transmitters (Gaba and glutamate) also play a role in interneuronal communication has not yet been established. Studies of iontophoretic application of Gaba in the periphery of ganglia of cockroach have been interpreted as showing that Gaba is participating in the synaptic transmission in the CNS neuropile (Callec and Boistel, 1966; Kerkut et al., 1969a, b; Pitman and Kerkut, 1970) but further evidence is necessary to verify this hypothesis.

Turning to aromatic amino acids, tyrosine is the amino acid precursor of the catecholamines. Any change in its concentration in brain affects the synthesis of catecholamines in vertebrates (Anderson, 1981). A depression in brain tyrosine level probably has

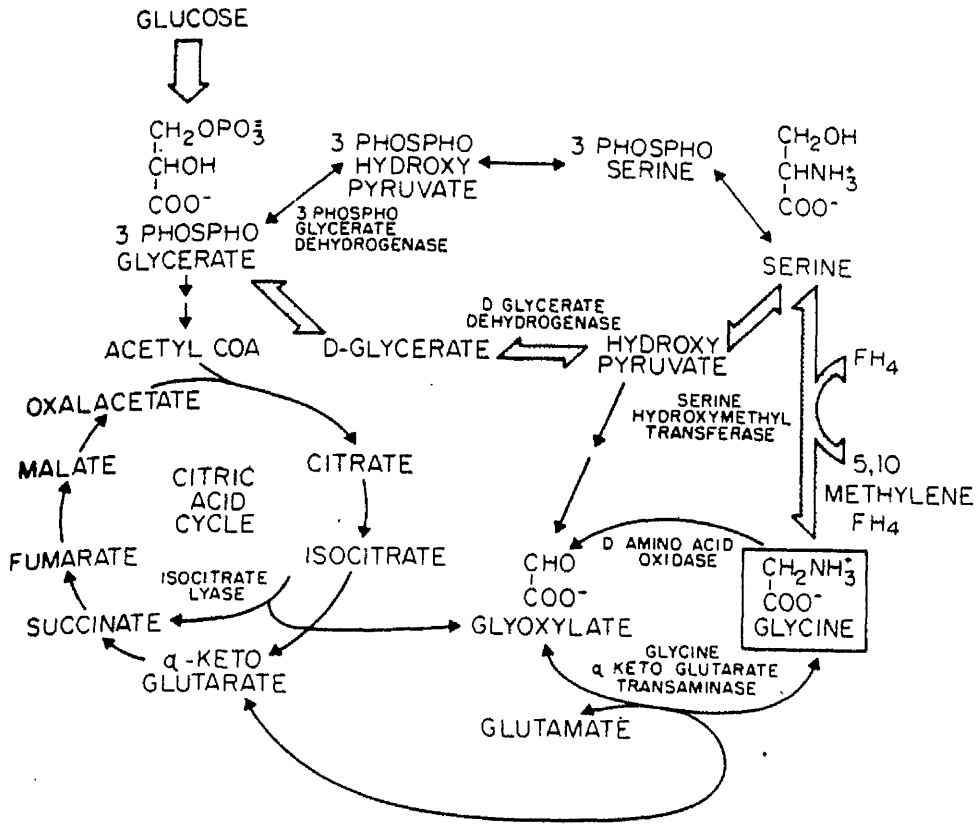


Fig.6. **Principal known reactions of glycine that may be operative in nervous tissue.**
(Siegal et al. 1972)

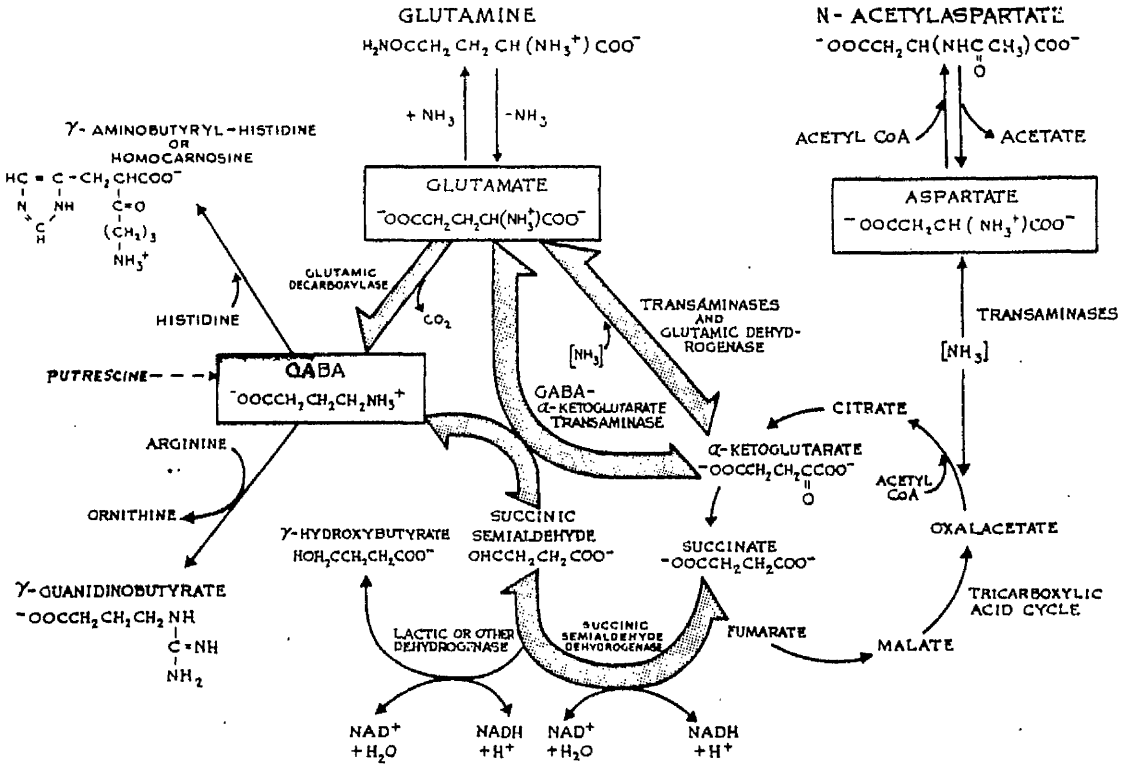


Fig 7 Principal known reactions of GABA, glutamate, and aspartate in the nervous system. The reactions pertinent to GABA metabolism are emphasized by the large arrows.

(Siegal et al. 1972)

the greater effect, but increased tyrosine may also inhibit the amine synthesis (Davidson, 1976). In the mammalian nervous system, tyrosine undergoes a series of chemical transformations resulting in the ultimate formation of dopamine, noradrenaline or adrenaline, depending upon the presence of dopamine-β-hydroxylase or phenylethanolamine, N-methyltransferase (Cooper et al., 1976) as shown in Fig. 8. Alternative routes by which tyrosine (and other amino acids) might be metabolized in brain include decarboxylation and transamination. If the amino acids are decarboxylated, the respective minor amines - phenylethylamine, tyramine and tryptamine - are formed from phenylalanine, tyrosine and tryptophan (Davidson, 1976).

The tyrosine metabolism in insect nervous system, on the other hand, culminates in the formation of N-acetyl derivatives of the monoamines as shown in Fig. 9 in section 1.6.1 (Mir, 1981). Phenylalanine is the precursor amino acid of tyrosine and tryptophan is metabolized to yield 5-hydroxytryptamine (5-HT), a neurotransmitter, as described in section 1.6.1.

1.5.2 Neurotransmitter function of amino acids in invertebrates

γ-aminobutyric acid (Gaba) has a potent inhibitory effect on insect neurones (Kerkut et al., 1969; Pitman and Kerkut, 1970). Iontophoresis of about 10^{-3}M Gaba onto cells in the cockroach metathoracic ganglion produces hyperpolarization mediated by an increase in chloride permeability. Glycine does have an inhibitory effect on these cells, but is around 100 times less active than Gaba (Kerkut et al., 1969). Evidence has been obtained for inhibitory Gaba receptors, and both inhibitory and excitatory glutamate receptors

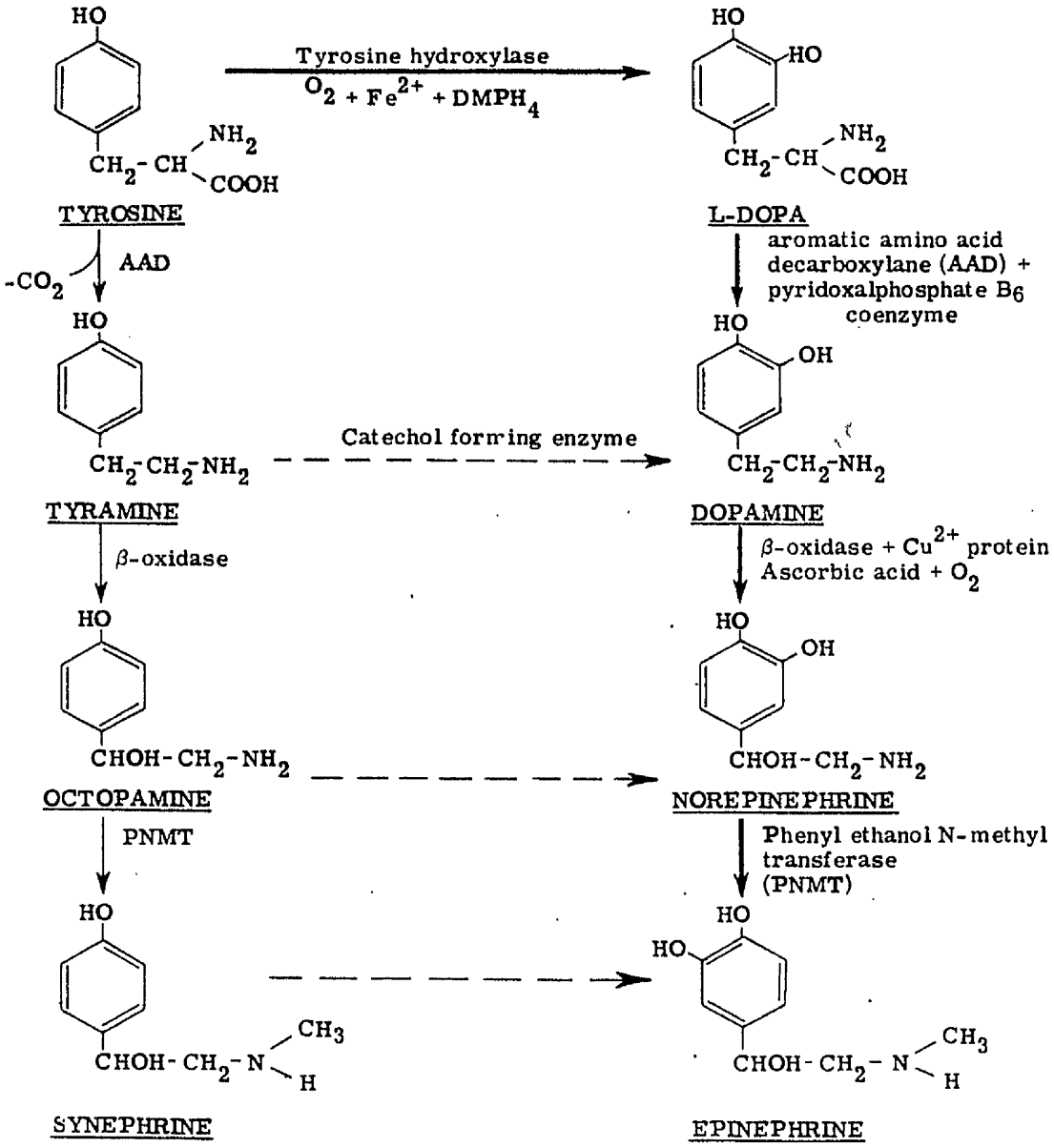


Fig. 8 - Metabolism of Tyrosine in vertebrate nervous tissue.

After Cooper, et al. (1976).

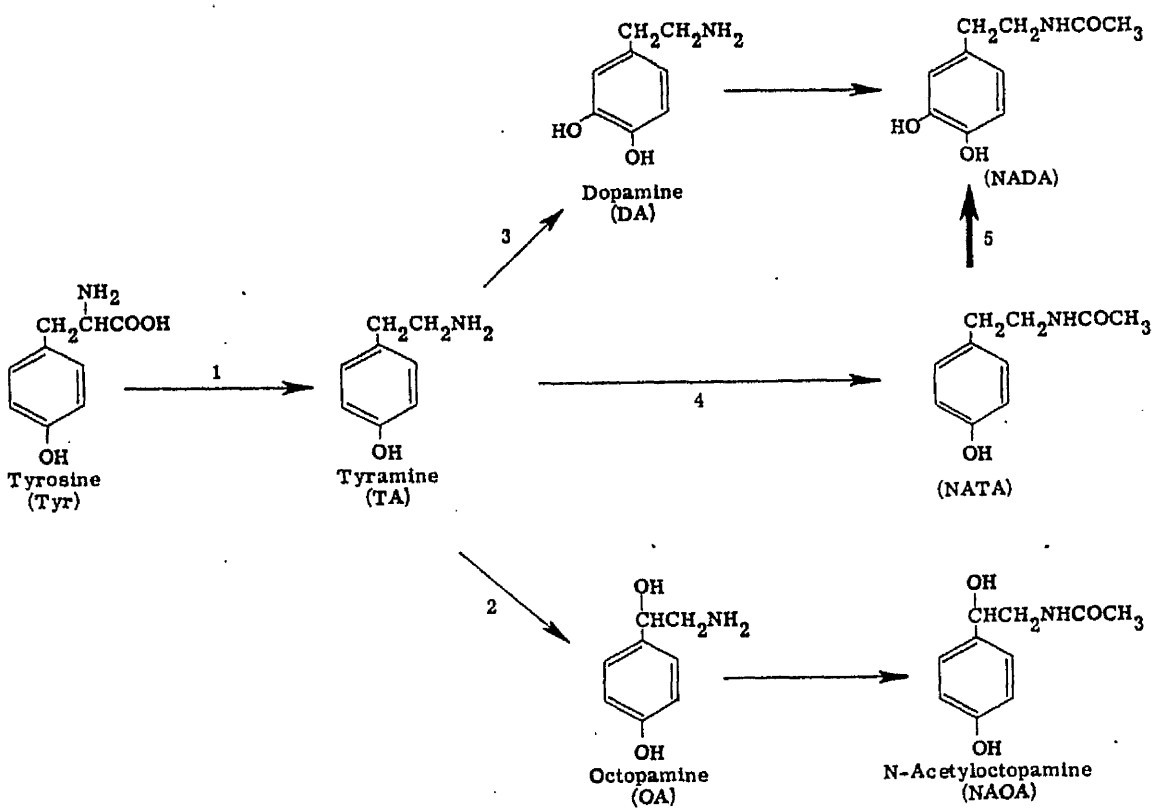


Fig. 9 - Metabolism of Tyrosine in locust (Schistocerca gregaria) nervous tissue.

1. Aromatic amino acid decarboxylase
2. tyramine-β-hydroxylase
3. o-hydroxlyase
4. N-acetyl transferase
5. o-hydroxylase

(After Mir (1981).

on central neurones of the horseshoe crab Limulus polyphemus (Walker and James, 1978). These authors observed that taurine and β-alanine also have inhibitory effect on these cells, but are about 100 times less potent than Gaba. Glycine does not appear to have any action on this invertebrate and glutamate inhibits some cells while exciting others.

Glutamate depolarizes cells and induces contractions in crustacean muscle (Robbins, 1958, 1959; Van Harreveld, 1959; van Harreveld and Medelson, 1959). Takeuchi and Takeuchi (1964) located neuromuscular junctions of the crayfish, Cambarus clorkii, aductor muscle using extracellular recording techniques and stimulating the nerve to produce ejps (excitatory junctional potentials). They observed that iontophoretic application of glutamate produces depolarization in the muscle and it is the junctional area which is sensitive to glutamate. The glutamate mimics the natural excitatory transmitter of crustaceans' skeletal muscle. It has also been shown to be released from a perfused leg of crab Carcinus meanas and of a cockroach following nervous stimulation (Kerkut et al., 1965).

Stimulation of inhibitory nerves to crustacean muscle give rise to chloride-mediated inhibitory junctional potentials (ijps) which can be mimicked by Gaba (Boistel and Fatt, 1958). Dudel et al. (1963) in a study of the lobster central nervous system found that three main compounds which possessed muscle blocking activity were Gaba, taurine and betaine (trimethylglycine) - while alanine and β-alanine were also present. Kravitz et al. (1963) looked for similar compounds in peripheral nerves, and again found that

Gaba and betaine made up most of the blocking activity in the nerve extracts. The Gaba concentration in the inhibitory nerve axons was as high as 0.5% of its wet weight, but it was absent from the excitatory axons. In fact, it was from the clear-cut inhibitory role of Gaba in invertebrates that deductions were made about its role in vertebrates.

Various attempts have been made to implicate aspartate as a transmitter or modulator of excitatory neuromuscular transmission in the crustacea (Shank and Freeman, 1975; Shank et al., 1975). There is approximately 3 times more aspartate than glutamate in lobster excitatory axons, but it is also noted that in the inhibitory axons there is about 5 times more aspartate than glutamate. Aspartate is about 10 times less active than glutamate, but a mixture of aspartate and glutamate are almost as potent as the amount of glutamate equal to the sum of the mixture. Similarly, Irving and Miller (1979, 1980) have found a transmitter role for aspartate and glutamate at 'fast' and 'slow' neuromuscular junctions. They observed that injection of both glutamate and aspartate into the haemolymph of Locusta affected motor activity, whereas injection of aspartate into Lucilia produced no detectable effect. The injections of aspartate and glutamate together had a synergistic effect. Shank et al. (1975) suggested that neuromuscular excitation may be mediated by a combination of glutamate and aspartate activity together. L-Aspartate, which after L-glutamate is the most effective natural amino acids at the insect excitatory nerve muscle synapses (Usherwood and Machili, 1968; Clements and May, 1974) was also shown by Dowson and Usherwood (1973) to increase the

frequency of the minimum excitatory postsynaptic potentials of locust extensor tibiae muscle fibres when applied at a low concentration (10^{-7}M). These observations would support an excitatory neurotransmitter role for aspartate.

Amino acids also induce contraction of the striated muscle in the vas deferens of Astacus leptodactylus. Murdock (1971) showed that L-glutamate was very potent, being more than 100 times more active than its D-isomer. L-Glutamine was also more active than D-glutamine. L-Aspartate and the γ-methyl and γ-ethyl analogues of glutamate were about 100 times less potent than L-glutamate. Gaba also caused muscle contractions but was less potent than glutamate. β-alanine was about 100 times less active than Gaba, and glycine was inactive. From a range of putative transmitters tested on this preparation, receptors for only glutamate and Gaba were found.

Following the observations that glutamate excited crustacean muscle, it was shown to induce contractions and depolarizations in insect muscles (Kerkut et al., 1965; Usherwood and Machili, 1966). Kerkut et al. (1965) using leg of Periplaneta americana perfused in situ suggested that glutamate might be the excitatory transmitter at this junction. Subsequent evidence suggested that glutamate could be acting presynaptically as well as postsynaptically (Usherwood and Machili, 1966; Kerkut and Walker, 1966, 1967). Beranek and Miller (1968) by applying glutamate iontophoretically onto the muscle of Schistocerca and Locusta located areas of high sensitivity that coincided with the neuromuscular junctions from which ejps could be recorded. A similar study by Dowson and Usherwood (1973) on the metathoracic tibiae and retractor ungius muscle of

Schistocerca, showed that iontophoresis of 10^{-7}M glutamate gave a 32% increase in miniature end plate potentials (mepp) frequency. Thus low concentrations of glutamate act presynaptically by facilitating spontaneous transmitter release as well as impulse-linked release. There is evidence of Gaba having a marked inhibitory effect on contractions elicited by both nerve stimulation and glutamate application (but not ACh) in the perfused cockroach leg (Kerkut et al., 1965). There is now clear evidence for inhibitory axons innervating insect muscles (Usherwood and Grundfest, 1964, 1965; Usherwood, 1968; Pearson, 1973). Stimulation of the inhibitory axons and neurones produces an inhibitory junctional potential (ijp) in the muscle, which is associated with an increase in permeability to chloride. Iontophoretic application of Gaba onto locust metathoracic adductor muscle fibre (Usherwood, 1973) produces prolonged depolarizing or hyperpolarizing potentials at sites corresponding to the glutamate-sensitive areas. The evidence indicates that Gaba is the peripheral inhibitory transmitter in insect skeletal muscle.

From the foregoing discussion it could be concluded that glutamate (and possibly aspartate) are excitatory neurotransmitters and Gaba is an inhibitory neurotransmitter in insects. Little evidence is available for transmitter function for glycine in the insects nervous system.

1.6 Metabolism and function of monoamines

1.6.1 Metabolism of mono-amines in the insect nervous system

a) Catecholamine (Dopamine and octopamine)

Considerable differences have been found in the synthesis and degradation of the catecholamines between vertebrate and invertebrate nervous systems. One of these differences is the major route of inactivation of monoamines in insects by their N-acetylation (Dewhurst et al 1972; Evans & Fox, 1975; Vaughan & Neuhoff, 1976; Hayashi et al 1977; Mir & Vaughan, 1978, 1981). This contrasts with the vertebrates in which the amines are metabolized by oxidation through the action of mono-amine oxidase (MAO) or methylation by catechol-o-methyltransferase (COMT), (Bachelard, 1974). In the insect haemolymph it has been demonstrated that tyrosine is metabolized to dopamine via dihyroxyphenylalanine (Dopa), which is subsequently acetylated to N-acetyl-dopamine and complexed with cuticular proteins for the sclerotinization process (Sekeris & Karlson, 1966; Anderson, 1971). However, the formation of DOPA as an intermediate in the biosynthesis of dopamine in insect nervous tissue has not been clearly demonstrated as yet. The studies of Vaughan & Neuhoff (1976), Mir & Vaughan (1978, 1981) have provided evidence that in Schistocerca americana gregaria nervous tissue, tyrosine is converted to tyramine by the action of amino acid decarboxylase and is subsequently converted to octopamine by β-hydroxylase (β-oxidase) as shown in Fig. 9.

b) Indolamine (serotonin, 5-hydroxytryptamine)

In vertebrates, serotonin is synthesized from tryptophan by a pathway similar to that of dopamine from tyrosine (Cooper et al 1976) as illustrated in Fig. 10. This pathway was first demonstrated in a number of insects by Welsh and Moorehead (1960), a finding later

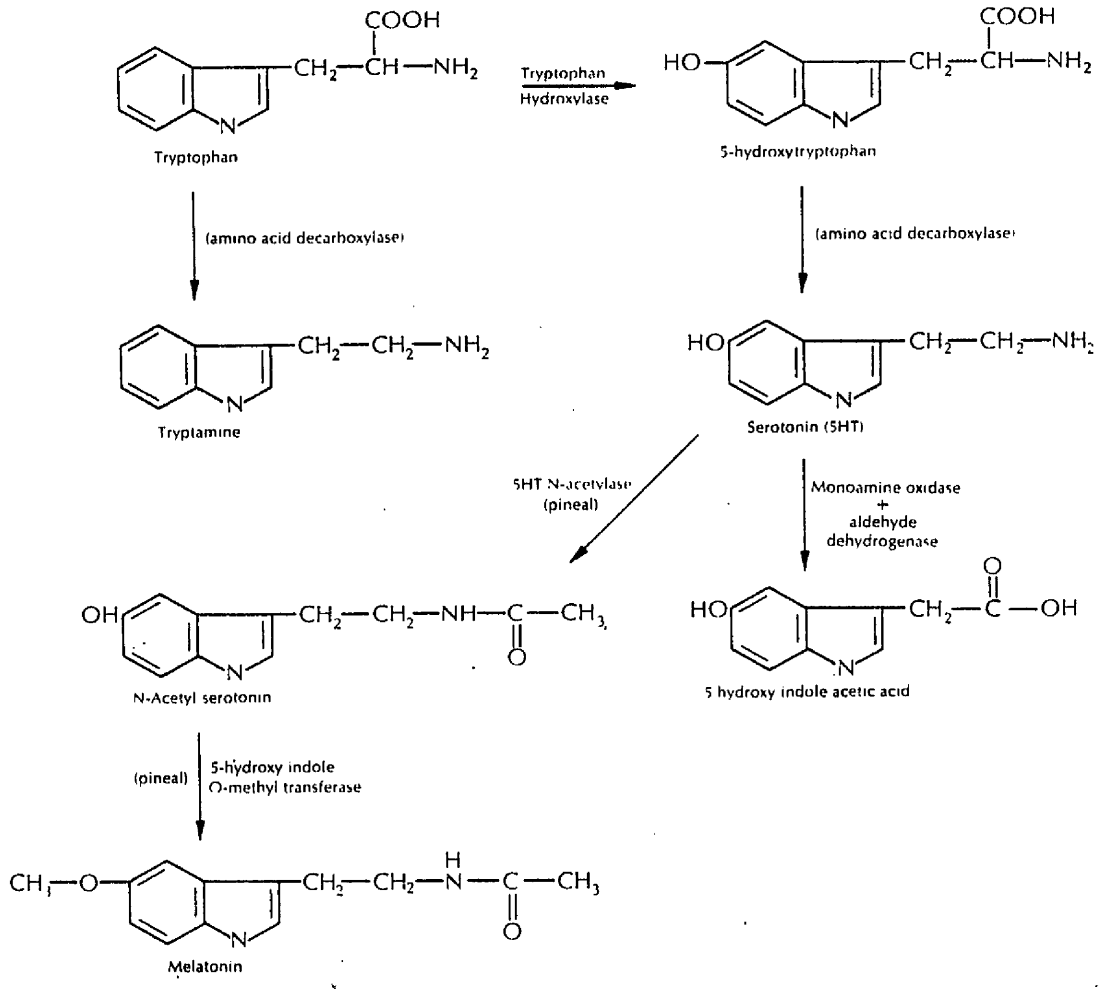


Fig 10. The metabolic pathways available for the synthesis and metabolism of serotonin.

(Cooper et al. 1976)

confirmed by other workers (Gersch et al 1961; and Colhoun, 1963). Cockroach brain and to a lesser extent other nervous tissue can synthesize this amine from 5-hydroxy-tryptophan (Colhoun, 1963). Cockroach nervous tissue does not show any monoamine oxidase activity (Blaschko et al 1961 and Colhoun, 1967) therefore, like catecholamines the 5-HT may be further metabolized to yield N-acetyl serotonin . This view is supported by the presence of an N-acetyl transferase in the neural and non-neural tissues of Drosophila which is active against dopamine, tyramine and 5-hydroxytryptamine (Dewhurst, et.al., 1972). Similarly Evans & Fox (1975), observed that dopamine, tyramine and 5-HT were transacetylated in brain homogenates of Apis mellifera. Hayashi et al (1977) have shown that N-acetyl transferase from different tissues of Locusta can utilize dopamine and 5-hydroxy tryptamine as substrates.

From these observations it is concluded that the inactivation of monoamines in the insect neural tissue is by the N-acetylation and these N-acetyl derivatives are physiologically inactive (Florey & Rathmayer, 1978).

1.6.2 Neurotransmitter function of mono-amines in invertebrates

In molluscs, particularly in the gastropods and lamellibranches there is evidence for dopamine (DA) as a central and peripheral transmitter respectively. DA is present in specific neurones in Helix, Planorbis and Lymnaea (Sedden et al 1968; Marsden & Kerkut, 1970; Winlow and Benjamin, 1977). Berry & Cottrell (1975) and Cottrell (1977) have presented conclusive evidence for dopamine as both an inhibitory and excitatory transmitter in Planorbis central nervous system. In the lamellibranches there is now good evidence for

DA having an inhibitory effect on lateral cilliated gill epithelium (Paparo & Aiello, 1970).

Dopamine has also been implicated in neuromuscular transmission at the anterior byssal retractor muscle (ABRM) of Mytillus. Dopamine has been shown to be present in Mytillus ganglia (Sweeney,1963). It relaxes catch tension and hyperpolarises the ABRM (Hidaka, 1969; Twarog & Cole, 1972; Hikada et al 1977).

In Aplysia dopamine is present in large amounts in the pedal ganglia and the posterior parapodial nerve. Smaller amounts occur in the cerebral, buccal and visceral ganglia (Carpenter et al 1971). Noradrenaline (NA) is not detected in significant amounts.

There is very little evidence for noradrenaline as a transmitter in any invertebrate phylum. Noradrenaline does occur in the insect nervous system (Frontanli & Haagendal, 1969; Klemm & Bjorklund, 1971), but at a lower concentration than dopamine. The situation is true for a number of other invertebrates (Robertson & Jurio, 1976).

Octopamine (OA) occurs in the insect CNS (Robertson & Steele, 1974; Robertson, 1976; and Evans, 1978) and many invertebrates (Robertson & Jurio, 1976; Axelrod & Saavedra, 1977; Evans, 1980). OA can be formed from labelled tyramine in the insect CNS (Robertson & Steele, 1974; and Mir, 1981). Octopamine causes a cyclic AMP-mediated activation of a phosphorylase in cockroach nerve cord, while DA and NA have no effect on phosphorylase activity (Robertson & Steele, 1972). The phosphorylase activation and the resulting glycogenolysis suggested a role for OA in regulation of energy supplies in the nervous system. This view is strengthened by the observations of Goosey & Candy (1980a,b) that OA concentration in the haemolymph of locust shows a

5 times increase with the onset of flight in this insect. Candy (1978) found that among a number of compounds, octopamine stimulated a most significant increase of oxidation of glucose, trehalose, butyrate and diacylglycerol by locust thoracic muscle in vitro and he concluded that OA has physiological significance in regulating metabolism in flight muscle and fat body during early periods of flight (Candy, 1981). Similarly Downer (1979) observed OA to cause hypertrehalosemia in the haemolymph of cockroach. There is sufficient evidence to suggest that these effects of OA are through the elevation of cAMP. Octopamine-sensitive adenylate cyclases have been shown to be present in insect nervous tissue (Nathansen & Greengard, 1973; Harmer & Horn, 1977; Kilpatrick et al 1980; Hiripi & S-Rosza, 1980; Bodnaryk, 1982) and in CNS of annelid (Robertson & Osborne, 1979).

It has also been suggested that firefly luminescence is regulated by octopaminergic nerves (Robertson & Carlson, 1976). Octopamine has been shown to be present in the brain of Helix (Walker et al 1972) and in single Aplysia neurones (Saavedra et al 1974). Specific octopaminergic receptors have been found in Aplysia (Carpenter & Gaubatz, 1974) and Helix (Batta et al 1977) neurones. Hoyle & Barker (1975) have shown that OA can be synthesized by the dorsal unpaired medium (DUM) neurones in the segmental ganglia of locust and their further experimentation showed that OA has a neurohumoral action on these cells. The general conclusion is that the amine can act as a humoral agent effecting metabolism in the nervous system.

The other important biogenic amine, Serotonin (5-HT), is found in a number of invertebrates (Welsh & Moorehead, 1960, Robertson & Jurio, 1976; and Evans,1980), and a neurotransmitter or neuromodulator role is often suggested for this indoleamine. For example the two giant

serotoninergic cells (GSC's) in the cerebral ganglia of Helix pomatia have been found to make synaptic connections onto a group of follower cells in the buccal ganglia and that following stimulation of GSC's, excitatory postsynaptic potentials (epsps) of 500-600ms duration can be recorded in three non-serotonergic buccal giant neurones (Cottrell & Macon, 1974).

Shimahara & Tauc (1975) have found evidence for 5-HT as the transmitter mediating heterosynaptic facilitation in Aplysia. Stimulation of a nerve trunk induces facilitation of an epsp recorded from the Aplysia left giant cell in the pleural ganglion as a result of stimulation of interneurones. Iontophoretic application of 5-HT can also induce an unusually long excitatory response in certain cells in the buccal and abdominal ganglia of Aplysia (Pellmar & Wilson, 1977).

5-HT also excites the heart of a wide range of molluscs (Welsh, 1953, 1957). Gaddum & Paasonen (1955) investigated the action of 5-HT on a range of molluscan and other invertebrates hearts in an attempt to establish a convenient bioassay method for 5-HT. These authors found that 5-HT excites the heart beat in Solen siliqua, Cyprins islandica, Mya areneria, Helix aspersa, H. pomatia and Spisula solida.

5-HT is the most potent amine to increase the rate of the beat of the denervated heart of american cockroach (Miller & Metcalf, 1968). The action has been studied in more detail on the heart muscle cells of the locust (S-Rosza et al. 1973). 5-HT (2×10^{-8}M) decreases the frequency of the heart muscle action potential (APs) while higher doses (10^{-7} - 10^{-4}M) increase the rate.

These observations and many more suggest that DA, OA and 5-HT are functioning as neurotransmitters or neurohormones in invertebrates. If they play a part in the symptoms of poisoning in insects (Section 1.9) it may be a hormonal effect due to excessive release from the carpus cardiacum. In addition to releasing the peptide hormones which control a wide range of physiological activities, including moulting, pigmentation and peristaltic movements of heart and the malpighian tubules, this organ contains, and may well release, amines which affect the nervous system. These include DA, 5-HT (Natalizi et al. 1970; Klemm, 1972), and more recently the presence of OA has been recorded (Evans, 1978). Certainly these amines are associated with the release of hyperglycaemic and adipokinetic hormones, which is one of the responses to poisoning by insecticides (Samaranayaka, 1974 & 1976).

1.7 Metabolism and function of the diamine, Putrescine

The presence of putrescine has been recorded in the CNS and other tissues of a number of invertebrates i.e. brain of Helix pomatia (Dolezalova et al.1973; Osborne,1971, 1972, Hiripi & Osborne, 1976), haemolymph, fat body and wings of silkmoth (Wyatt et al 1973) and other nervous tissues of molluscs (Gould & Cottrell, 1974), and haemolymph of corn earworm (Heliothis zea) larvae (Cheung et al 1982). So far no report is available on the occurence of this amine in insect nervous tissue. Putrescine occurs widely in living organisms i.e. microbes, plants and vertebrates (Tabor & Tabor, 1972), and has gained much attention due to its enhanced metabolism in fast growing tumour cells (Russel, 1973; Raina et al. 1980; Seiler et al. 1978). Generally the polyamines (putrescine, spermidine and spermine) are all strongly cationic and are associated with the nucleic acids, proteins, and anionic sites of the membrane (Seiler, 1981). Current information

suggests that polyamines (a) stimulate protein synthesis in vivo and in vitro, (b) bind tightly to substrates such as tRNA and ATP and (c) bind to ribosomes and stabilize the ribosomal structure and function (Tabor & Tabor, 1972; Cohen, 1971; Stevens, 1970; Raina & Janne, 1975; Tabor & Tabor, 1976).

Although in the brain of snail (Helix pomatia) the levels of putrescine have been reported to increase after electrical stimulation (Osborne et al 1971). Gould and Cottrell (1974) have ruled out any neurotransmitter function for putrescine as perfusion of the isolated nervous tissue with 10^{-8} - 10^{-4}M putrescine had no effect on the nervous activity. Similarly to date no evidence is available for neurotransmitter role in vertebrates (Seiler, 1981).

The interest of neurochemists in putrescine lies in the fact that it is a potential precursor of Gaba in mammalian and other vertebrate brain tissue (Seiler et al.1971, 1973; Seiler & Al-Therib, 1974; Seiler & Eichentopf, 1975; Seiler et al.1979). In rat brain the suggested pathway (Seiler & Eichentopf, 1975) for the catabolism of putrescine comprises acetylation of putrescine, oxidative deamination of monoacetyl putrescine to N-acetyl-γ-amino butyrate and transformation of N-acetyl-γ-amino butyrate to γ-aminobutyric acid (Gaba).

As the insect nervous tissue has no or very little monoamine oxidase (MAO) and in the light of reported low activity of glutamic acid decarboxylase (GAD), 20nmol/mg protein/hour, in this tissue (Bradford et al 1969), it would be interesting to explore the possible contribution of putrescine in the synthesis of Gaba in insect CNS either by the above mentioned pathway or by the pathway first realized in micro-organisms such as bacteria (as shown in Fig. 11).

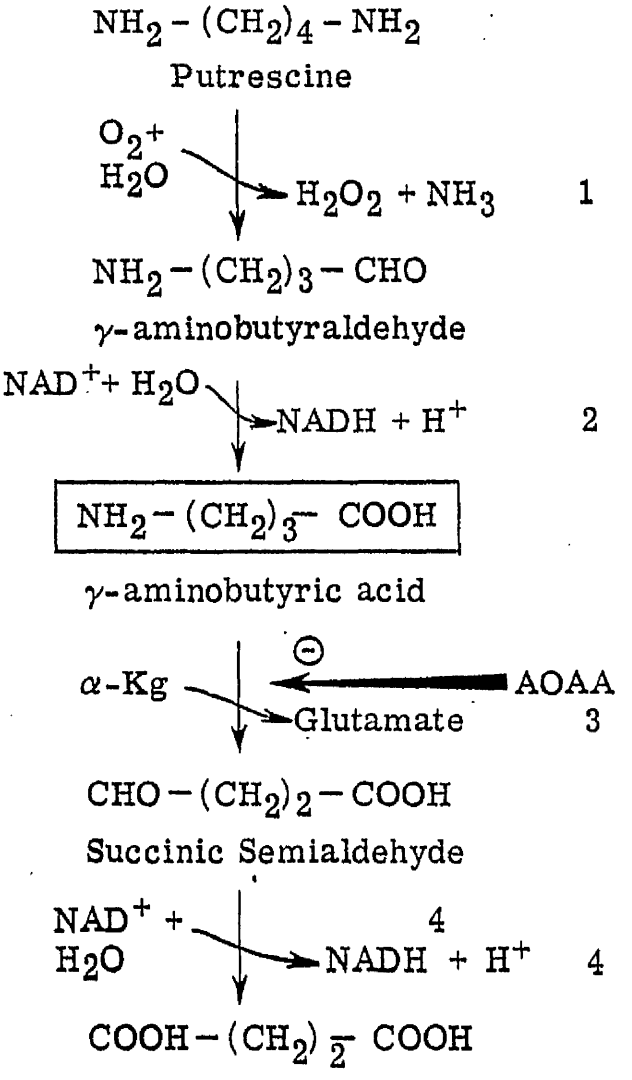


Fig. 11 Metabolism of putrescine in microbes (Bacteria).

The point of inhibition by aminooxyacetic acid (AOAA) is shown by the arrow. The various enzymes of the pathway are 1. Diamine Oxidase, 2. Aldehyde dehydrogenase, 3. Amino transferase and 4. Succinic semialdehyde dehydrogenase.

1.8 Release of amino acids and amines from nervous tissue

A wealth of information is accumulating on the release of neurotransmitter and non-neurotransmitter substances both in vertebrates and invertebrates after the electrical or chemical stimulation of the nervous tissue *in vivo* and *in vitro*. It would be quite useful to try to ascertain the specificity of the release (quantitatively if possible), after the application of certain stimuli, for the elucidation of mechanism of neutrotransmission.

In the vertebrates, in pioneering experiments, Jasper et al (1965) analyzed the amino acid effluent with respect to electroencephalographic (EEG) signals in the exposed cerebral cortex of unanaesthetized cats. They noted an inverse relationship between Gaba release and cortical stimulation as judged by the EEG. Thus preparations showing an activated EEG showed a 40% rise in glutamate release, and a decrease in Gaba release of approximately two thirds compared with those from cortex with a synchronized "sleeping" EEG. These results were confirmed later by Jasper & Koyama (1969) who showed in addition that electrical stimulation of midbrain reticular formation caused significant increases in the release of glutamate, aspartate and glycine, while substantial increase in taurine release was noted in some experiments.

In the studies with the rat cerebral cortex *in vitro*, Edwardson and his colleagues (1972) have demonstrated that the electrical stimulation of synaptosomal preparation causes the release of a number of amino acids. They observed that after stimulation the transmitter amino acids (e.g. glutamate, aspartate and Gaba) were present as 59, 62 and 58% respectively in the bathing medium as compared to glutamine 30%, serine 24%, glycine 31% and alanine 28%.

Similarly in the experiments on the release of transmitter substances from central nervous system of invertebrates, Gerschenfeld et al (1978) have observed the release of endogenous 5-HT following the stimulation of the two giant serotonergic cells (GSC's) in Aplysia. Using a radioenzymic microassay of 5-HT they found that spontaneous release of 0.4-1.2 pmole of 5-HT into the medium bathing the buccal ganglia was increased by up to 100% on stimulation of the GSC's but only in the presence of 1-10μm chlorimipramine (5-HT uptake blocker). The duration and amplitude of the excitatory post-synaptic potentials (epsp's) and inhibitory postsynaptical potentials (ipsp's) evoked in the buccal follower neurones by GSC stimulation was also enhanced in the presence of chlorimipramine.

Octopamine, another transmitter candidate in invertebrates, is released from the lobster nervous system following incubation with labelled tyramine or tyrosine (Evans et al.1976). Moreover, pulsing with 100mM K^+ causes an increase in the release of labelled material. Thus, these observations show that the amino compounds are released from the central nervous system in reponse to external or internal stimuli.

This phenomenon is not restricted to the central nervous system only, the peripheral nervous system has also been shown to release various substances e.g. glutamate efflux is recorded from perfused leg of the crab (Carcinus maenas) and of a cockroach (Periplaneta) following nervous stimulation (Kerkut et al 1965). The amount of glutamate in these perfusates is proportional to the number of stimuli given. In a similar experiment on an isolated nerve muscle preparation from lobster, Kravitz et al.(1970) showed that nerve stimulation induced a small increase in glutamate release superimposed on a large

background efflux. Daoud & Miller (1976) analyzed the perfusate from crab (and locust) legs and found that stimulation of leg caused a release of many amino acids including glutamate, but there was no evidence of a specific glutamate efflux related to synaptic function. These authors emphasize the difficulty of trying to locate a putative neurotransmitter at a synapse, particularly when that compound exists also as a general metabolite.

The release of the amino compounds has been a focal point in the study of normal or pathogenic circumstances(due to, for instance, insecticide treatment or other stresses) in the life of animals. In this respect it would be worth mentioning the observations of Sternburg et al.(1959), Colhoun (1960) that a number of amino substances are released following the electrical, insecticide or other chemical treatment of insect nervous system. Similarly Tashiro et al.(1975) identified one of these substances as isoamylamine (a decarboxylated product of leucine) as being released from nerve cord of cockroach into the perfusate containing insecticides. Therefore, this release of neuroactive substances is going to be the major subject matter of present investigation and will be reviewed in more detail in the next pages.

1.9 Insect CNS and insecticides

The paramount importance of the nervous system to the overall functioning of animals makes it an extremely sensitive target for the action of poisons. A discrete lesion in metabolism at one site may be lethal to an animal through the far reaching consequences of general disruption of nervous integrity (Shankland, 1976). Since the nervous system constitutes the major, and in many cases the only target site for insecticides, some yardstick against which to measure their effects on

this system is critical to establishing the precise mechanism by which they act. Even so, studies in this field have not attained the same level of popularity as several other areas of research on insecticide action and disposition (Narahashi, 1976).

1.9.1 Action of insecticides on the nervous system

It is well established that organophosphates and carbamates possess anticholine esterase (AChE) activity (O'Brien, 1967; Koelle, 1970, Narahashi 1976). Malathion is a poor inhibitor of AChE in cockroach and other insects (Cornwell, 1976). Recently, Gripois and his colleagues (1977) reported that insecticides like dieldrin and chlordimeform inhibit the monoamine oxidase (MAO) activity of the locust brain. This seems unlikely as many reports indicate that there is no MAO in insect CNS (Blaschko et al 1961; Colhoun, 1967). The insecticide chlordimeform and its N-dimethyl derivative mimic the action of OA in the intact firefly light organ (Hollingworth & Murdock, 1980) and at the locust neuromuscular junction (Evans & Gee, 1980) indicating that the pesticidal and pestistatic properties of the formamidines may result from action on octopaminergic systems (Hollingworth & Murdock, 1980).

For some insecticides the target is not an enzyme but neuronal membrane. The insecticides bind to macromolecules in post-synaptic membranes and supress the postsynaptic potentials, presumably in a manner similar to curare and other natural neurotoxins (Osborne, 1979).

The precise mechanism of action of most insecticides is still obscure. Some insecticides such as DDT and pyrethroids directly affect the transmission of action potentials (Osborne, 1979).

Synaptic transmission is disturbed due to induced multiple spiking in the case of DDT, or the block of axonal conduction caused by pyrethroids. Nicotine, γ-BHC and chlordimeform are all known to have some sort of central effect. The various possible sites of insecticidal action at synapses are shown in Fig. (12).

To date no insecticide has been reported which specifically affects the release or metabolism of neurotransmitter amino acids. Nevertheless, as already mentioned, insecticides (DDT, dieldrin) and the natural neurotoxin (tetrodotoxin) have been shown to cause the release of amino compounds from the insect nerve cord (in vitro) (Sternburg et al 1959, Tashiro et al. 1972, 1975). These compounds have been shown to cause hyperstimulation in the isolated nerve cord of the cockroach as discussed in the following section.

1.9.2 Insecticide and stress syndrome

It is not a recent suggestion that paralysis and subsequent death of stressed insects results from autotoxicity due to the production of some neurotoxin within the insect itself. It has further been proposed that this may be common to all sorts of stress whether "natural" or due to insecticides. It was observed that insect Periplaneta americana, after subjection to prolonged mechanical immobilization often became paralyzed (Beament, 1958). Similar symptoms appeared in cockroaches after 4-12 hours of enforced activity. Moreover, when the blood from these paralyzed cockroaches was injected into normal insects the symptoms of paralysis often developed. An apparently identical type of paralysis occured in the submissive male cockroach Nauphoeta cinerea (Oliv) that had been exposed to an aggressive

Figure 12. Schematic diagram to show the various steps in synaptic transmission (numbered rectangles). The action potential (1) arrives at the presynpatic terminal and increases calcium influx (2). This triggers the release of transmitter (3) which combines with postsynpatic receptor molecules (4) producing the postsynaptic potential (5) which instigates an action potential in the postsynpatic axon (6). Transmitter in the cleft is inactivated either by an enzyme (7), by its uptake into the presynaptic terminal possibly by pinocytosis (8), by uptake into the postsynaptic cell (9) or by uptake into the glial cell (10) for ultimate return to the presynaptic termina. Mitochondria (m) can be involved in synthesis or degradation of the transmitter (11) and microtubules (t) apparently transport transmitter and membrane precursors from the soma to the presynpatic terminal (12). Pinocytotic activity is thought to give rise to coated vesicles (cv) which coalesce into cisternae (c) from which new vesicles (sv) are formed. Dotted lines indicate steps in synaptic transmission affected by particular insecticides. Question marks indicate possible sites of insecticidal attack which have not yet apparently been exploited. (From Osborne, 1979).

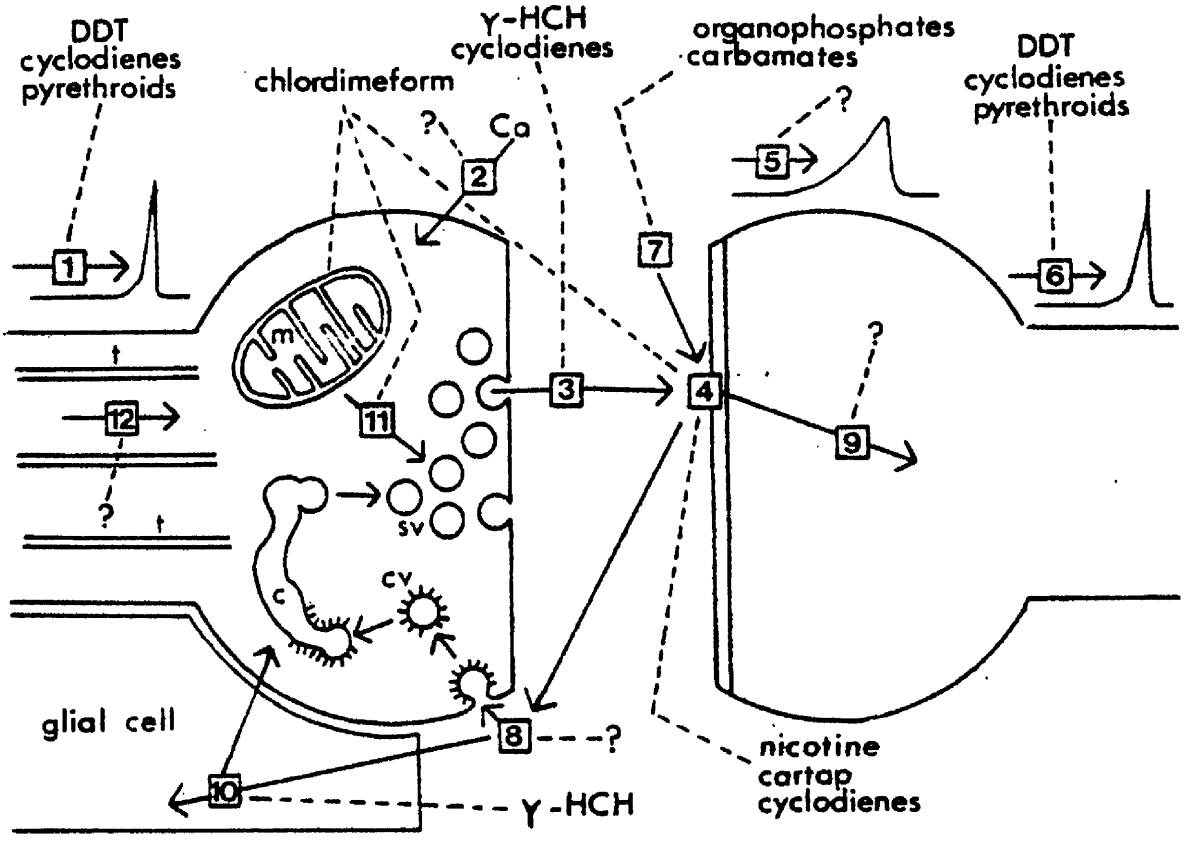

DDT
cyclodienes
pyrethroids
γ-HCH
cyclodienes
organophosphates
carbamates
DDT
cyclodienes
pyrethroids
chlordimeform
?
Ca
2
1
5
?
7
6
m
3
4
12
11
9
?
sv
cv
c
glial cell
8
?
nicotine
cartap
cyclodienes
10
γ-HCH

Figure 12.

encounter with other males (Ewing, 1967). A similar phenomenon occured in field cricket, Cyribus integer, after social stress (Sanford, 1971). The symptoms leading to stress paralysis are nearly identical, whether cockroaches are subjected to physical or chemical (DDT-intoxication) Stress (Hes⁀lop & Ray, 1959). Other chemicals too (e.g. TEPP, Dieldrin), produced the same sequence of events that lead to paralysis (Colhoun, 1960). Thus a variety of stimulii of sufficient strength and duration can trigger a stress syndrome and death. This process of autointoxication has been reported to arise from excessive release of pharmacologically active substances from the central nervous system (Sternburg et al.1959, Cook et al. 1969) and neuroendocrine complex (Davey, 1963). Although neuropharmacological changes associated with paralysis arising from body stress in cockroach have been studied in detail (Cook & Holt, 1974), very little is known about the biochemical events causing the release of neuroactive substances into the haemolymph which lead to paralysis and ultimate death of the insect.

1.9.3 Identity of possible neurotoxins

Neurotoxic factors have been reported to be produced by the central nervous system in reponse to hyperstimulation and to be released into the haemolymph, but their identity has not yet been ascertained unequivocably (Hawkins & Sternburg, 1964; Tashiro et al.1972; Cook et al.1969; Gardener & Brady, 1977). Suggestions as to the nature of these compounds have ranged from an unidentified esterified aromatic amine (Sternburg, 1963), a catecholamine (Colhoun, 1960) to leucine (Tashiro et al.1972) and isoamylamine derived from

leucine by decarboxylation (Tashiro et al, 1975). No certain identification has been made. It has been reported, however, that these factors can be produced by the stimulation of nervous system in vitro (Brown & Sternburg, 1964).

As well as the ganglia of ventral nerve cord, another tissue which has been implicated in the response of pesticide poisioning is the corpus cardiacum, the main neurohaemal organ of the insect. It is well known that factors from the corpus cardiacum influence the rate of spontaneous activity of the isolated nervous system, either increasing or inhibiting it according to their concentrations (Ozbas & Hodgson, 1958; Colhoun, 1959).

A number of substances in abnormal amount are found in the haemolymph of cockroach after treatment with DDT, TEPP or Lindane (Colhoun, 1960). As already mentioned among these was a "catecholamine" which increased most significantly and was biologically active against nerve and heart preparation of the normal cockroaches. Colhoun (1960) thinks the main source of this amine is corpus cardiacum gland. Insecticides (γ-BHC and Permethrin) cause the release of neurohormones and hypertrehalosalmic factors from corpus cardiacum in the insects, Schistocerca and Calliphora (Samaranayaka, 1974; 1976; Normann & Samaranayaka-Ramasamy, 1977; Normann, 1979). Kater (1968) demonstrated that a proteinaceous heat stable substance was released from the Corpus cardiacum after electrical stimulation of the brain of Periplaneta americana. Similarly Cook (1967) obtained a neuromuscular excitatory substance (Factor S) from insects and crustacea. Later Cook et al, (1969) observed that this factor showed several properties of a typical neurohormone and suggested that it has an involvement in the mode of action of insecticides.

From the forgoing reports it is apparent that as the corpus cardiacum is involved in the release of so many peptide hormones, it is impossible to dismiss the possibility that the neurotoxic factors may also be such a hormone. There is evidence, however, that whatever the nature of these agents, these are not inactivated by the action of proteolytic enzymes (Milburn & Roeder, 1962; Brown, 1965).

Clearly the response to stress and the chemical effectors of the central nervous system and its associated neurohaemal organs is complex, and is likely to involve amino acids, amines and their derivatives, and possibly also hormones, but the identity of these neuroactive substances is far from clear so far and merits further investigation.

1.10 Estimation of amino compounds

In order to have a better understanding of the intricate and diverse functions of the brain, it is of prime importance to develop very sensitive, specific and accurate assays for the separation, identification and quantitation of brain amines and amino acids. Since the endogenous concentrations of biogenic amines are in the picomolar range while those of the amino acids in nanomolar range in the insect nervous tissue, and the amount of tissue available from one insect is only a few milligrams, the need for the development of very sensitive method becomes more pronounced.

Amino acids occur in higher concentrations than amines in biological fluids and tissues, and have mostly been estimated by automated amino acid analyzers using ninhydrin reagent introduced by Moore and Stein (1948). Several procedures in use for quantitation of indole and catecholamine include: bioassay, colorimetry, fluorimetry, liquid chromatography, radio enzymic assays and dansylation followed

by thin layer chromatography. These are briefly discussed below.

a) Bioassay

Bioassay used as early as 1912 by Elliott, was perhaps the first real attempt to measure the amount of adrenaline in adrenal glands. Afterwards, bioassays were widely employed for estimating catecholamines by various workers (Barsoum & Gaddum, 1935; De Jalon et al 1945; Page & Green, 1948; von Euler, 1948; Crawaford & Outshoorn, 1951; and de la Lande & Harvey, 1965). As these were time consuming and not very accurate, where possible these methods have been replaced by colourimetric and spectrophotometric procedures.

b) Colourimetric and spectrophotometric methods

Of several colourimetric procedures, that of von Euler & Hamberg (1949) for estimating N.A. and A was probably the most successful. Later Ehringer and Harnykiewier (1960) used similar methods for estimating DA in human brain. The spectrophotometric methods are based on the absorption of monochromatic light by the catechol ring in the ultra violet region.

c) Spectrophotofluorometric methods

The development of sensitive fluorescent techniques for the assay of tissue catecholamines has certainly been a major factor in the rapid development of knowledge of these compounds (Cooper et al.1976). Ion exchange resins have been employed to isolate amines e.g. N.A., D.A. and 5-HT which are strongly absorbed to cation exchange resins from the tissue (Bartler et al.1958; Haggendal, 1962). After elution these can be measured by native fluorescence or the fluorescence induced by coupled chemical reactions. Two types of chemical reactions form the basis of these fluorometric assays.

1) Trihydroxy indole procedure (Van Eular 1959; Vandsalu, 1960; Haggendal, 1963; Laverty & Taylor, 1968); 2) 1,2-diaminomethane condensation procedure (Natelson et al.1949; Montagy, 1957; von Eular & Lishajko, 1957; Laverty & Sharman, 1965).

d) Ion-exchange chromatographic methods

Liquid column chromatographic techniques permit the measurement of various amines and amino acids (Martin & Cohen, 1973). Although simultaneous estimation of various compounds is achieved, the conventional means of detection by fluorescence is not sensitive enough for the small quantities of amines in biological materials. Moreover, the procedure is time consuming. Recently an advance was made with the advent of high pressure liquid chromatography (HPLC) for detecting NA and DA in the pmole range (Platskey et al. 1977; Keller et al.1976) which has the advantage of speed and greater sensitivity due to the use of electrochemical detectors. A better resolution of catecholamines (i.e. A, NA, DA, DOPA, HVA and other compounds, with comparable sensitivity to HPLC, in less than 10 minutes, has been achieved by reverse phase "soap chromatography" (Knox & Jurand, 1976).

e) Gas chromatographic and mass spectrometric method

The sensitive and specific combination of gas chromatography and mass spectrometry has found little application so far. Though used by various workers for estimating NA, DA, OA, 5-HT (Clark et al 1967; Koroum et al.1972; Martin & Ansel, 1973; McAdoo & Coggeshall, 1976), it has not yet been utilized to its fullest capacity mainly due to inherent problems of electron capture.

f) Radioenzymic analysis

Recently some very sensitive and specific radioenzymic assays have been developed for estimating catecholamines in biological fluids and tissues (Saelens et al.1967; Nikodjviez et al.1969; Cuello et al.1973; Fry, 1974; Palkovitz, 1974).

These assays have the limitation that only one compound may be estimated at a time from one sample.

g) Dansylation reaction

The application of the dansylation reaction followed by TLC for the determination of amino compounds, first introduced by Seiler & Weichmann (1966, 1968) for the estimation of amines and amino acids, allows the simultaneous estimation of as many as 33 compounds in very low concentration in a single biological sample (Osborne, 1973). The quantitative accuracy of the procedure has been greatly enhanced by the use of double isotope labelling (Brown & Perham, 1973; Joseph & Haliday, 1975; Recasense et al.1977).

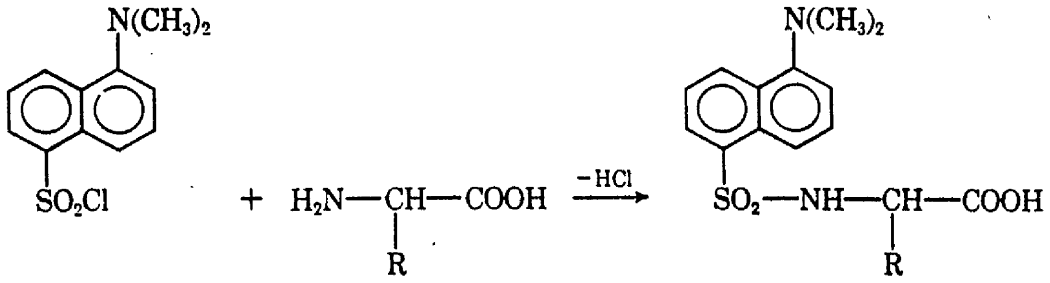
To conclude the general survey,the specificities and sensitivities of various analytical procedures commonly in use are summarised in Table 3. It can clearly be observed that most sensitive procedures are specific for a limited number of compounds. In an investigation involving so little tissue as is available from the insect nervous system, one needs a method which covers a broad spectrum of compounds without sacrificing sensitivity. Preferably it should also be economical in terms of cost per assay. Dansylation seems to fulfil all these criteria. This procedure involving the formation of dansyl derivatives, their separation by TLC and estimation by double labelling is more sensitive than fluorimetric methods, less expensive than those using mass spectrophotometric and radioenzymatic methods, and it has

Table 3. Analytical procedures commonly in use to estimate amines and amino acids in nervous tissue

	Methods	Sensitivity		Specificity	References
1	Bioassay	5-500 p-mole	1-100 ng	Catecholamines (CAs)	Cooper et al. (1976)
2	Fluorometric	0.1-1 n-mole	25-250 pg	A, DA, NA, NM	Laverty & Taylor (1968)
		50 p-mole	10 ng	5-HT, 5-HIAA	Curzon & Green (1970)
3	Radioenzymic	5 p-mole	1 pg	A, NA	Peular & Johnson (1977)
		30 p-mole	6 pg	DA	Peular & Johnson (1977)
		f-mole		A, DA, NA	DA Prada & Zurcher (1976)
		10 f-mole	(1.7) pg	NA	Hajendahl et al (1979)
		5 f-mole	(0.9) pg	A	Hajendahl et al (1979)
			10-20 pg	5-HT	Boiream et al (1976)
4	Liquid column chromatography (Ion exchange)	p-mole		Arg. agmatine, cadavarine, DA, diamino propane, ethanolamine, 5,6,dihydroxytryptamine, 5-HT, HTP, His, Histamine, 5-methoxytryptamine, TP, hexamethylene diamine, NA, LYS, PUT, phenylalanine, spermidine, spermine	Villanueva and Adlakha (1978)
		p-mole		ACh. Ch. DA, NA, 5-HT, 5-HTP, 5-HIAA, Try, Tyr, Gly, Asp, Glu, Ala and Gaba	Smith et al (1975)
5	HPLC	0.5-1 p-mole	75-150 pg	DA, DOPA, DOPAC, HVA	Westerink & Mulder (1981)
		3 p-mole	500 pg	NA	Westerink & Mulder (1981)
		50 f-mole	35 pg	A, DA, NA	Hajendahl et al (1979)
		p-mole		DA, NA	Plotsky et al (1977)
6	Gas chromatographic	10-100 f-mole		DA 5-HT, Gaba, NA, OA	McAdoo & Coggeshall (1976)
7	Dansylation (double label)	20 p-mole		Lys, HIS, ARG, ASP, Thr, Ser, Glu, Pro, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe	Brown & Perham (1973)
		<10 p-mole		a wide range of amino acids	Jabbar and Strang (1979)
		0.1-1 p-mole		TYR, A, DA, DOPA, 5-HT, NA, NMN	Recasense et al (1977)
	(Single label)	<1 p-mole		a number of amino acids	Weise & Oken (1978)
		5 p-mole		amino acids, monoamines, polyamines, peptides	Seiler (1968)

the great advantage of permitting the measurement in the same brain tissue of many amines and amino acids which may be of interest. Coupled with autoradiography it is quite useful for obtaining qualitative and comprehensive picture of otherwise new and undetectable amino compounds.

The various steps of the reaction sequences are shown in Fig. 13. Over the past decade this procedure has been greatly improved to suit the analysis of nervous tissue of a number of invertebrates. It has been successfully applied in the analysis of various tissue of Helix pomatia (Osborne et al 1971), nervous tissue of cockroach (Osborne & Neuhoff, 1974), CNS of annelid, Lumbricus terrestris (Robertson & Osborne, 1979), CNS of spiders (Meyer et al.1980), CNS of snail (Dolezalova et al 1973). Osborne (1973, 1978) has made extensive use of this technique to study the serotonin (5-HT) at cellular levels in the giant serotonergic cells (GSC's) of Helix.



Dansyl-chloride Amino acid Dans-amino acid

Fig 13. The reaction of dansyl-chloride with amino acids.

1.11 Aims of Present Investigations

The constant aim behind the work in this thesis was to examine in a comprehensive and quantitative way the relationship between the function of the insect CNS and the amino compounds occuring within it, whether by biosynthesis in the tissue, or by absorption from haemolymph.

The main analytical procedure chosen to pursue this overall aim was that of formation of dansyl derivatives of these amino compounds. The method seemed to combine simplicity, sensitivity and cheapness. It also offered the possibility of discovering any novel compound which might be produced by the nervous system under some pathogenic condition.

As it was initially intended to investigate both amines and amino acids a great deal of effort was put into clearly separating all the compounds, which on the basis of previous information might be encountered. Previously used methods were compared and if possible improved.

It was hoped also to apply the method in a much more rigorously quantitative way than previously had been the case in similar studies. This preparatory and essentially empirical work was largely successful, but it became apparent that practical considerations made it impossible to examine in detail the amines of the insect CNS. Their concentrations in the tissue were too low, and the cost of the labelled standards too high, to allow further quantitative work with them. Consequently, the emphasis thereafter was on the amino acids.

A prime intention was to follow reports that stress produced neuronal amino compounds in the CNS from which they were released into the haemolymph. Careful studies *in vitro* were made with a view to determine the normal release of amino compounds from the nervous

system, so that any abnormal release might be observed.

The effects of stress produced by insecticides and enforced activity were then tested in vitro and in vivo. It was hoped to confirm previous reports of neuroactive compounds in the blood of stressed insects. The main insect used was the desert locust, but some of the work reported was on cockroach, partly because many of the previous reports relate to this insect. All the electrophysiological work, too, was done with CNS from cockroach. The main reason for this was that the author experienced a severe allergic reaction to locusts. The lengthy contact with the insects required for electrophysiological work made it impossible to use the locust for this procedure.

The preliminary work with the amino acids in vitro posed a few interesting questions about the metabolism of amino acids under what might be considered normal aerobic and anaerobic conditions.

Another aspect of the study, therefore, was to look at the question of the role of amino acids in the general function, and particularly energy metabolism of the thoracic ganglia of locust. Activities of important enzymes were investigated and the rate of uptake of O_2 by both whole tissue and mitochondrial preparation estimated.

Alanine is an amino acid whose accumulation is associated with both aerobic and anaerobic metabolism. It was with a view to find the reasons for its accumulation under anaerobic conditions, that such conditions were imposed on the tissue. It was hoped that by estimating the concentrations of various metabolites, we could find out why alanine accumulated, and also would help in determining the importance of shuttle mechanisms for transporting reducing power across the mitochondrial membranes.

It was stated previously that after preliminary studies no further interest was taken in amines. An exception to this was putrescine (a diamine) which was found in high concentration in the locust haemolymph and CNS. The possible metabolism of putrescine in the thoracic ganglia was investigated by several procedures, with the aim to study the amine as a potential precursor of Gaba.

Behind all the experimentation used to pursue the various aims, lay the general tenet that results obtained *in vivo* should as much as possible be reproduced *in vitro*.

SECTION TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Radiochemicals

All the radiochemicals specified below were obtained from Amersham International Ltd (The Radiochemical Centre), Amersham, England.

[U-^{14}C] - protein hydrolyzate, specific activity (S.A) 57 m Ci/milliatom carbon.

A typical analysis has:

L-Alanine	9.5%	L-lysine	4.8%
L-Arginine	6.9%	L-Methionine	0.6%
L-Aspartic acid	10.0%	L-Phenylalanine	7.4%
L-Glutamic acid	9.0%	L-Proline	5.3%
Glycine	5.8%	L-Serine	3.2%
L-Histidine	1.6%	L-Threonine	5.7%
L-leucine	12.7%	L-Tyrosine	5.8%
L-Iso-leucine	5.8%	L-Valine	5.9%

[^{3}H]-Inulin	S.A. 22 Ci/m mol
[1,4-^{14}C]-Putrescine dihydrochloride	S.A 116 m Ci/m mol
[N-methyl-^{14}C]-Dansyl Chloride	S.A. 43.9 m Ci/m mol
[G-^{3}H]-Dansyl Chloride	S.A. 13.5 Ci/m mol
L-[U-^{14}C]-Glutamine	S.A. 42 m Ci/m mol
[Ethylamine-2-^{14}C]-Dopamine hydrochloride	S.A. 60 m Ci/m mol
4-Amino-n[U-^{14}C]-butyric acid	S.A. 224 m Ci/m mol
[U-^{14}C]-Taurine	S.A. 113 m Ci/m mol
[^{35}S]-Methionine	S.A. 1140 Ci/m mol

2.1.2 Fine Chemicals

The fine chemicals were obtained as specified below:

CHEMICAL	SUPPLIER
Dopamine hydrochloride (DA)	BDH Chemical Co., Poole, England
Dihydroxyphenylalanine (Dopa)	
Dansyl Chloride (Dans-Cl)	
γ-amino butyric acid (Gaba)	
Taurine (Tau)	
Succinic acid	
Fumaric acid	
Glycerol	
Folin and Ciocalteau Reagent	
Hyamine 10-X hydroxide in methanol	
Amino oxyacetic acid (AOAA)	Sigma Chemical Co., London, England.
Iso-amylamine (IAA)	
Tyramine (TA)	
Putrescine dihydrochloride	
Spermine tetra hydrochloride	
Dimethylsulphoxide (DMSO)	
Glycerol-3-phosphate (α-GP)	
Bovine serum albumen	
Octopamine hydrochloride (OA)	Aldrich Chemical Co., Gillingham, Dorset, England.

CHEMICAL	SUPPLIER
5-hydroxytryptamine(5-HT)	Ralph N. Emanuel Ltd, Wembley, England.
Spermine	Koch Light Labs. Ltd., Cambridge, England.
Ninhydrin	
2,5-diphenyloxazole (PPO)	
1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP)	
Methylcellosolve	May & Baker Ltd., Dagenham, England.
L-Malic acid	Fluka A.G., Glossop, Derbyshire, England.
TritonX-100	Rohin and Haar, Croydon, England.

N-acetyldopamine, N-acetyloctopamine and N-acetyltyramine were synthesized by the method of Anderson (1971) by the reaction of respective amine with acetic anhydride and purifying the product.

CHEMICAL	SUPPLIER
Adenosine-triphosphate (ATP)	Boehringer-Mannheim Corporation, London, England.
Adenosine-diphosphate (ADP)	
Nicotinamide-Adenine dinucleotide (NAD^+)	
Nicotinamide adenine dinucleotide (reduced) [NADH]	
Nicotinamide adenine dinucleotide phosphate ($NADP^+$)	
Coenzyme A (Co-A)	
Guanosine triphosphate (GTP)	

2.1.3 Enzymes

Lactate dehydrogenase	E.C. 1.1.1.27	Boehringer-Mannheim Corporation, London, England.
Fumarase	E.C. 4.2.1.2	
Malate dehydrogenase	E.C. 1.1.1.37	
Pyruvate kinase	E.C. 2.7.1.40	
Succinyl Co A synthetase	E.C. 6.2.1.5	
Glycerol-3-phosphate dehydrogenase	E.C. 1.1.1.8	
Glycerol-kinase	E.C. 2.7.1.30	

2.1.4 Thin-layer chromatographic material

Polygram-Sil G	Layer thickness	0.25mm	Cam-lab, Cambridge
Polyamide	" "	0.25mm	B.D.H., England
Cellulose	" "	0.1mm	E. Merck, Germany

2.1.5 Films for autoradiography

Kodak X-Omate XR P5 13x18 cm	Kodak Ltd., England

2.1.6 Electrical Apparatus

Specification	Model	Supplier
Preamplifier	Type A101	Isleworth Electronics, Bucks, England
Cathode ray Oscilloscope	Telequipment-D66A	Tektronix, U.K. Ltd, Southgate, England
Electrical stimulator	DS9	Digitimer Ltd,
Spike processor	D130	Herts, England
Pen recorder	PM 8220	Phillips Electrical Co.
Perpex peristaltic pump	AB 516125	LKB Products, Sweden.
Casette recorder		Sanyo, Japan.
Continuous recording Camera	PC 2A	Nikon Kohden, Kogyo Co. Ltd, Japan.
Soniprobe	Type 7530A	Dawe Instruments Ltd., England.

2.1.7 Insecticides

The insecticides shown in Fig. 14 (except Nicotine) were the kind gift of Dr. J.F. Donnellan, Shell Bioscience Laboratory, Sittingbourne, Kent, England.

2.2 Preparation of standard solutions

2.2.1 Amino Acid Solution

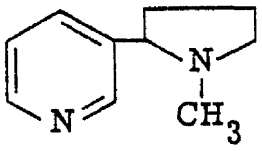
The amino acid mixture used in qualitative and quantitative estimates by dansylation was prepared in 0.05M $NaHCO_3$ (Osborne, 1973) so that each amino acid had a final concentration of $\sim 10^{-3}$M. This is referred to as "STANDARD AMINO ACID MIXTURE" in the text.. The mixture contained; tyrosine, proline, valine, γ-aminobutyric acid, alanine, glycine, glutamic acid, aspartic acid, iso-leucine, leucine, phenylalanine, methionine, histidine, arginine, lysine, tryptophan and taurine.

2.2.2 Amine and N-acetylamine solutions

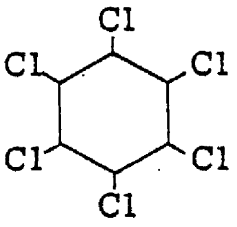
Mixtures of dopamine, octopamine, tyramine, N-acetyldopamine, N-acetyltyramine and N-acetyloctopamine were prepared in 0.05M $NaHCO_3$ and traces of sodium bisulfite were added to prevent oxidation of dopamine and N-acetyldopamine (Chaulis, 1967). Whether the amines were separate or mixed the concentration of each was kept at 10^{-3}M. The solution of dopamine, octopamine and tyramine is referred to as "AMINE MIXTURE" and that of N-acetyl-dopamine, N-acetyloctopamine and N-acetyltyramine as "N-ACETYLAMINE MIXTURE".

2.2.3 DOPA and 5-HT solutions

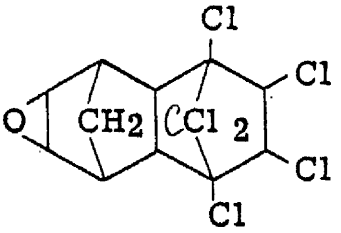
Similarly, 10^{-3}M solutions of these compounds were prepared in 0.05M $NaHCO_3$. Traces of sodium bisulfite were added to solutions of DOPA to prevent oxidation.



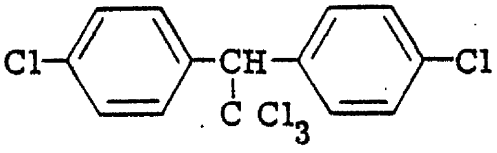
Nicotine



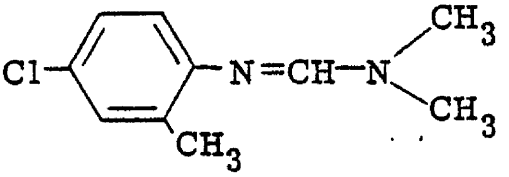
Lindane(γ-BHC)



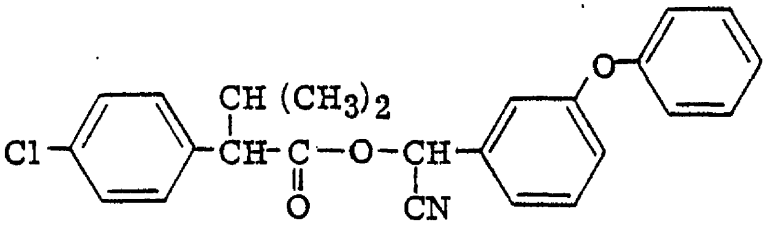
Dieldrin



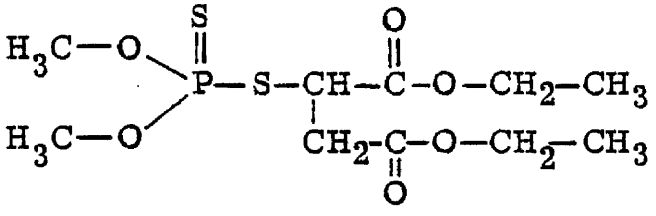
DDT



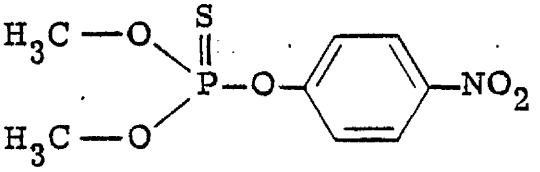
Chlordimeform



Fenvalerate



Malathion



Methyl-Parathion

Fig 14. Molecular structure of insecticides.

All these solutions were kept at $4^{o}C$ while not in use. These solutions were prepared afresh after 10-12 weeks.

2.2.4 Dans-Cl solution

Solutions of Dans-Cl ($1x10^{-3}$ and $5x10^{-3}M$) were prepared in acetone and kept in the dark in sealed vials to prevent evaporation when not in use. Similarly solutions ($10^{-3}M$) of radioactive Dans-Cl (either [^{3}H]- or [^{14}C]-Dans-Cl) were prepared. As the manufacturer supplied the compound in benzene, this was evaporated in a stream of warm air, and an appropriate amount of unlabelled Dans-Cl solution in acetone was added to yield a final concentration of $10^{-3}M$ Dans-Cl with a specific activity of 500μ Ci/ml for [^{3}H]-Dans-Cl, and 100μ Ci/ml for [^{14}C]-Dans-Cl.

2.2.5 Stains for electropherograms

a. Ninhydrin reagent (Block et al 1955) was sprayed to stain the amino acids, while

b. Sulphanilic acid diazo dye (Sandler and Ruthven, 1969) was sprayed to make visible the phenolic compounds i.e. catecholamines and their N-acetylated derivatives.

2.3 Insects

2.3.1 Locusts : Schistocerca americana gregaria were purchased from the Larujon Locust Suppliers, c/o Welsh Mountain Zoo, Colwyn Bay, North Wales. They were kept in cages at $30^{o}C$ on a wheat bran diet.

2.3.2 Cockroaches : Periplaneta americana, the kind gift of Dr. A. Lackie, Department of Zoology, Glasgow University, were bred and reared in the laboratory in the above department. They were fed on a diet of cereal cakes.

2.4 Methods

2.4.1 Weight of Locusts. The locusts were weighed in a pan using micro-balance type BE 22 supplied by Mettler Instruments, Greifense, Zurich, Switzerland. To overcome the variation in weighing due to body movement of the insects they were pre-cooled at -20°C for 15-20 minutes. Subsequently, slight exposure to the heat from a 60W electrical bulb restored their normal activity. Insects so treated readily recovered and showed no perceptible ill effects.

2.4.2 Estimation of volume of haemolymph

The volume of haemolymph in the living insects was estimated by the indirect isotope dilution procedure of Loughton and Tobe (1969). A known amount (1μ Ci) of aqueous solution of radiolabelled [^{3}H]-inulin was injected into the thoracic cavity in volume of 10μl. 30 minutes after the injection, when the inulin had become distributed throughout the body cavity, the insect was either bled (see following section) or the thorax was punctured and samples of haemolymph (10-50μl) were taken from the ventral side near the hindmost leg. The radioactivity was measured in four 1μl samples of the haemolymph. The total haemolymph volume was worked out from the dilution of radioisotope.

2.4.3 Collection of haemolymph

Locust haemolymph was collected in larger volumes by making an incision round the neck, cutting off the end of the abdomen and gently eviscerating the insect by detaching the head from the body.

The decapitated and eviscerated locusts were placed in conical, 15ml centrifuge tubes, thorax downwards. Centrifugation of the body at 5-10g for 10 minutes was sufficient to drain off the haemolymph (Strang and Clement, 1980). In this way 50-100µl of haemolymph could be obtained from each locust. Any sample contaminated with gut contents was discarded. The haemolymph was kept on ice and deproteinized by adding an equal volume of acetone as soon as possible after its collection to avoid the oxidation and enzymic degradation of metabolites.

2.4.4 Preparation of nervous tissue for in vitro studies

Much of the work in this study was carried out on the cerebral, meso- and meta-thoracic ganglia of adult desert locust, without making any discrimination between male and female insects unless otherwise mentioned. Locusts were immobilized at -20°C for 15-20 minutes and the ganglia were dissected out under a stereomicroscope, by removing the cutical from the ventral side of the thorax. The cerebral ganglia were dissected by making longitudinal incision through the centre and sides of the head and exposing the ganglia. After freeing them from adhering tracheal tissue, the ganglia were taken out and kept in ice cold insect saline (see Table 4) prior to incubation, or, alternatively, immediately frozen in tubes kept on dry ice prior to the measurement of endogenous concentrations of metabolites.

In the electrophysiological studies which employed the abdominal nerve cord of cockroach the entire abdominal nerve cord was dissected out of the insect, by removing the cutical from the dorsal surface and freed from the tracheal material. The stereomicroscope was used throughout the dissection. The nerve cord was transferred to a small dish containing 0.5ml insect saline kept in electrophysiological chambers at room temperature.

2.4.5 Composition of incubation medium

The insect saline used in these experiments was that of Hoyle (1953) except that $MgCl_2$ was omitted and $NaHCO_3$ replaced by a phosphate buffering system (Clement, 1979). The saline contained approximately the same ion concentrations as present in locust haemolymph. Glucose was added as an exogenous energy source and sucrose was occasionally included to adjust the osmolarity to approximate to that of the haemolymph. The composition of various media employed in the study is presented in Table 4.

The stock solutions of salines were kept at $4^{o}C$ while not in use. Carbohydrates were added immediately prior to the experiment. No sterilization of the salines was carried because it was considered unnecessary for the short time course of the reported experiments.

2.4.6 Procedures for incubation of the nervous tissue

2.4.6.1 Incubation to follow the release of amino compounds

Three pairs of thoracic ganglia were incubated at $37^{o}C$ in 0.5ml of fully oxygenated saline (unless otherwise specified) for 30-60 minutes. In preliminary experiments to determine how release varied with time, samples of 50µl were withdrawn at 5,10,15,20,30,45 and 60 minutes of incubation. The volume of incubation medium was made up to 0.5ml after each removal of the sample so as to maintain a constant volume. At the end of the experiment both the tissue (finally washed rapidly three times in fresh saline), and the incubation medium were analyzed quantitatively for the presence of various amino compounds. In calculating totals, corrections were made for the samples removed.

2.4.6.2 Incubation in aerobic and anaerobic conditions

To ensure that ganglia have the maximum possible supply of oxygen in vitro, the incubation medium was kept saturated with oxygen by

Table 4. Composition of the media used for incubation of nervous tissue from locust (Schistocerca americana gregaria).

Component	Insect saline*	Iso-osmolal insect saline	Insect saline + KCN	Insect saline + glucose	Iso-osmola insect saline + amino acid
	mM	mM	mM	mM	mM
KCl	10	10	10	10	10
NaCl	140	140	140	140	140
Na_2HPO_4	6	3	6	6	3
NaH_2PO_4	4	2	4	4	2
$CaCl_2$	2	2	2	2	2
Glucose	-	10	-	10	10
Sucrose	-	100	-	-	100
KCN	-	-	10	-	-
Amino acids	-	-	-	-	†

* Reference: Hoyle, (1953).

† Amino acid composition (mM) is as follows: Proline 10; Valine 2; Alanine 1.5; Glycine 10; Glutamic acid 0.5; Aspartic acid 1; Iso-leucine 1; Leucine 1; Phenylalanine 1.5; and Tyrosine 1.5. The pH of the media was adjusted to 6.8.

bubbling the gas throughout the experiment (Clement and Strang, 1978). On the other hand, when anaerobic conditions were required, either the reaction tubes were kept in sealed reaction vessels in which the air had been replaced by N_2 or, alternatively, N_2 gas was bubbled continuously through the medium containing the nervous tissue.

2.4.6.3 Incubations with drugs and insecticides

The insecticides were applied to the ganglia by incubation in 10^{-3}M solution of insecticides in iso-osmotic saline containing 2% ethanol or acetone (This concentration of organic solvent was adequate to dissolve the rather hydrophobic insecticides). Controls containing ethanol or acetone was carried through the same procedure.

Similarly other drugs were dissolved in appropriate concentrations in the iso-osmotic saline, and ganglia incubated as described in Section 2.4.6.2.

2.4.6.4 Incubation with putrescine

The possible formation of Gaba from putrescine was followed by incubating a pair of locust thoracic ganglia at 37°C in 20µl of iso-osmotic saline containing 10mM putrescine. The metabolism of putrescine was also studied by incubating the homogenate of one pair of the thoracic ganglia in 10mM putrescine in the presence and absence of 10mM NAD+ and 10mM amino oxyacetic acid (AOAA).

2.4.6.5 Incubation with [^{3}H]-tyrosine

To study the biosynthesis of mono-amines, one pair of thoracic ganglia was incubated at 37°C in 10µl insect saline containing 3µ Ci [^{3}H]-tyrosine. The incubations were carried out at ambient O_2 concentration because saturation of medium by bubbling oxygen through such a small volume resulted in evaporation of the media.

2.4.7 Studies With live insects

2.4.7.1 Metabolism of [^{14}C] - Putrescine in vivo

The metabolism of putrescine in living insects was followed by injecting 20µl of [^{14}C] - putrescine (1µCi) in water into the thoracic cavity of locusts with the aid of 25µl Hamilton Syringe. The insects thereafter were kept at room temperature (25°C) in glass containers. The haemolymph and nervous tissue extracted from different insects either 6 or 26 hours after injection was analyzed for the presence of various radiolabelled metabolites.

2.4.7.2 Metabolism of [^{35}S] - methionine in vivo

In order to investigate whether the insects are able to synthesize taurine from methionine, 5µl [^{35}S] - methionine (30µCi) were injected into locusts. The insects were killed after 5 or 24 hours, and nervous tissue, fat body and haemolymph removed for analysis as given in Section 2.4.12.4.c.

2.4.7.3 Treatment of insects with insecticides in vivo

One mg of DDT and 100µg of other insecticides, each dissolved in 10µl DMSO and 10µl of a 10^{-4}M aqueous solution of nicotine were injected into the thoracic cavity of locust and cockroaches using 25µl Hamilton Syringe. As a control 10µl DMSO, without any drug, were injected into the other insects. The insects were kept at room temperature for 3-6 hours. The haemolymph and nervous tissue were removed and analyzed for amino compounds as given in Section 2.4.9.3.

2.4.7.4 Subjection of insects to physiological stress

The insects were subjected to mechanical stress by forcing them to walk continuously, following the procedure of Cook et al. (1969). A glass jar containing the insects was placed on its side and continuously rotated for a period of 8-24 hours. In this way the insectswere forced to walk continually to keep from falling over. A considerable dessication was observed in the locusts after 24 hours of this treatment. At the end of experiment the haemolymph and nervous tissue were analyzed for the presence of amino compounds. Section 2.4.9.3.

2.4.8 Extraction of metabolites from nervous tissue

2.4.8.1 Amino acids and amines

The procedure of Osborne (1973) was followed for the extraction of amino acids and amines from the nervous tissue and haemolymph.

Nervous tissue (2^{-10}mg fresh weight) was homogenized in 20-100µl of extracting reagent in a thick walled glass tube in ice. The glass tube was 50mm in length and of 3mm internal diameter. The homogenization was achieved with the aid of a dental drill, fitted with a "teflon" pestel. A variety of reagents were tried for the extraction of amino compounds.

a) 0.1M HCl

The use of 0.1M HCl as extracting agent and protein precipitant did not yield satisfactory results because of the difficulty encountered in the homogenization. The tissue often became like a paste at the bottom of the homogenizing tube.

b) 0.3M $HClO_4$

Use of 0.3M $HClO_4$ resulted in a better homogenization and precipitated the proteins at the same time. But as the quantitation with the dansylation procedure had to be carried out at an alkaline pH, it was necessary to remove the acid. Neutralization of $HClO_4$ with $KHCO_3$ resulted in excessive formation of $KClO_4$ which always interferred with the final chromatography of dansyl derivatives on polyamide sheets. Evaporation of $HClO_4$ under reduced pressure does not remove the non-volatile $HClO_4$. Moreover, the $HClO_4$ has oxidizing properties and it could not be used in the analysis of easily oxidizable substances, such as the phenolic amines.

c) Insect saline

Bearing in mind the above-mentioned difficulties, the extraction was carried out in two steps by homogenizing the tissue in insect saline and later precipitating the protein with acetone. This procedure was carried out at a low temperature ($0^{o}C$) to minimize any metabolic changes. The preparation of the nervous tissue has already been given in Section 2.4.4, and further processing of the extract and quantitation of the amino compounds using the dansylation procedure is given in Section 2.4.9.3 and summarized in Figure 15.

NERVOUS TISSUE

2-8 ganglia + 20-100 µl 0.05M Na HCO_3

↓

Homogenized — with dental drill for 3 minutes → (5µl) Protein estimation

↓ (1µl) ↑

Centrifuged — at 8000g for 15 minutes ← (20µl) (Haemolymph)

↓

Supernatant — treated with equal volume of acetone

↓

Precipitation — of proteins completed by keeping at -20°C for 30 minutes

↓

Centrifuged — at 8000g for 15 minutes ← (50µl) Incubation medium

↓

Supernatant — (5µl from tissue extract and incubation medium and 1µl haemolymph)

↓

taken to dryness at reduced pressure

↓

Residue dissolved in 4µl 0.05M $NaHCO_3$

(For quantitative estimations ^{14}C-standards added)

↓

Reacted with 4µl 5mM Dans-Cl

(1 or 5mM ^{3}H- or ^{14}C-Dans-Cl wherever mentioned)

↓

Reaction completed by keeping at 37°C for 30 minutes

↓

Sample (1µl) chromatographed on polyamide sheets (5x5cm)

↓

Various derivatives analyzed by Scintillation counting or autoradiography

Fig. 15 - Flow diagram summarizing the extraction and estimation of amino compounds from nervous tissue, haemolymph and incubation medium

2.4.8.2 Intermediates of energy metabolism

The extraction of various metabolites was achieved by following the procedure of Bergmeyer (1974). After incubation in appropriate media three pairs of thoracic ganglia were homogenized in 100µl 3M perchloric acid. After centrifugation for 15 minutes at 10,000g 100µl of ice-cold distilled water was added to the supernatant to increase the volume of extract. 30 minutes later 200µl of 2M $KHCO_3$ (the volume found suitable after studying various proportions) was mixed to neutralize the acid. The contents were agitated for 5-10 minutes to remove the CO_2 and kept in ice for one hour. Centrifugation for 15 minutes was carried out to remove the potassium perchlorate. The supernatant was divided into 3 portions of 100µl each for the assay of 1) succinic acid, 2) malic and fumaric acid (both in same sample), and 3) glycerol-3-phosphate. The estimations were carried out using the enzymatic methods of Bergmeyer (1974) as detailed in Section 2.4.12.6.

2.4.8.3 Metabolites of [^{14}C] - putrescine

The thoracic ganglia dissected from locust (as described in Section 2.4.6.4) were homogenized using a dental drill. The contents were centrifuged and the supernatant and haemolymph were deproteinized with an equal volume of acetone. The extract was subjected to electrophoresis for the resolution of radiolabelled metabolites using the method of Seiler and Al-Therib (1974) as described in Section 2.4.9.1.b.

2.4.8.4 Metabolites of [^{3}H] - tyrosine

At the end of the incubation of nervous tissue described in Section 2.4.6.5, 50µl of 0.3M $HClO_4$ were added to stop the reaction by denaturing the enzymic proteins. The tissue was homogenized and the contents centrifuged for 10 minutes at 10,000g. The clear supernatant was applied to cellulose sheets for further separation of metabolites using electrophoresis as given in Section 2.4.9.1.a or loaded onto a column for resolution by means of HPLC (Section 2.4.9.5).

2.4.9 Separation of metabolites of the nervous tissue

2.4.9.1 Electrophoresis

a) Amino acids and amines

Samples (10µl) of deproteinized supernatant were applied to 10 x 10 cm plastic-backed cellulose sheets. They were then subjected to electrophoresis for 15-30 minutes in a 0.75M formic acid buffer of pH 2.0 (HVE Guide, Savant Instruments, New York), at a voltage of 400v and a current of 0.8mA/cm using Shandon power pack no. SAE 2761, supplied by Shandon Southern Ltd.

Standards run in parallel with the tissue extract were then stained with ninhydrin reagent followed by sulfanilic acid dye to make visible respectively the compounds with primary amino groups and acetylated derivatives of phenolic amines.

Corresponding unstained portions of the layer were then scraped off into 2ml plastic centrifuge tubes for elution of tissue solutes with 200µl acetone/0.1N HCl (9:1 v/v). After the elution, the extracts were taken to dryness under reduced pressure. Residues

were redissolved in 5µl 0.05M $NaHCO_3$ for further analysis by formation of dansyl derivatives.

b) Polyamines

The procedure of Seiler and Al-Therib (1974) was followed for the resolution of polyamines. The deproteinized extracts of tissue and haemolymph were applied to silica gel (5 x 20cm) plates. The plates were wetted by brushing with a pyridine/acetic acid/H_2O (5:5:390 v/v/v) buffer of pH 4.8. Electrophoretic separation was achieved by using the same buffer at a constant current of 2mA/cm and a voltage of 450v for 45 minutes.

After electrophoresis the plates were autoradiographed.

2.4.9.2 Phase separation (Seiler and Weichmann, 1965)

Standard solutions of amines and amino acids and deproteinized extracts of the nervous tissue of locust (50µl) were reacted with an equal volume (50µl) of 1mM Dans-Cl. After completion of the reaction, 100µl of ethyl acetate was added to the mixture and the contents were kept for 1 hour at room temperature. The upper ethyl acetate layer was transferred to another tube and dried under the stream of air. The residue was redissolved in 10µl of acetone and 1µl portions applied to the polyamide sheets for chromatography. Acid (100µl of 0.1M HCl) was added to lower the pH of the aqueous phase, which was then extracted with 200µl of ethyl acetate. The Dans-amino acids were further processed as described above for Dans-amines. For a quantitative check on the recovery of separated Dans-amines, either the standard cold amines were reacted with

[^{3}H] -Dans-Cl or [^{3}H]- dopamine was reacted with the unlabelled Dans-Cl and carried out through the same extraction procedure.

2.4.9.3 Thin Layer Chromatography of Dansyl Derivatives on Polyamide Layers

The unknown compounds and standards (where appropriate, labelled with ^{14}C) were dansylated with [^{3}H]-Dans-Cl according to a modification to the method of Brown and Perham (1973). Samples (1µl) of the reaction mixture were then chromatographed in two dimensions on polyamide sheets (5 x 5cm). The usual solvents (unless otherwise stated) for the first and second dimension were 3% formic acid and toluene/acetic acid (9:1 v/v) respectively (Seiler, 1970).

2.4.9.4 Ion-exchange column chromatography

When sufficient material was available the separation of amino acids from the haemolymph and nervous tissue was achieved by running 50µl of deproteinized haemolymph and extracts of nervous tissue through an amino acid analyzer, type JLC-5AH, supplied by Japan-Electron Optics, Co. Ltd., Tokyo. A typical trace of standard amino acids is shown in Fig. 16 to illustrate the separation achieved.

2.4.9.5 High performance liquid chromatography (HPLC)

The metabolites of [^{3}H]- tyrosine were separated by the procedure of Mir (1981). The deproteinized supernatant was injected onto a "partisil-10 ODS" column (Whatman Ltd., 25cm x 4.6mm id.), protected by a "Copell-ODS" guard column (Whatman Ltd., 6cm x 4.6mm id.), via a six part rotary injection valve (Model 7120, Rheodyne Instruments Ltd., U.S.A.) filled with a 100µl sample loop. Separation of metabolites

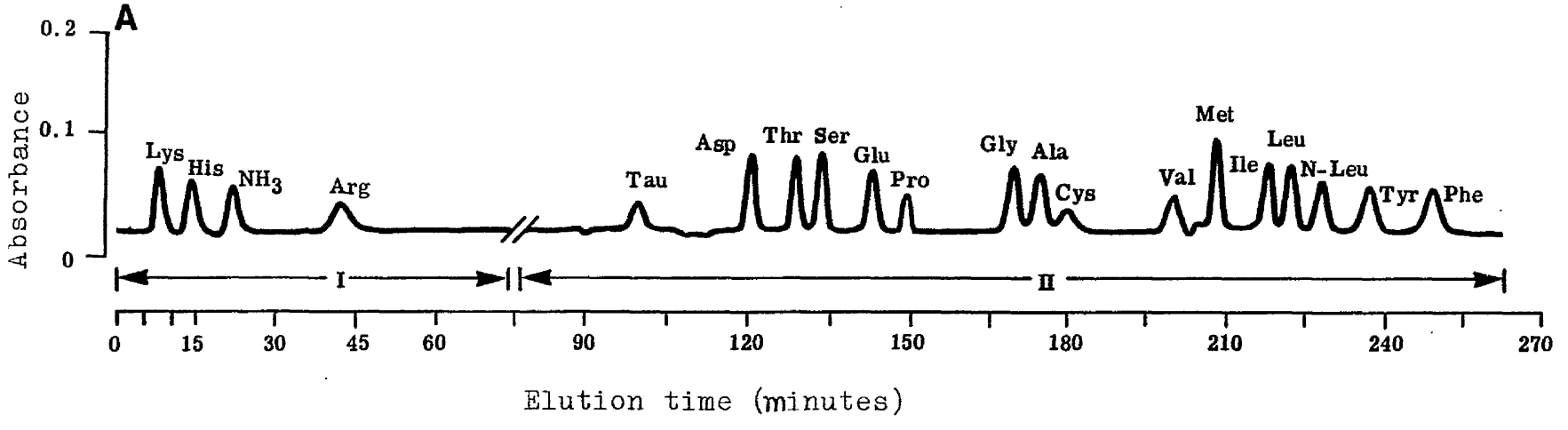


Figure 16 Resolution of standard amino acids by liquid column chromatography. Separation was achieved on automated amino acid analyzer using cation exchange (I) short column at pH 5.25 and (II) long column at pH 3.30 and 4.25.

was achieved by using ion pair, reverse phase chromatography (Knox and Jurand, 1976), with a modified mobile phase of methanol/water/cH_2SO_4/SLS (20:80:0.40:0.12; v/v/v/w). The mobile phase was filtered through millipore filters (0.47μm pore size) before use continuously degassed by helium and delivered at a constant flow rate of 1.5ml min^{-1}, using an LC 3-XP pump (Pye Unicam Ltd., Cambridge, U.K.). The U.V. absorbance of the eluate was monitored at 280 nm with an LC-UV detector (Pye Unicam Ltd.). Samples were collected manually as each separate U.V. absorbing peak (Fig. 17). The radioactivity in 0.5ml portions of each sample was measured by liquid scintillation spectrophotometry.

2.4.10 Determination of Radioactivity by Liquid Scintillation Spectrometry.

After resolution of the Dans-derivatives of amino compounds by thin-layer chromatography (Section 2.4.9.3), the spots were eluted into 0.3ml hyamine hydroxide for 24 hours, and the radioactivity was measured by liquid scintillation spectrometry in 3ml of toluene scintillant containing PPO (8g/l) and POPOP (0.8g/l) on Philip scintillation counter programmed to determine ^{3}H and ^{14}C separately in same sample. Radioactivity associated with the metabolites of [^{35}S] - methionine and [^{3}H] -tyrosine was measured by adding 5ml toluene/triton x-100 (2:1/v/v) containing PPO (5g/l) and POPOP (0.5g/l) as scintillant (Turner, 1968) to samples (1ml) of eluted fractions of ion-exchange column.

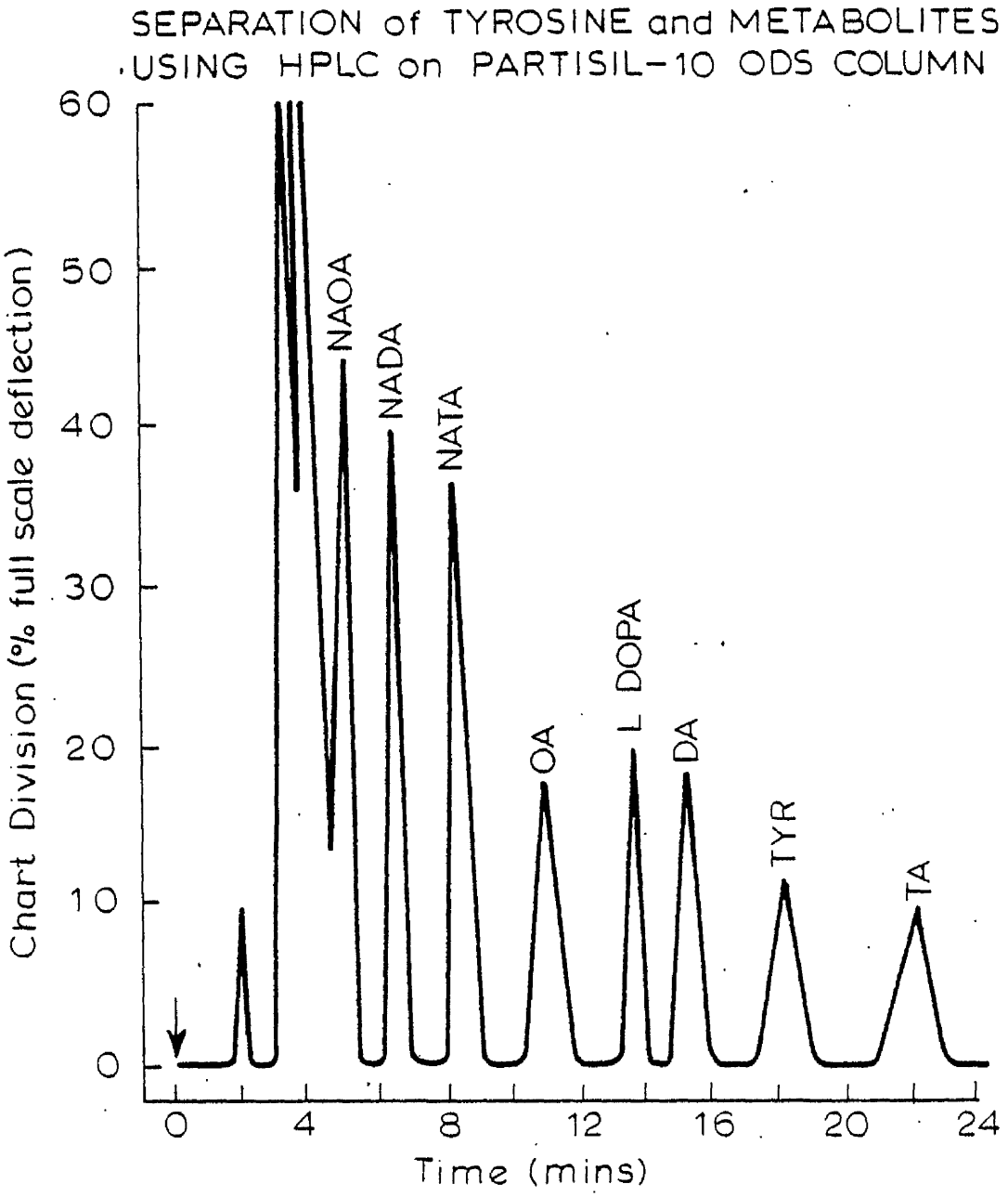


Fig.17 Separation of TYR and metabolites added as carriers to $HClO_4$ extract of 4 ganglia on Partisil-10 ODS column using HPLC. Sensitivity 0.04 absorbance units full scale deflection (a.u.f.s), 20mV chart recorder: flow rate 1.5ml/min; pressure drop 2247 psi. Solvent, methanol,/ water,/ c.H_2SO_4 / sodium lauryl sulphate (20:80:0.04:0.12; v/v/v/w). 2μg N-acetyloctopamine (NAOA), N-acetyl dopamine (NADA), N-acetyl tyramine (NATA), 4μg octopamine (OA), L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), tyrosine (TYR) and tyramine (TA).

(After Mir, 1981)

2.4.11 Autoradiography of [^{14}C] -Dans-derivatives

Thin-layer polyamide chromatograms of the [^{14}C]-Dans-derivatives of the extract of nervous tissue, haemolymph or incubation medium were placed facing the photographic film between two glass plates firmly held together with powerful paper clips, to ensure close contact, and wrapped in tin foil to exclude light. The whole process was carried out in subdued red light. The films were developed after an exposure of 60-72 hours. Silica sheets (containing metabolites of [^{14}C] -putrescine) after electrophoresis were autoradiographed in a similar way.

2.4.12 Quantitative estimations

2.4.12.1 Estimation of protein

The nervous tissue (1-3 pairs of ganglia) was homogenized in 40-100 µl insect saline and 5-10 µl were transferred to a tube containing distilled water and made up to a final volume of 50 µl. The protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin (B.S.A.) as standard. The quantitation was carried out with reference to a calibration curve (Fig. 18) obtained from the known amounts of standard (B.S.A.) protein.

2.4.12.2 Estimation of NH_3

Free ammonia in 10 µl of deproteinized tissue extract or incubation medium was estimated by the chemical method of Henry (1966) and quantitation was carried out with reference to known amount of standard ammonium ions processed similarly.

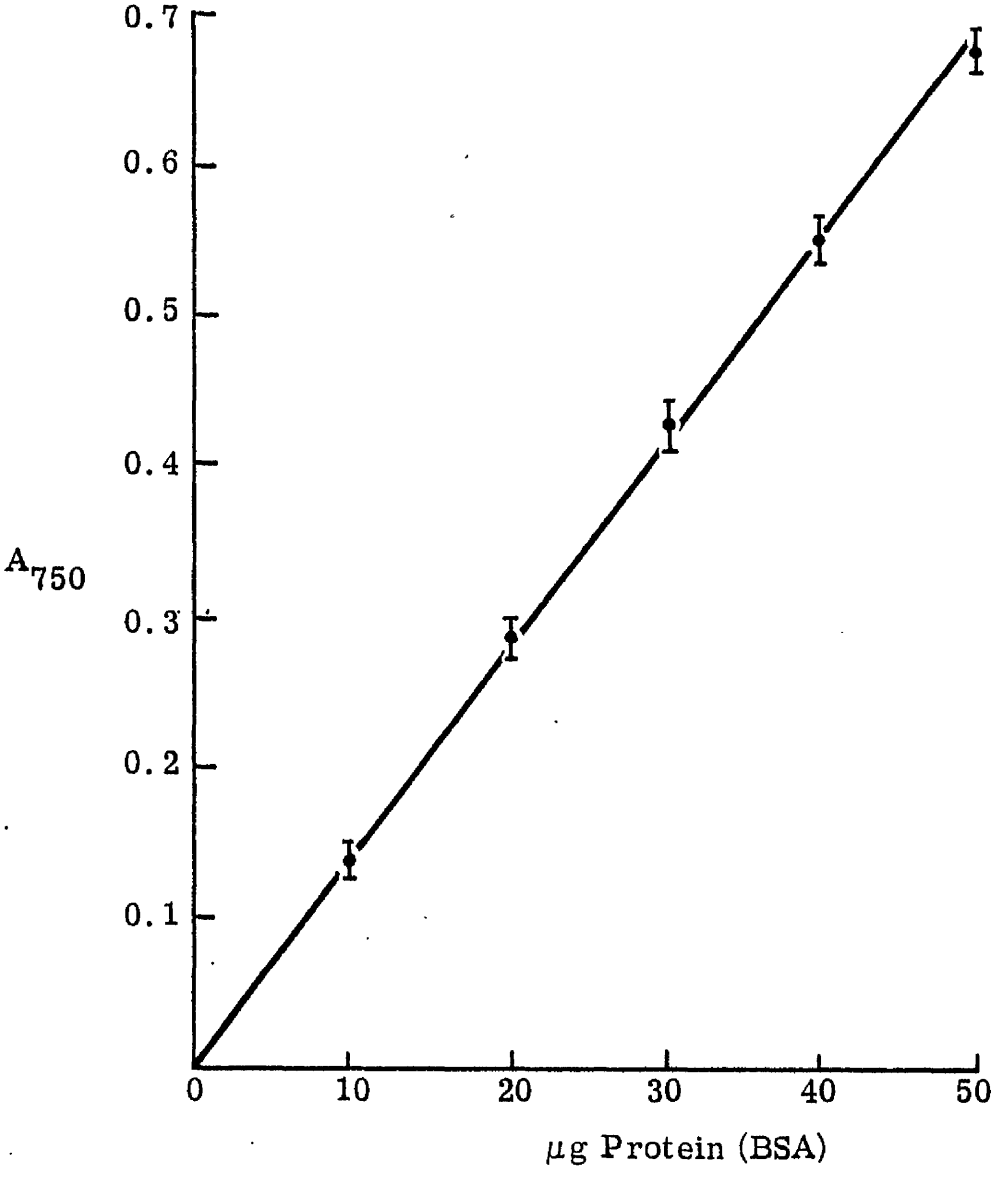


Fig.18. Standard curve for protein estimation. BSA, Bovine Serum Albumin. Values are expressed as means ± S.D. (n = 4).

2.4.12.3 Estimation of the total amino nitrogen

Total amino nitrogen was estimated using the procedure of Yemm and Cocking (1955), whereby the purple colour formed by the reaction of amino acids in acid solution with ninhydrin was measured spectrophotometrically at 570 nm.

2.4.12.4 Estimation of amino compounds in the nervous tissue of locust by:-

a) Dansylation with [^{3}H]-Dans-Cl as the only radiolabel

Quantitation of amino compounds was attempted by following the procedure of Osborne (1973) as described in flow sheet diagram in Section 2.4.8.1. Briefly, the extract of nervous tissue (5µl) was reacted with (5µl) 1mM [^{3}H]-Dans-Cl of specific activity as 500µCi/ml. The dansyl derivatives in 1µl of reaction mixture were resolved on polyamide sheets mixed with the larger amounts of unlabelled Dans-amines and Dans-amino acids as carriers to help in the localization and identification of the spots. Visible spots of known compounds were cut out and transferred to the minivials and radioactivity measured as given in Section 2.4.10. The compounds were estimated on the basis of the total counts of ^{3}H incorporated.

b) Dansylation with two radiolabels

Quantitation of amino compounds with double isotope dansylation was carried out as above, except that a known amount of [^{14}C]-amino acids were mixed with the tissue extract prior to reaction with [^{3}H]-Dans-Cl. The estimates were made with reference to standard curves of ^{3}H / ^{14}C obtained for the known amounts of standard amino acids which had also been subjected to chromatography (Brown and Perham, 1973) (Fig. 19).

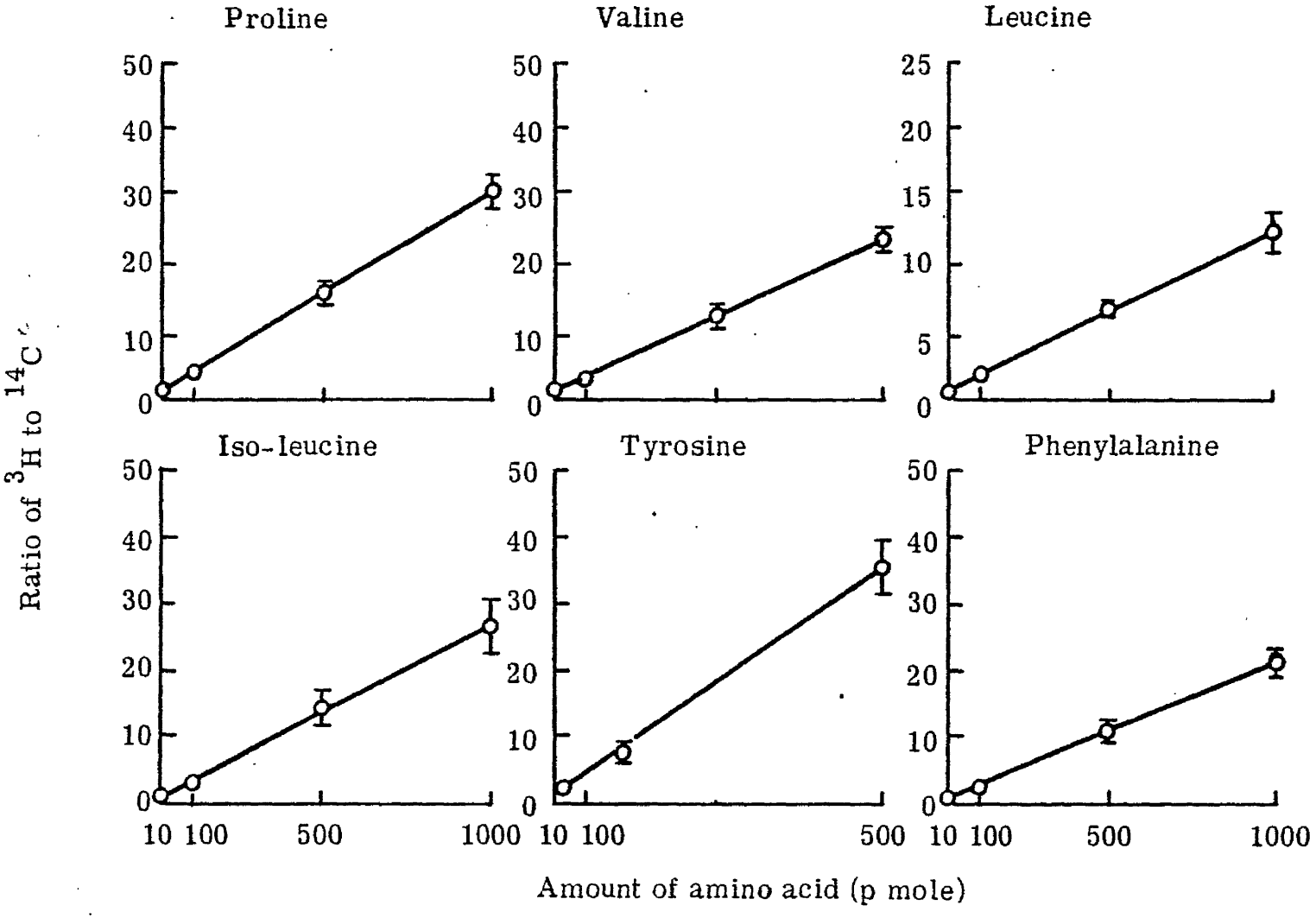


Figure 19a Standard curves of $^{3}H/^{14}C$ for estimation of Dans-amino acids. Each point is the average of 4 values and the standard deviation is shown by vertical bars.

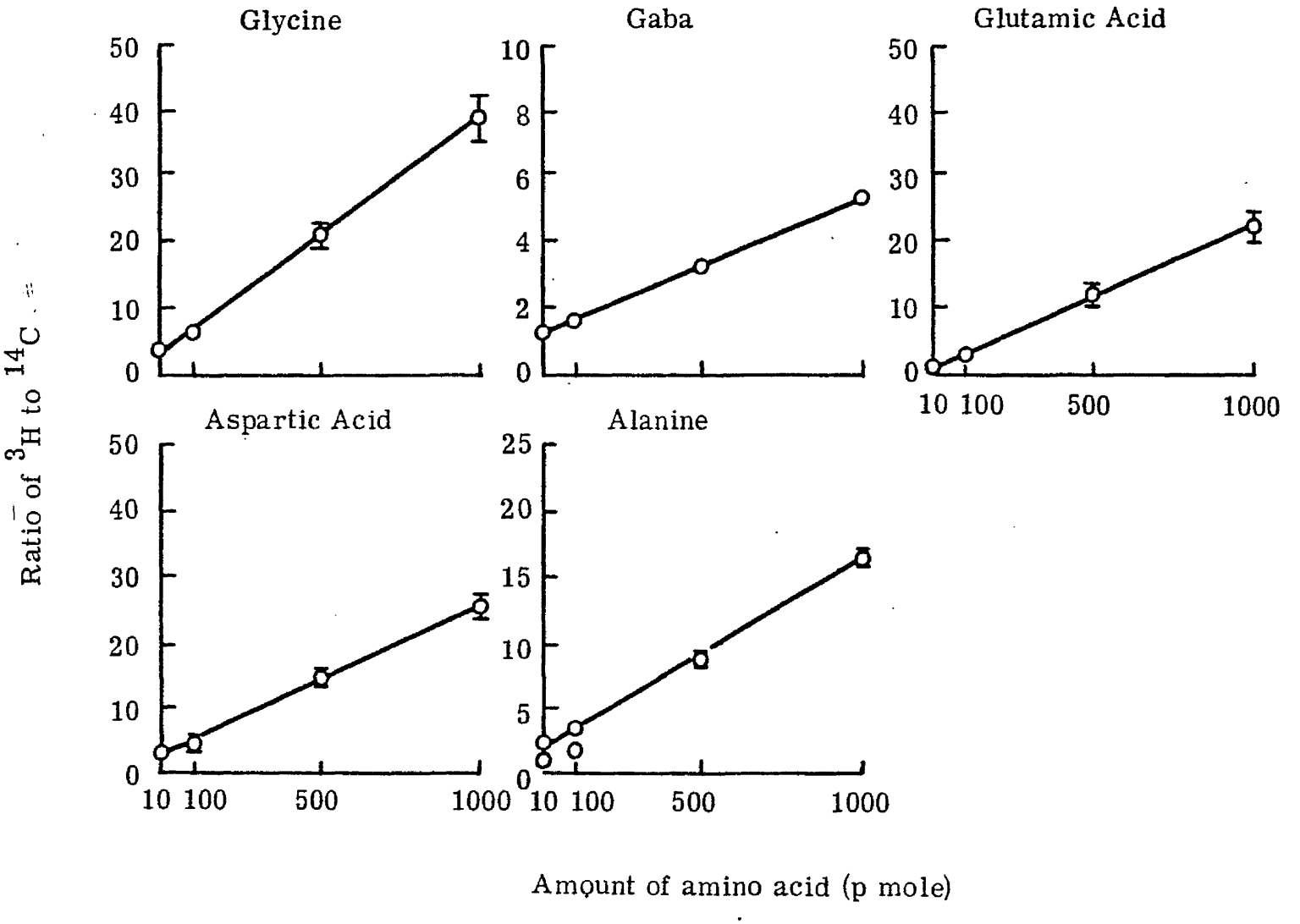


Figure 19b. Standard curves of $^{3}H/^{14}C$ for estimation of Dans-amino acids. Each point is the average of 4 values and the standard deviation is shown by vertical bars.

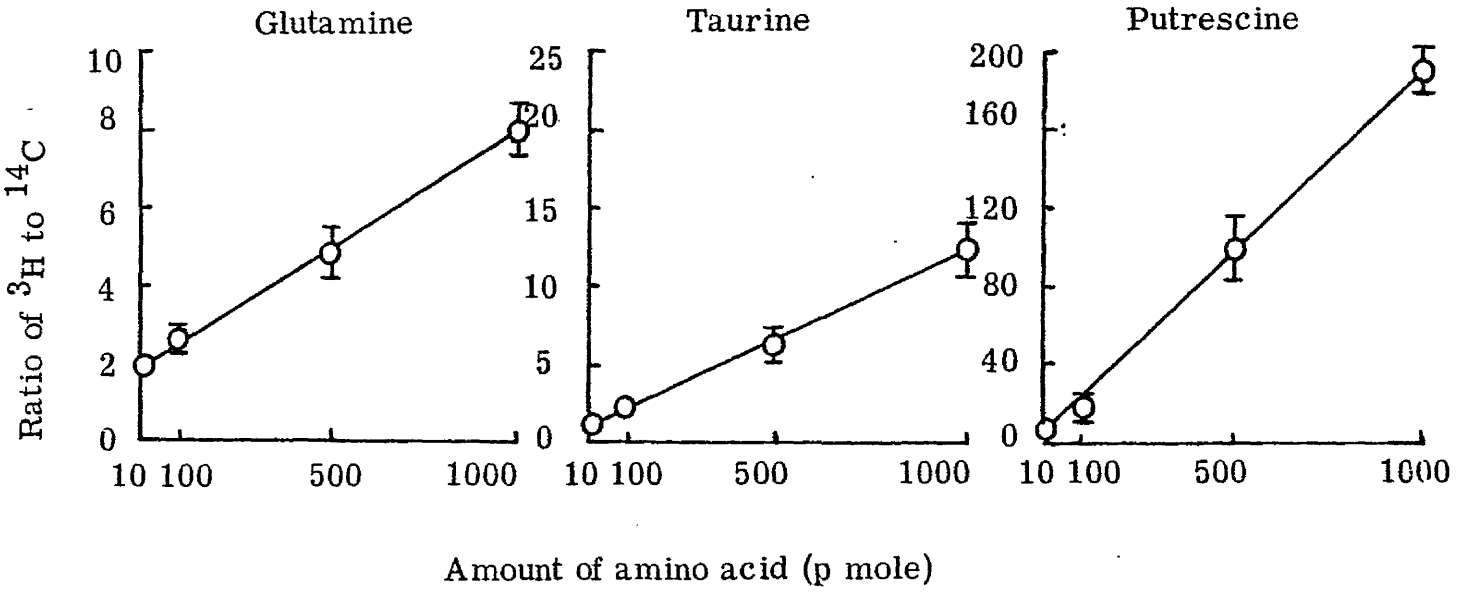


Figure 19c. Standard curves of $^{3}H/^{14}C$ for estimation of Dans-amino acids. Each point is the average of 4 values and the standard deviation is shown by vertical bars.

c) Ninhydrin reagent

The effluent from the ion-exchange column was reacted with the ninhydrin reagent and the change in absorbance monitored continuously and recorded on a chart. The area under the curves corresponding to the substances represented the amounts of each compound. Quantitative estimates were made by comparison with known amounts of standards run under similar conditions. To the experimental samples a known amount of nor-leucine was added as internal standard to check recovery from the column.

2.4.12.5 Metabolites of [^{35}S]-methionine

Radiolabelled compounds from the haemolymph, fat body and nervous tissue were resolved on an ion-exchange column using an autoanalyzer. The effluent was split into two portions. One portion was reacted with ninhydrin as mentioned in the previous section and the other portion was collected in 1.6ml fractions over 2 minutes each, whose radioactive content was estimated by scintillation counting (Section 2.4.10) using Intertechnique Liquid Scintillation Spectrometer SC30.

2.4.12.6 The metabolites of intermediary energy metabolism

The preparation of the tissue and the extraction of metabolites is given in Section 2.4.8.2.

Various metabolites i.e. succinate, fumarate and malate, and glycerol-3-phosphate were all measured in 100µl of the protein-free extract by the enzymatic methods given by Bergmeyer (1974), summarized as follows:-

a) Succinate

Outline: Succinate + GTP + CoA $\underset{\text{Thiokinase}}{\overset{\text{Succinate}}{\rightleftharpoons}}$ Succinyl CoA + Pi + GDP

GDP + PEP $\xrightarrow[\text{Kinase}]{\text{Pyruvate}}$ GTP + Pyruvate

Pyruvate + NADH + H^+ $\xrightarrow[\text{Dehydrogenase}]{\text{Lactate}}$ L-lactate + NAD^+

Assay conditions:

Triethanolamine buffer pH 7.4

(42.2mM triethanolamine; 8.4mM Mg^{++}; 4.2mM EDTA)

CoA mixture (0.11mM CoA; 21mM GTP; 1.1mM PEP)

50µM NADH

2.1 U/ml lactate dehydrogenase

630 mU/ml Pyruvate kinase

The contents were mixed and the absorbance recorded at 340nm and 90 mU/ml succinate thiokinase were added.

After 30 minutes the change in absorbance was measured. Blanks in which sample was replaced with water were treated similarly through the same procedure.

b) L-Malate and Fumarate

Outline:

Malate + NAD^+ + Hydrazine $\underset{\text{Dehydrogenase}}{\overset{\text{Malate}}{\rightleftharpoons}}$ oxaloacetate hydrazone + NADH+H^+

H_2O + Fumarate $\underset{}{\overset{\text{Fumarase}}{\rightleftharpoons}}$ Malate

Assay conditions;

Hydrazine buffer pH 9.0

(0.1M hydrazine; 0.2mM EDTA and 60 M NAD^+)

2.8 U/ml malate dehydrogenase

700mU/ml fumarase

The increase in the concentration of NADH as measured by increase in fluorescence, excitation wavelength of 360 nm and emission wavelength of 420 nm, using Aminco Bowman Spectrophotofluorometer supplied by American Instruments Company Inc. Maryland, U.S.A., was proportional to the amount of the malate and fumarate present.

c) Glycerol-3-phosphate (α-GP)

Outline;

$$\text{L-(-)Glycerol-3-phosphate} + NAD^+ \underset{\text{Dehydrogenase}}{\overset{\alpha\text{-GP}}{\rightleftharpoons}} \text{Dihydroxyacetone phosphate} + NADH + H^+$$

Assay conditions:

Glycine hydrazine buffer pH 9.5

(0.189 M glycine; 0.47M hydrazine; 2.7mM EDTA)

2.31mM NAD^+

4U α-GP dehydrogenase

The change in fluorescence as a measure of the metabolite was recorded as for malate and fumarate.

2.4.13 Estimation of the maximum specific activities of enzymes associated with the amino-acid metabolism in the thoracic ganglia of the locust

2.4.13.1 Preparation of tissue homogenate

Thoracic ganglia were dissected from locusts and carefully freed of fatty and tracheal tissue in ice-cold locust saline. The nervous tissue was either from freshly killed locusts or from those which had been frozen at -20°C immediately after death, until required (tests with various enzymes revealed no differences in enzymic activity between frozen and fresh tissue).

After cleaning, the tissue was blotted dry and rapidly weighed on a small piece of aluminium foil. For each estimate ganglia from 6-12 locusts were pooled, resulting in a total wet weight of from 10-20mg.

The tissue was then homogenized in 200µl of 100mM Tris/HCl buffer pH 7.1 in a small conical centrifuge tube by means of a specially made teflon pestel driven by a dental drill as given in Section 2.4.8.1. During the homogenization and all subsequent operations until the assay of enzymic activity, the tissue was kept on ice.

With the aid of 100µl washes, the homogenate was quantitatively transferred to a plastic vial, so that the final volume was 500µl. The disruption of the tissue was completed by means of 30 secs. of ultrasonic vibration (Soniprobe Type 7530 A.).

Estimates of activity were then carried out on the complete homogenate as soon afterwards as possible.

2.4.13.2 Assay of enzyme activity

All assays were made at 37°C and at pH 7.1. When possible, the activity of the enzyme was followed continuously on a recording Pye Unicam SP 800 spectrophotometer by the oxidation or reduction (either directly or by means of a coupled enzyme system), of the appropriate nicotinamide cofactor. The change in optical density at 340 nm was followed. The temperature was maintained by circulation of water through the cell housing.

As will be described for the relevant enzymes, it was not always possible to estimate the activity by a continuous method. In these cases the assay mixture was kept in ice until complete, at which time a 0 time sample (usually 100µl) was taken, before the mixture was incubated at 37°C in a water bath. Subsequent samples were taken after 10 and 20 minutes of incubation. To stop the enzyme activity, samples were immediately pipetted into 100µl 3M $HClO_4$. The precipitate of protein which formed was removed by centrifugation at 10,000g for 5 min. The acid supernatant was neutralized with 200µl 2M $KHCO_3$. After leaving in ice for 60 minutes to ensure reasonable precipitation of $KClO_4$, which was removed by further centrifugation, samples (100-500µl) of the neutralized supernatant were taken to estimate the product.

The estimate of enzyme activity were carried out in volumes ranging from 1000-1500µl. In the case of those followed directly by spectrophotometry, silica semi-micro cells (path length 1cm), were used.

Appropriate blanks were run for each assay. In these either homogenate, substrate, cofactors or auxiliary enzymes were omitted. Volumes of homogenate added to each assay ranged from 10-50µl. Under the conditions used, such volumes contained 1-3mg wet weight of tissue per assay.

In the assays monitored continuously, the activity was estimated over a 5 min. period. In all cases the activities of the enzymes involved were linear over that period. In the case of those measured discontinuously, the higher rate of the two ten minute periods was used.

When auxiliary enzymes were employed, addition of the appropriate substrate to the full assay mixture ensured that these enzymes were in adequate excess.

All acid substrates were titrated to pH 7.1 in the course of preparation. Solutions of oxaloacetate were made up immediately prior to their use in assays, as this acid rapidly and spontaneously decarboxylates to form pyruvate.

In deciding what concentrations of substrate to use to obtain the maximum specific activity of an enzyme under the conditions employed, this was based where possible upon published work with the enzyme. In general, concentrations of at least 5 times the Km were used when information allowed this. In each case higher concentrations were used to check the assumptions. In a few cases in which the Km of the enzymes for a substrate was particularly high, a range of concentrations were used, and the maximum activity estimated by extrapolation to infinite substrate

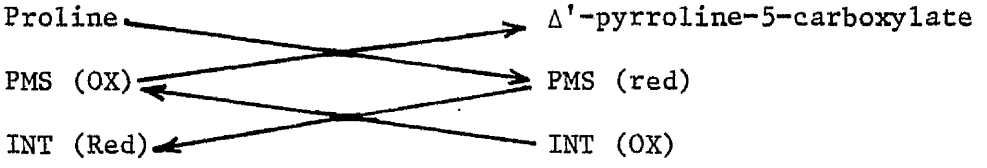
concentration of a Lineweaver-Burk plot.

2.4.13.3 Assay conditions for individual enzymes

a) Proline dehydrogenase (No EC. No.)

The method followed was that of Crabtree and Newsholme(1970) except that the reaction for 'Oxidizing NADH' (with acetylaldehyde-alcohol dehydrogenase system) was omitted, as it did not affect the rate.

Outline:



The change in colour of INT was measured.

PMS : phenazine methosulphate

INT : 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl monotetrazolium chloride.

Assay mixture.

100mM Tris/HCl buffer pH 7.1

100mM L-proline

1mM KCN

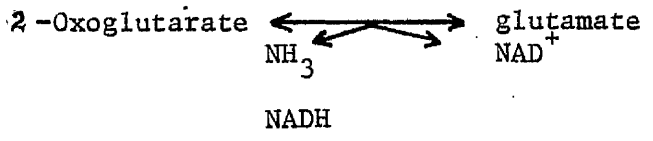
1mg/ml PMS

0.7mg/ml INT

b) Glutamate dehydrogenase (E.C. 1.4.1.2)

The method was that of Sudgen and Newsholme (1975), except that KCN rather than antimycin was used to prevent the oxidation of NADH by mitochondrial fragments.

Outline:



Assay mixture:

100mM Tris/HCl buffer pH 7.1

10mM 2-oxoglutarate

100mM ammonium acetate

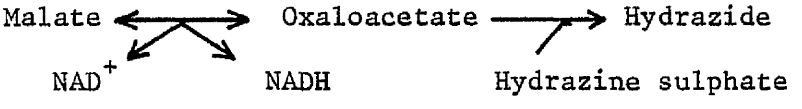
2mM ADP

0.2mM NADH

c) Malate dehydrogenase (E.C. 1.1.1.37)

The enzyme was assayed in the direction of the formation of oxaloacetate according to a modification of the method of Weeda et al. (1980).

Outline:



By forming a hydrazide with the oxaloacetate formed, the presence of hydrazine sulphate in the assay prevented the reaction reaching equilibrium. In its absence, the equilibrium is rapidly reached with only the reduction of a very small amount of NAD^+. Under the conditions used linearity of enzyme activity wasachieved for periods of up to 15 min., without any inhibition of activity due to the hydrazine.

Assay conditions:

100mM Tris/HCl buffer pH 7.1

50mM L-malate

2mM NAD^+

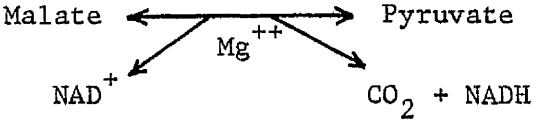
40mM Hydrazine sulphate

d) Malate dehydrogenase (Decarboxylating), ("Malic" Enzyme)

i) NAD^+ dependent (E.C. 1.1.1.39)

The method was that of Weeda et al. (1980) except for the omission of purified malate dehydrogenase. The high endogeneous activity of this enzyme made this unnecessary.

Outline:



Assay conditions:

100mM Tris/HCl buffer pH 7.1

50mM L-malate

5mM NAD^+

After the equilibrium had been reached between malate and oxaloacetate, the reaction of the decarboxylating enzyme was started with 10mM $MgCl_2$.

ii) $NADP^+$ dependent (E.C. 1.1.1.40)

The method was the same as for the NAD^+ dependent enzyme, except that $NADP^+$ was used, and no initial equilibration of malate dehydrogenase was required.

e) Alanine dehydrogenase (E.C. 1.4.1.1.)

The method used was that according to Yoshida and Freese (1965). The reaction was measured in the direction of alanine formation.

Outline:

$$\text{Pyruvate} + NH_3 + \text{NADH} \longleftrightarrow \text{Alanine} + H_2O + NAD^+$$

Assay conditions:

100mM Tris/HCl buffer pH 7.1

6mM pyruvate

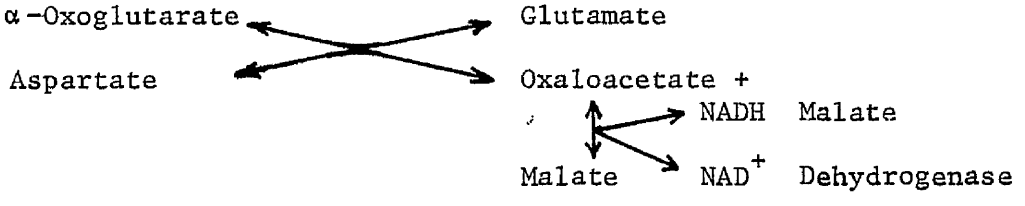
0.2mM NADH

In the absence of added NH_4^+ ions, the initial rate was taken to be due to lactate dehydrogenase. Once this had been established, NH_4Cl was added to a final concentration of 100mM. Any increase in the rate was due to alanine dehydrogenase.

f) Aspartate aminotransferase (E.C. 2.6.1.1)

The method used was that of Sudgen and Newsholme (1975). As the Km values of the enzyme have been previously found to be high, different concentrations of aspartate were used, and the V_{max} found by extrapolation.

Outline:



Assay conditions:

100mM Tris/HCl pH 7.1

10-50mM L-aspartate

10mM α-oxoglutarate

1mM KCN

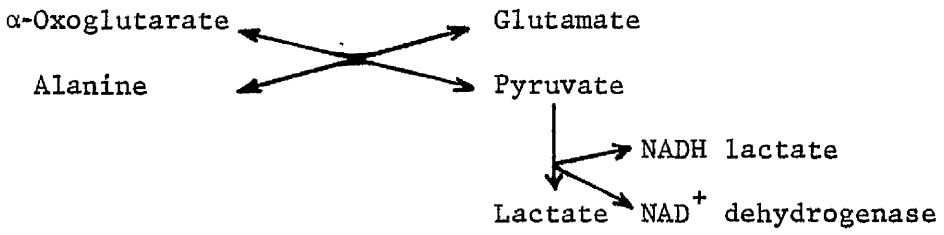
0.6U Malate dehydrogenase

(Addition of pyridoxyl phosphate was found to have no affect upon the rate of reaction).

g) Alanine aminotransferase (E.C. 2.6.1.2.)

The method was similar in principle to that for aspartate aminotransferase.

Outline:



Assay conditions:

100mM Tris/HCl buffer pH 7.1

10mM Oxoglutarate

10-100mM L-alanine

0.2mM NADH

1mM KCN

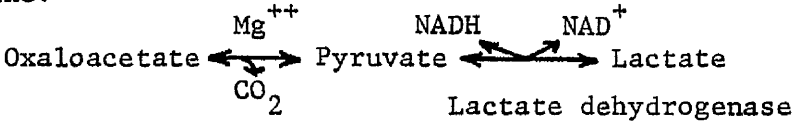
0.3U Lactate dehydrogenase

It was found necessary to add only the minimum of lactate dehydrogenase compatible with sufficient excess, as the specificity of the enzyme is wide enough to use the α-oxoglutarate as substrate.

h) Oxaloacetate decarboxylase (E.C. 4.1.1.3)

Due to the high activity of endogeneous malate dehydrogenase it was not possible to measure the activity of this enzyme continuously by the production of pyruvate from oxaloacetate and the subsequent reduction of that pyruvate in the presence of lactate dehydrogenase and NADH. Instead a two-step assay system was used as outlined in the introduction.

Outline:



Assay 1:

100mM Tris/HCl buffer pH 7.1

10mM Oxaloacetate

10mM $MgCl_2$

100 µl samples of this assay mixture were tested for the presence of pyruvate in a total volume of 1ml of the following:

Assay 2:

100mM Tris/HCl buffer pH 7.1

0.1mM NADH

0.3U Lactate dehydrogenase

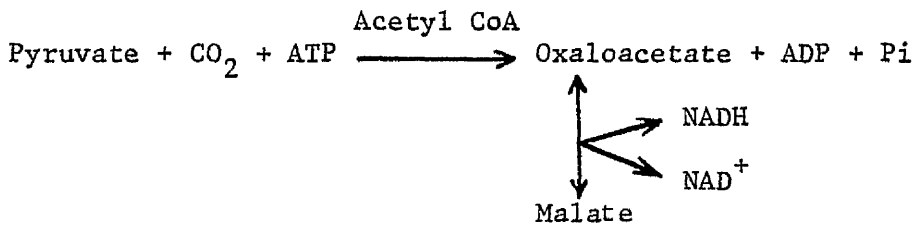
Oxidation of NADH was followed at 340 nm. The addition of known amounts of pyruvate to the second assay ensured that it was functioning correctly.

i) Pyruvate carboxylase (E.C. 6.4.1.1.)

Due to the endogeneous activity of lactate dehydrogenase it was impossible to follow the activity of this enzyme continuously by the coupled reaction below (Weeda et al., 1980).

As before a two-stage assay was used.

Outline:



Assay 1:

100mM Tris/HCl buffer pH 7.1

10mM $MgCl_2$

2mM ATP

10mM $KHCO_3$

10mM Pyruvate

0.2mM Acetyl CoA.

Samples from this assay were tested for the accumulation of oxaloacetate in the following assay system in a volume of 1ml.

100mM Tris/HCl buffer pH 7.1

0.1mM NADH

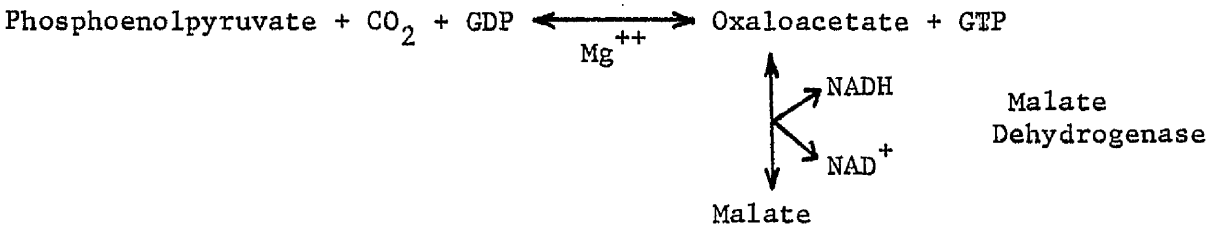
0.6 U Malate dehydrogenase.

As before the oxidation of NADH was followed at 340 nm and additions of oxaloacetate to the assay ensured that it was functioning correctly.

j) Phosphenolpyruvate carboxykinase (4.1.1.32)

Initially this was measured in the direction of the formation of oxaloacetate according to the method of Holten and Nordlie (1965), except that GDP was substituted for IDP.

Outline:



Assay conditions:

100mM Tris/HCl buffer pH 7.1

1mM Phosphoenolpyruvate

1mM GDP

10mM $MgCl_2$

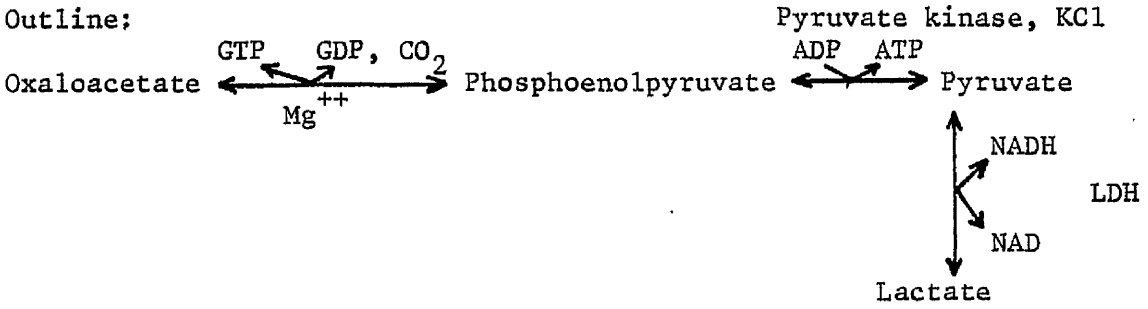
100mM $KHCO_3$

0.2mM NADH

0.6 U Malate dehydrogenase

As the above authors remark that the specific activity of the enzyme is about 7 times as fast in the direction of the formation of phosphoenolpyruvate, as compared to the formation of oxaloacetate, attempts were also made to measure this rate.

Outline:



Assay conditions:

100mM Tris/HCl buffer pH 7.1

1mM Oxaloacetate

2mM GTP

10mM $MgCl_2$

10mM KCl

1.6 U Pyruvate kinase

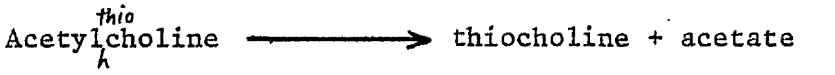
Samples of this assay mixture were tested for the accumulation of pyruvate in the same way as already recorded for oxaloacetate decarboxylase. To ensure that the reaction catalyzed by pyruvate kinase was active, some assays contained known amounts of phosphoenolpyruvate.

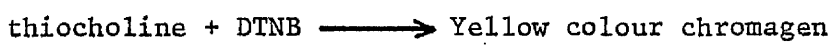
k) Acetylcholinesterase (E.C. 3.1.1.7)

This enzyme was used as a marker for nerve cell plasma membrane (Marchbanks, 1975; Donnellan et al., 1976).

The method used was that of Ellman et al. (1961). The thiocholine released by the action of acetylcholinesterase reacts with dithio-bis-nitrobenzoate (DTNB) to produce a red product whose absorbance is followed at 412 nm. The molar extinction coefficient was taken as 1.3×10^4 1. mol^{-1}. cm^{-1}.

Outline:





The final assay mixture was as follows:

100mM Phosphate buffer pH 7.9

0.03mM DTNB

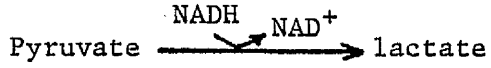
0.5mM ATC

Blanks omitted either, ATC, DTNB or homogenate.

1) Lactate dehydrogenase (E.C. 1.1.1.27)

This enzyme was used as a cytoplasmic marker, although it is in very low activity in locust nervous tissue compared to the mammalian brain. The methods employed in its assay was that of Crabtree and Newsholme (1972).

Outline:



The assay conditions were as follows:

100mM Tris-HCl buffer pH 7.5

0.2mM NADH

2mM KCN

1.0mM Pyruvate

The change in absorbance at 340nm was followed continuously and the molar extinction coefficient of reduced NADH taken to be 6.2×10^3 1. mol^{-1}. cm^{-1}.

m) Succinate dehydrogenase (E.C. 1.3.99.1)

This was taken as a mitochondrial marker, and was assayed by the method of Pennington (1961), based upon the reduction of dye INT in the presence of succinate.

The final assay conditions were:

50mM Phosphate buffer pH 7.5

2mM KCN

50mM Succinate

1mg/ml INT solution in H_2O

1mg/ml PMS

The incubation was carried out in a water bath at 37°C for 15 minutes and the reaction then stopped by the addition of 1ml of 10% TCA. The reduced dye was then extracted into 3ml ethylacetate by vortexing for about 30 seconds. After centrifugation for 5-10 minutes at 1000 rpm to break the dispersion, the formazan in ethylacetate was estimated at 490 nm. The molar extinction coefficient was taken as 20.1×10^3 1. mol^{-1}. cm^{-1}. (Crabtree and Newsholme, 1970).

2.4.14 Preparation of mitochondria from locust nervous tissue

Mitochondria were prepared according to the methods advocated for the preparation of brain mitochondria by Marchbanks (1975). Cleaned thoracic and cerebral ganglia from about 12 locusts were homogenized in a medium consisting of 0.32M sucrose in 2mM Tris-HCl buffer pH 7.2. Homogenization was achieved by means of a glass motor-driven pestel with a clearance of 0.2mm, at a speed of 1000-1200 rpm. for 1 minute. During the homogenization, the tube was kept in ice.

The volume was then made up to 1.5ml with ice-cold medium, and 0.5ml removed as a sample for enzymic analysis. The remainder was centrifuged at 1000g for 10 minutes to remove the cellular debris and nuclei. The resulting pellet (P_1) was washed with 0.5ml of medium, and the washing added to the main bulk.

The supernatant was subjected to centrifugation at 10,000g for 15 minutes in the cold room. The pellet from this run (P_2) was the crude mitochondrial pellet. It was washed with 0.5ml of medium, and the washing added to the final supernatant (S). Both pellets were resuspended in 0.5ml of medium (full scheme shown in Fig. 20). Samples(20-200µl)were taken from all three fractions and from the initial homogenate for the enzymic analysis (Section 2.4.13.3). The assay medium in which the O_2 uptake studies were made consisted of 20mM phosphate buffer; 0.20M mannitol and 0.05M sucrose, pH 7.1.

2.4.15 Uptake of O_2 by nervous tissue

The experiments following the O_2 uptake by the intact thoracic ganglia of the locust and also by mitochondrial preparations from the tissue, were carried out in a water-jacketed O_2 electrode (Rank Bros., Bottisham, Cambridge, U.K.), at 37°C in 2ml of the assay buffer. The experiments with the mitochondrial fraction were performed at the concentration of O_2 in equilibrium with the atmosphere, whereas in those with the whole ganglia the medium was saturated with the gas (ie. $[O_2] > 700\mu M$) by bubbling moist, prewarmed O_2 through it.

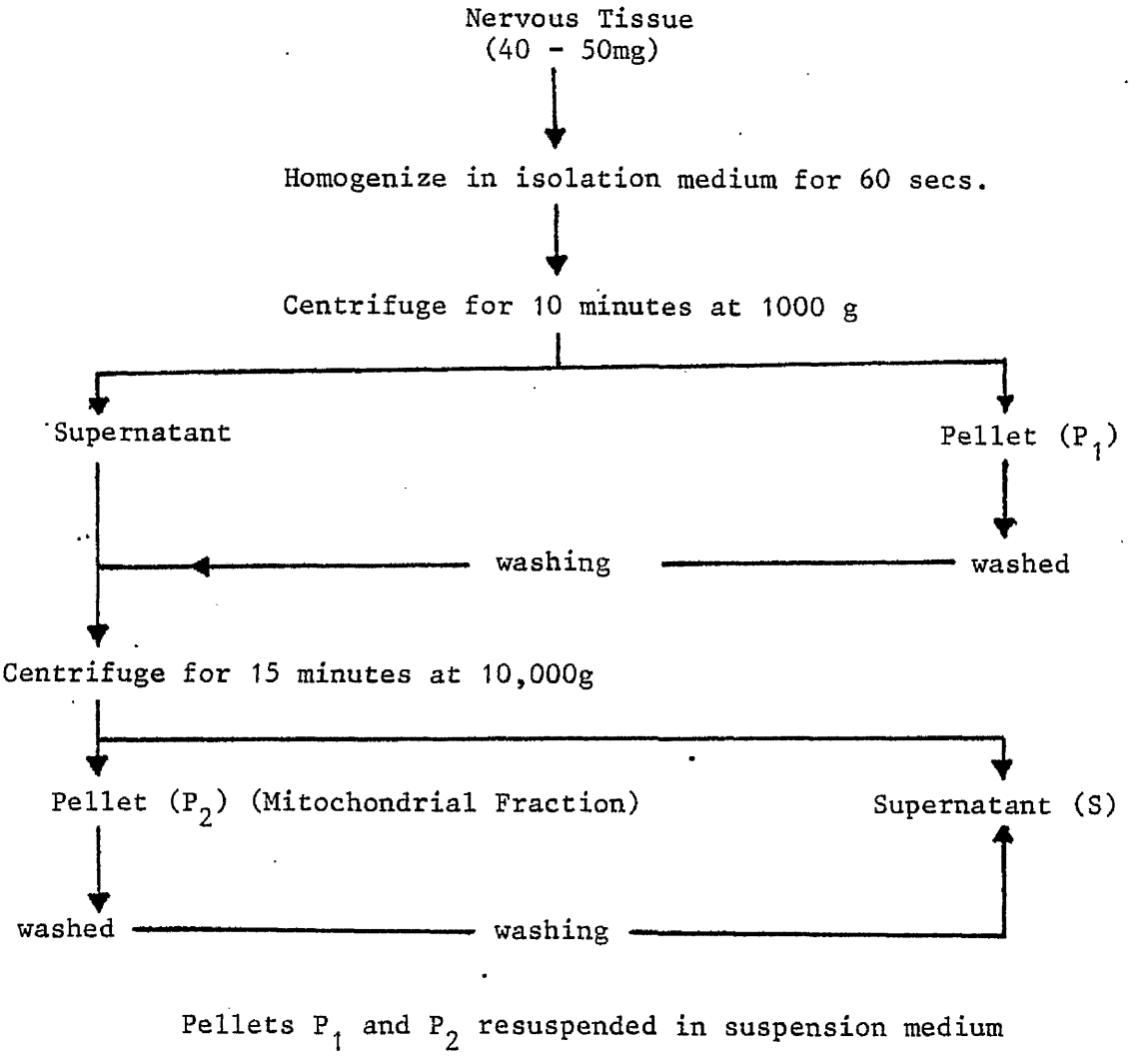


Fig. 20 - Flow diagram for the isolation of mitochondria from nervous tissue of locust

Samples (100µl) of the mitochondrial preparation, or 6 pairs of intact thoracic ganglia were added to the assay medium of pH 6.8 to make the total volume to 2ml. During the recording of the O_2 uptake by the tissue the medium was stirred by means of a magnetic follower. The signal from the O_2 electrode passed to a chart recorder, and the O_2 uptake measured (Clement and Strang, 1978). The chart was calibrated using phenazine methosulphate (PMS) and following the quantitative disappearance of O_2 after the addition of a known amount of NADH (Robinson and Cooper, 1970). Additions of substrates were made into the tissue preparations in the sealed electrode, after equilibration, by means of a 25µl Hamilton syringe. Additions did not exceed 1% of the volume of medium in the electrode. In general the final concentration of potential substrates was 10mM.

2.4.16 Electrical stimulation of thoracic ganglia in vitro

The thoracic ganglia (meso-and metathoracic ganglia intact) were dissected out of the locust and after a thorough rinse in ice-cold insect saline, were placed in small plastic dish containing 50µl of iso-osmotic saline. The dish was transferred to the Faraday cage. Stimulation was carried out at room temperature. A silver wire hook was placed under one of the connectives (as shown in Fig. 21) and adjusted to make a firm contact with the nerve cord. An indifferent electrode was placed in the saline to act as reference. The electrodes were connected to the stimulator (D.S.9, Digitimer Ltd.). A continuous repeated

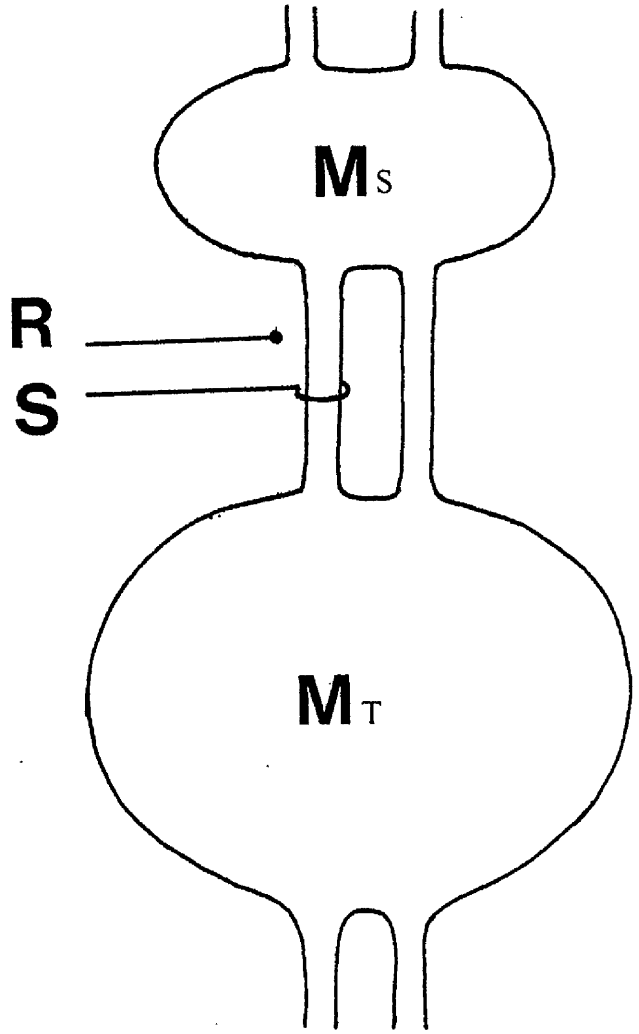


Figure 21 Diagram showing position of electrodes for in vitro electrical stimulation of the thoracic ganglia of locust.

Ms: mesothoracic ganglia; Mt: metathoracic ganglion, S: stimulating electrode; R: reference electrode.

stimulus of 1 millisecond duration with an amplitude of 5 volts at 50 stimulii/sec. was applied for 30 minutes (J.A. Miyan, personal communication). At the end of the experimental period the nerve cord was removed and washed three times in fresh saline. The amino acids in the nerve cord and in the bathing solution were estimated as described in Section 2.4.12.4.b.

2.4.17 Recording of spontaneous nervous activity

The assay of neuroactivity was carried out at room temperature i.e. 25 ± 2°C by recording the spontaneous discharges in the isolated abdominal nerve cords of American cockroach. The nerve cords dissected out of the unanaesthetized cockroach with the use of minimal chill comma (by keeping insects at -20°C for 10 minutes) procedure were placed in a small glass dish containing 1ml of iso-osmotic saline. The cord was fixed with the aid of two needles which pinned the connectives at each end (shown in Fig. 22). A small portion of cord between the 2nd and 3rd ganglia was kept out of the solution and placed on top of two silver wire hooks (one serving as recording and other as reference electrode). The part of the cord outside the saline was covered with a drop of vaseline to prevent drying of the cord and to reduce the interference of "noise". The saline bathing the nerve cord could be replaced by test solution through plastic tubing by the use of peristaltic pump. Usually the flow rate was kept at 1ml/minute. Therefore, the complete replacement of saline could be completed in 2-3 minutes.

Figure 22 Experimental layout used in following the spontaneous electrophysiological activity of the abdominal nerve cord of cockroach.

A: Audio speaker; C = Chart record; D, Digital display; N, Nerve cord preparation; O, Oscilloscope trace; T, Cassetted tape recorder; 1 and 2, Input to channel 1 and 2 respectively.

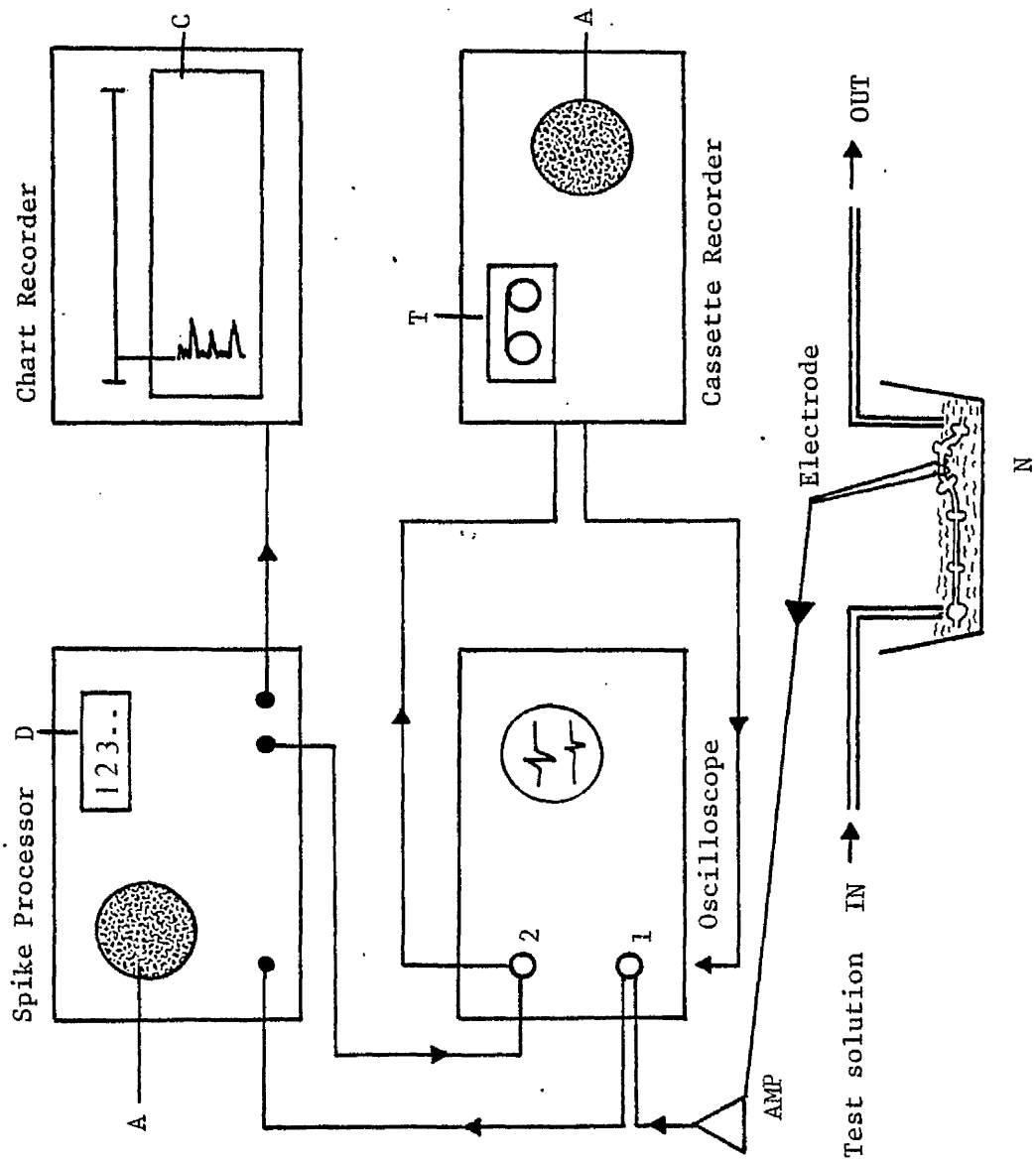


Chart Recorder
C
Spike Processor
D
123..
A
Cassette Recorder
A
T
Oscilloscope
2
1
AMP
Electrode
OUT
IN
Test solution
N

Figure 22.

The preamplified signal was either fed directly to the cathode ray oscilloscope and/or to the spike processor which had the provision of lower and upper signal level discriminator, audio output by means of a loudspeaker, digital analogue display for quantitative measurement of nerve impulses in term of action potential/unit time, and output signal for chart recorder. The screened signal from the spike processor was fed to the 2nd channel of the oscilloscope for visually monitoring the discrimination levels, and was further connected to the cassette recorder for a permanent record using TDK C90 cassettes supplied by TDK Elec. Co. Ltd., Japan. The layout of all the apparatus is shown in Fig. 22.

A continuous recording camera was used to photograph oscilloscope traces on Kodak linograph paper film 1930 obtained from Eastman Kodak Co., Rochester, N.Y. For photographs of various selected levels of spontaneous activity, tape records were first made of the activity, and these, in turn, played back into the oscilloscope. Photographs could then be made of selected portions of these "play backs".

2.5 Statistical analysis

The statistical analysis was carried out using "Student's t-test". The difference of $P<0.05$ only was considered significant.

SECTION THREE

RESULTS

PART ONE

OPTIMIZATION OF ANALYTICAL PROCEDURES, ESTIMATION OF AMINO ACIDS AND AMINES, AND STANDARDIZATION OF INCUBATION CONDITIONS.

3.1.1 Complexity of the two dimensional chromatograhy of Dans-derivatives

Since the first application of the dansylation procedure for the estimation of amines and amino acids of the nervous tissue (Casola et al 1969, Neuhoff et al 1969; Neuhoff, 1970 and Neuhoff & Weise, 1970), the method has been constantly refined to suit the needs of individual tissues and experimental conditions. Figure 23 illustrates the difficulty encountered when chromatography of the mixture of dansyl derivatives of amines and amino acids is attempted by the most commonly used solvents, according to the methods of Seiler (1970), Osborne (1973) and Gould & Cottrell (1974). There are many points of overlap between the compounds so that several spots lie so close to each other to prevent their clear identification. Also, as the Dans-Cl reacts with the ring hydroxyl groups as well as the amino groups of the amines (DA, OA, TA) more than one derivative is formed (Osborne, 1973), thus increasing the possibility of overlap. While single development in the second dimension on polyamide is sufficient to separate the Dans-amines, it leaves many amino acids insufficiently resolved. This is particularly true of tyrosine and taurine, alanine and ammonia and glutamic acid and aspartic acid.

Moreover, the thin layer method works best in separating various substances differing in concentrations, by at the most one or two orders (Dolezalova et al 1973). The range of concentrations of amino compounds encountered in the insect nervous system is likely to exceed this (see Tables 1 and 2, Section 1.4). It was in order to lessen this complexity of chromatograms, and to overcome the drawbacks of variable concentrations of amines and amino acids

Fig. 23 - Two-dimensional chromatograms of standard dansyl amino acids (A), and dansyl amines and dansyl N-acetylamines (B) on polyamide sheets (5 x 5cm). Numbered arrows indicate the solvents and direction of development. The solvents used were: 1. 3% formic acid; and 2. toluene/acetic acid, (9:1 v/v). A cross indicates the position of the origin. The identities of the dansyl compounds are as follows: (A) 1. tyrosine, 2. dihydroxyphenylalanine, 3. tryptophan, 4. isoleucine, 5. leucine, 6. phenylalanine, 7. lysine, 8. taurine and tyrosine, 9. methionine, 10. proline, 11. valine, 12. γ-aminobutyric acid, 13. dansyl-hydroxide, 14. alanine and ammonia, 15. glycine, 16. glutamate and aspartate, 17. serine and arginine, 18. dihydroxyphenylalanine, 19. uknown. (B) 1, 9, 13. tyramine, 2, 11, 14. octopamine, 3, 5. serotonin, 4, 8, 10. dopamine, 6. N-acetyltyramine, 7. N-acetyloctopamine, 12, 15. dansyl-hydroxide.

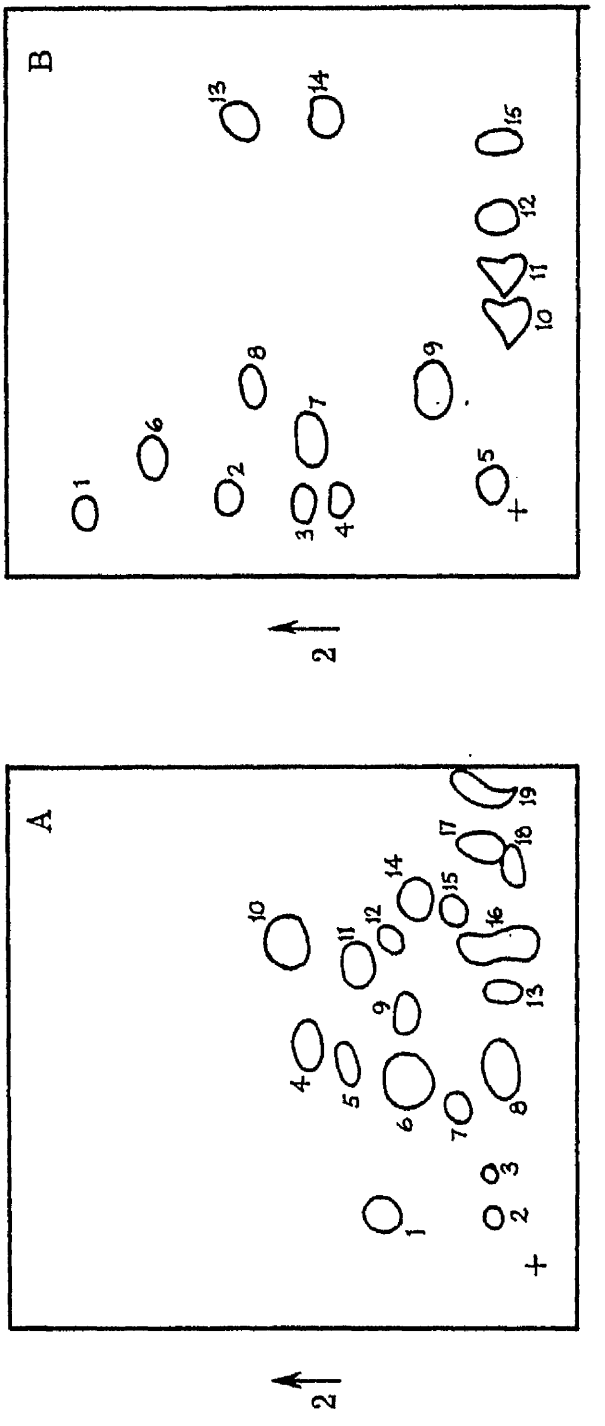


Figure 23.

in the insect nervous tissue, that a procedure was sought to achieve a preliminary separation of the various classes of compounds, particularly amines and their derivatives from amino acids (the last are present in much higher concentrations). The following section gives the results obtained from the various methods tried.

3.1.2.1 Preliminary separation by thin-layer chromatography on silica gel

The two-step thin-layer chromatographic procedure on silica plates reported by Recasens et al (1977) was followed to achieve the separation of Dans-amines and Dans-amino acids. The resolution of various Dans-derivatives is shown in Figure 24. For comparison, the digrammatic representation of chromatograms of dansyl derivatives of amines and amino acids from extract of rat brain as shown by Recasens et al. (1977) is also included. Most of the Dans-amino acids were found to run behind the Dans-OH (Fig. 25). Although the above-mentioned workers did not indicate the position of Dans-amino acids (except Dans-cysteic acid) on the chromatogram, they stated that only Dans-amines run with the solvent front. The material along the solvent front was, therefore, eluted with ethyl acetate. After reducing the volume by evaporation, the derivatives were applied to silica gel sheets (5x5cm) for further resolution (Fig. 26).

One dimensional chromatographic separation of dansyl derivatives from locust nervous tissue and standard amino acids is shown in Figure 27. It is evident that a great proportion of dansyl derivatives of amino acids is also found with the solvent front.

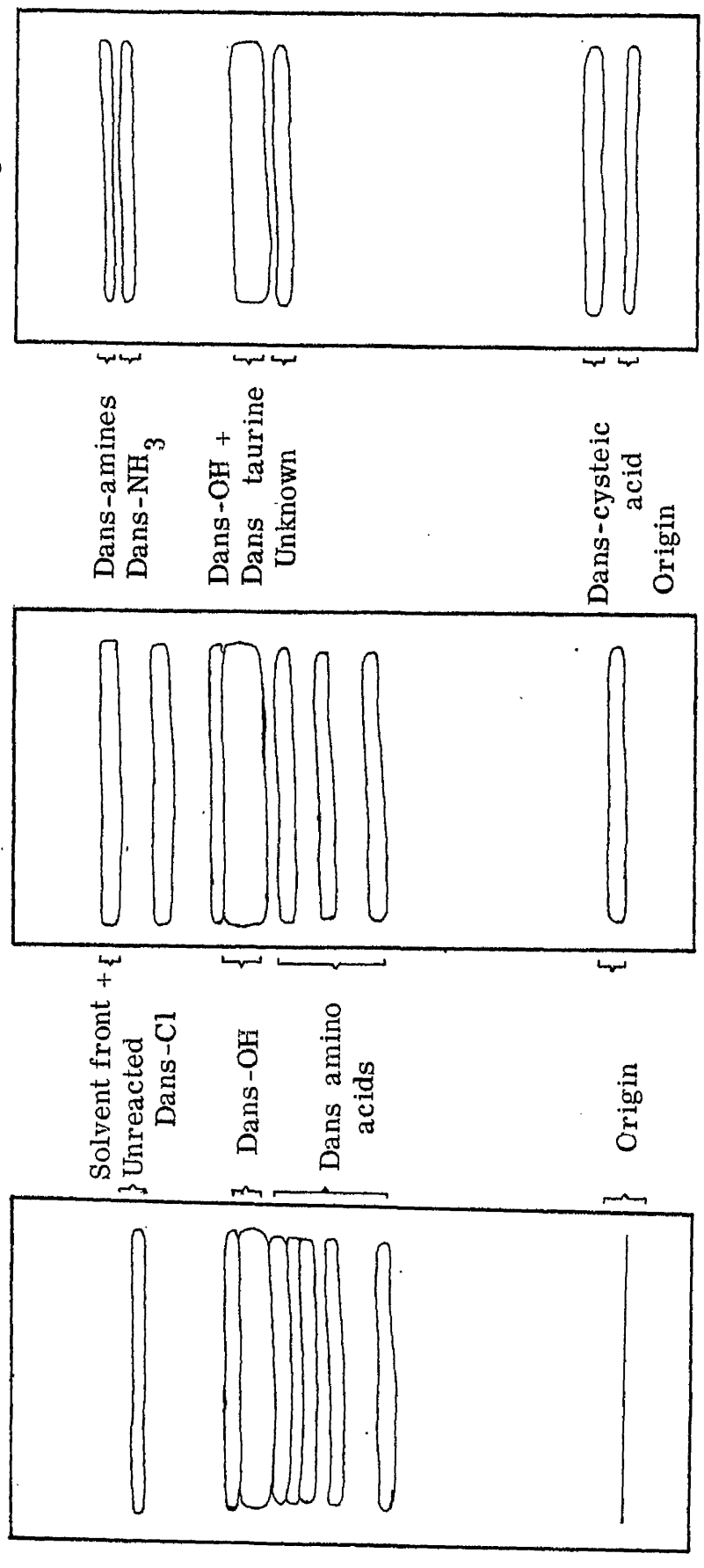


Figure 24. Single dimension chromatography of Dans-derivatives of (A) standard amino acids and (B) extract of locust nervous tissue on silica gel using ethyl acetate/2-propanol/25%NH_3 (9:6:4 v/v/v). Figure (C) from Recasens et al. (1977) is reproduced for comparison.

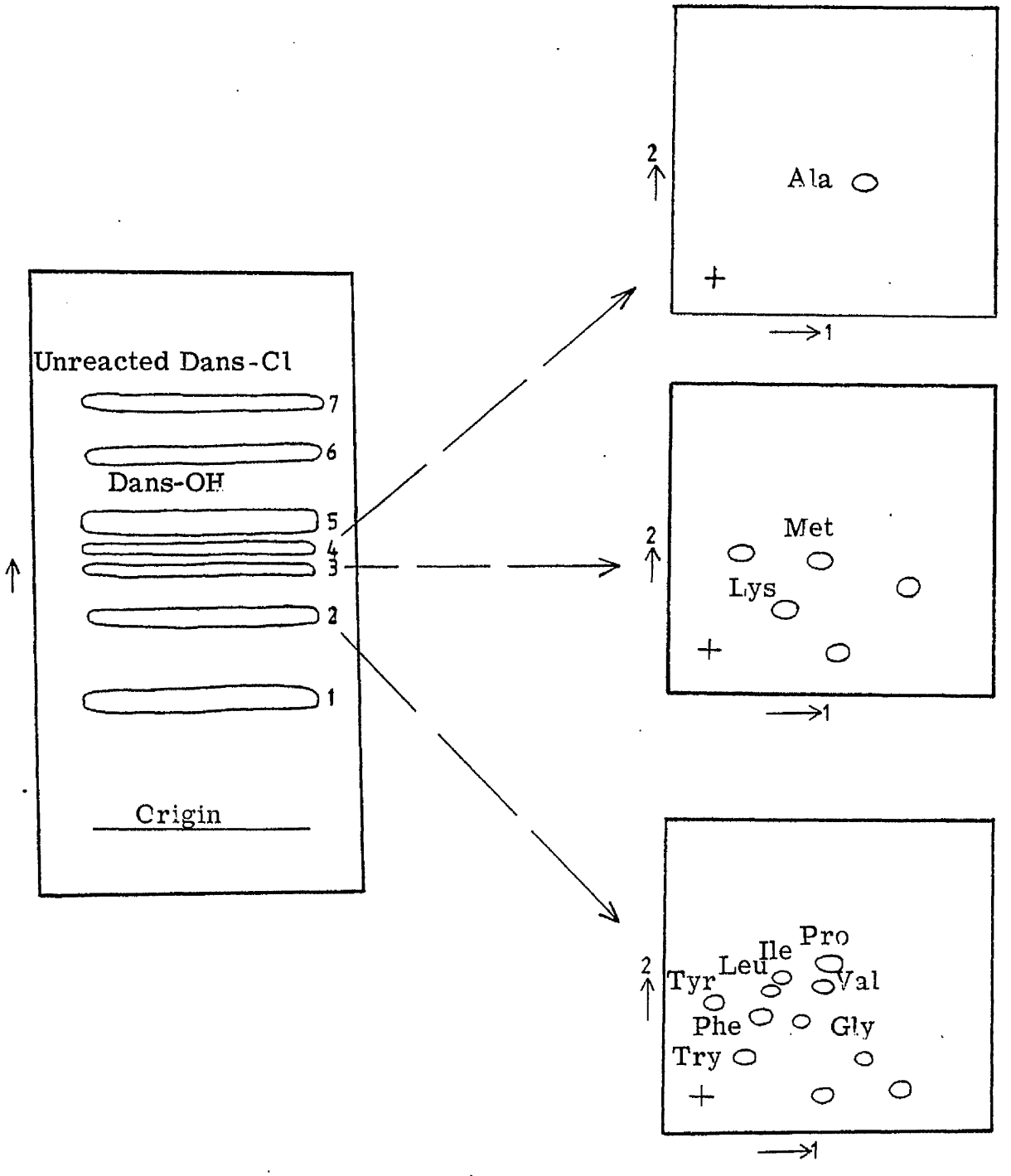


Figure 25. Chromatography of Dans-amino acids on silica layer using ethyl acetate/ propanol/NH_3 (9:6:4 v/v/v). Material running at bands number 2, 3 and 4 was eluted with ethyl acetate and further chromatographed on polyamide sheets in (1) 3% FORMIC ACID and (2) toluene/acetic acid (9:1 v/v).

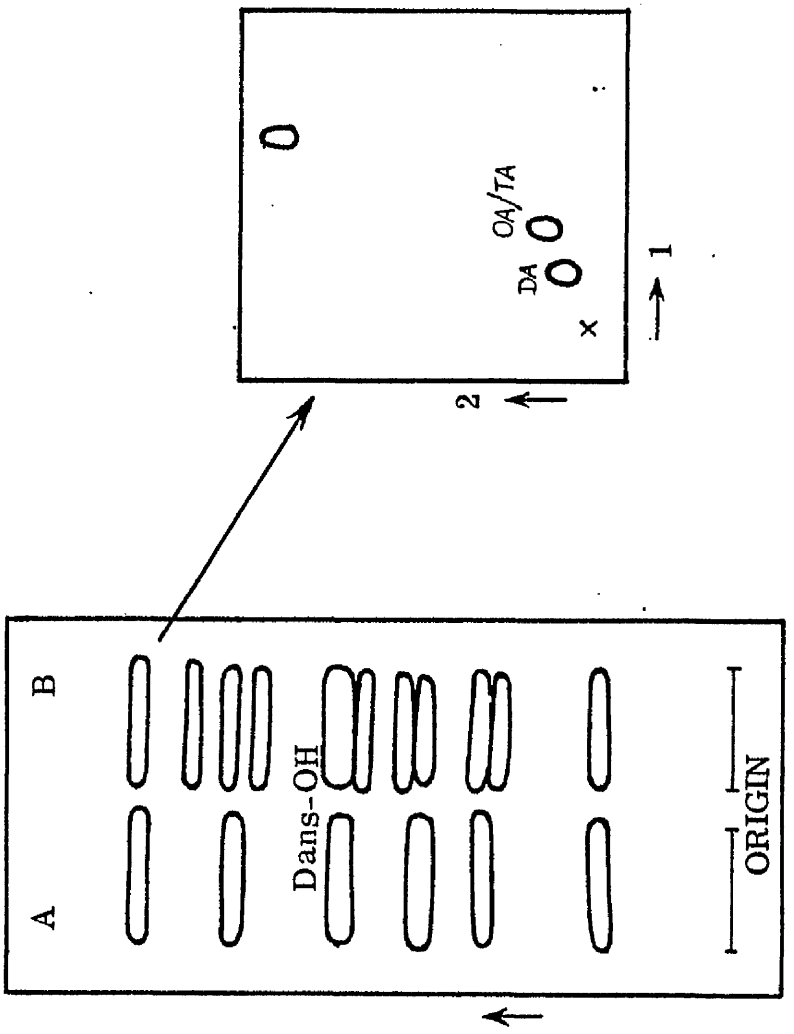


Figure 26. Chromatography of Dans-amino acids (A) and Dans-amino acids + Dans-amines (B) on silica layers using ethyl acetate/2 propanol/ NH_3 (9:6:4 v/v/v). Material running at the solvent front was eluted with ethyl acetate and further chromatographed in (1) Toluene/pyridine/acetic acid (150:15: 0.5 v/v/v) and (2) benzene/triethylamine/NH_3 (100:20:0.1 v/v/v).

Fig. 27 - Photograph showing the resolution of Dans-derivatives on silica gel sheet using ethyl acetate/2-propanol/25% NH_3 (9:6:4 v/v/v) as solvent.
Trace I; Dans-derivatives of the extract from locust ganglia. Trace 2; Dans-derivatives of the standard amino acid mixture. The composition of the mixture is given in Materials and Methods Section.

Figure 27.

Moreover, as the further two dimensional chromatography on silica gel did not adequately resolve various derivatives of the Dans-catecholamines i.e. OA from TA and Tyr from Dopa (Fig. 28), a different procedure was sought to obtain a better separation.

3.1.2.2 Phase separation of Dans-amines and Dans-amino acids

The separation of dansyl-derivatives is often achieved by their differential solubility in suitable organic solvents (Seiler, 1970). The extraction of Dans- amines by phase separation (Seiler & Weichmann, 1965) using ethyl acetate as eluent was tried (Fig. 29). The dansyl-derivatives of monoamines have no ionizable group and therefore will dissolve in solvents of low polarity such as ethyl acetate. As shown in Fig. 29a and 29b these amine-derivatives could be easily extracted with ethyl acetate. More polar solvents than ethyl acetate or benzene are needed for the extraction of Dans-amino acids (Seiler, 1970) which are charged under these conditions. Dans-amino acids were subsequently extracted from the aqueous phase by lowering the pH with the addition of equal volume of 0.1N HCl to eliminate the anionic charge. The Dans-amino acids could then also be extracted by the ethyl acetate in this way (Fig. 29c & d). The quantitative analysis of the separated phases showed that, although, when [^{3}H]-Dans-Cl was used nearly 90% of the radioactivity associated with the Dans-amines could be extracted with the ethyl acetate (Table 5), but experiments with the radiolabelled DA indicated that ethyl acetate extraction did not yield a complete extraction into the organic phase of the amine present in the reaction mixture. A number of reasons could be considered as the cause of this incomplete extraction. Possibly the Dans-amine may

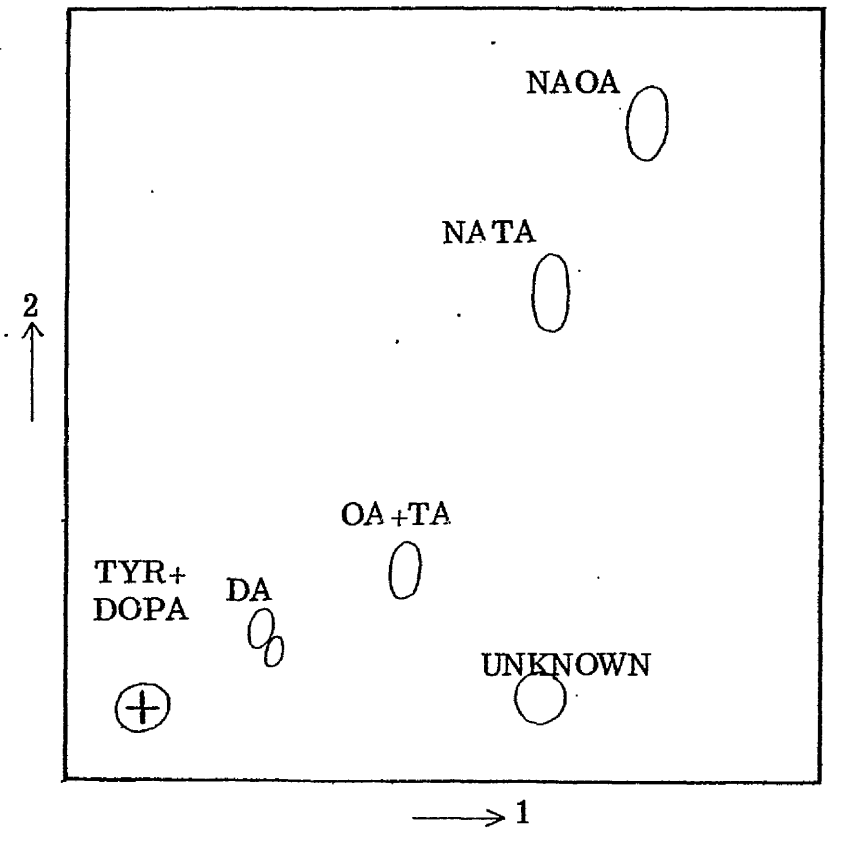


Figure 28. Two dimensional chromatography of Dans-derivatives of monoamines, N-acetylamines and tyrosine& Dopa on 5x5 cm silica gel sheets using toluene/pyridine/acetic acid (150:15:0.5 v/v/v) and benzene/triethylamine/NH_3 (100:20:0.1 v/v/v) as solvent in 1st and 2nd dimension. Chromatograms were developed twice in 1st direction.

Fig. 29 - Photograph under U.V. light showing the resolution, on polyamide sheets, of Dans-derivatives after extraction into ethyl acetate.

A and B represent respectively standard amines and amines from locust nervous tissue extract after extraction by ethyl acetate.
C and D represent respectively the amino acid derivatives from the standard mixture and locust nervous tissue extracted with ethyl acetate after acidification of the aqueous phase. Arrows indicate the direction of solvent development.

1 = 3% formic acid

2 = toluene/acetic acid (9:1 v/v)

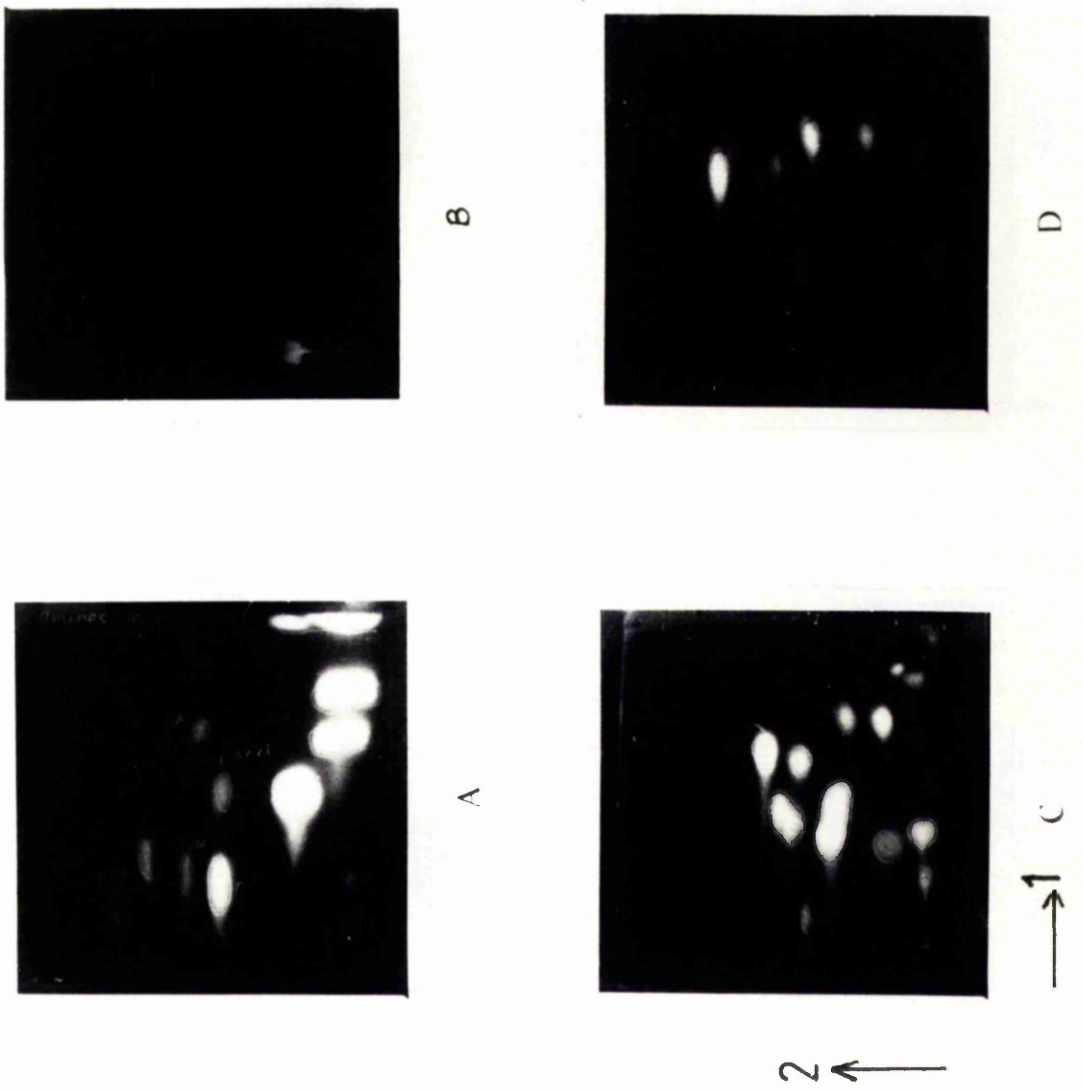


Figure 29

Table 5. Extraction of Dans-amines with ethyl acetate

No.	Dans-derivative	Phase	Total radio-activity (dpm)	% of total present
1	[^{3}H]-Dans-amines	Ethyl acetate	42611	91.84
		Aqueous	3786	8.16
2	Dans-[^{3}H]-dopamine	Ethyl acetate	587898	65.47
		Aqueous	310074	34.53

[^{3}H]-Dans-Cl was reacted with unlabelled amines (1), and unlabelled Dans-Cl was reacted with [^{3}H]-Dopamine (2). After the reaction of amines with Dans-Cl, 100 µl of ethyl acetate was added to the reaction mixture. The contents were thoroughly mixed and the phases allowed to separate. The upper organic phase was transferred to another vial and the aqueous phase was again washed with ethyl acetate and wash added to the ethyl acetate extract. The radioactivity in each phase was estimated.

have not been fully extracted by the ethyl acetate as some of it may have distributed itself in both phases, but the results using [^{3}H]-Dans-Cl indicate that this is not a great problem. A more likely reason could be that reaction of Dans-Cl with the amines and amino acids was far from complete due perhaps to the presence of water in the medium and other reactions of Dans-Cl as well (Osborne, 1973) as illustrated by the following reactions:

$$AA\text{-}NH_2 + Dans\text{-}Cl \longrightarrow AA\text{-}NH\text{-}Dans + HCl$$

$$H_2O + Dans\text{-}Cl \longrightarrow Dans\text{-}OH + HCl$$

$$AA\text{-}NH\text{-}Dans + Dans\text{-}Cl \longrightarrow Dans\text{-}NH_2 + \text{other products}$$

Because of this third reaction there was a possibility that some other reaction products and/or unreacted DA constituted much of the radioactivity in the lower phase. Though a reasonable separation (at least for qualitative purposes) of Dans-amines from Dans-amino acids could be achieved by the phase separation, the main reason for not proceeding with this method was that it was not suitable when working with very small volumes (10µl) of the reaction mixture (usually the nervous tissue from single locust is processed in such a volume).

3.1.2.3 Separation of amines and amino acids by electrophoresis

Ionophoretic separation of the amines, amino acids and N-acetylamines prior to dansylation was investigated because no satisfactory results could be obtained with previously applied methods of separating dansyl-derivatives. Under acidic conditions amines are likely to be more ionized than neutral and basic amino acids, whereas the N-acetylamines have no charge at all. Cellulose and silica gel were used as supporting media at pH 6.5 (Fig. 30).

Figure 30 Electrophoretic separation of amines, amino acids and N-acetylamines on cellulose (I and II) and silica (III and IV) at pH 6.5 (I and III) and pH 2.0 (II and IV). The electropherograms were stained first with ninhydrin (A) and then sulphanilic acid diazo dye (B).
Detailed description of components of various mixtures is given in Fig. 31.

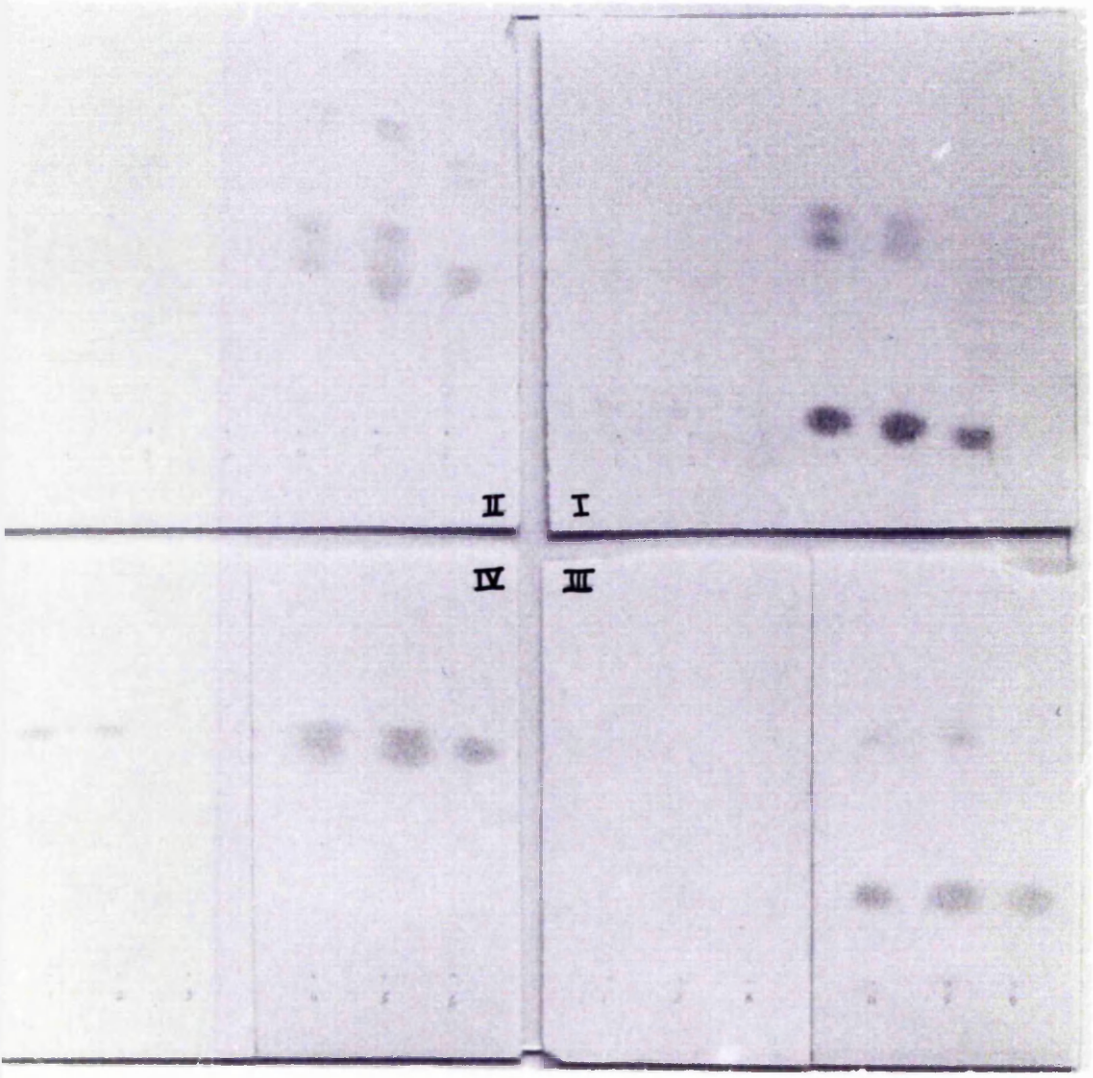


Fig. 30 A.

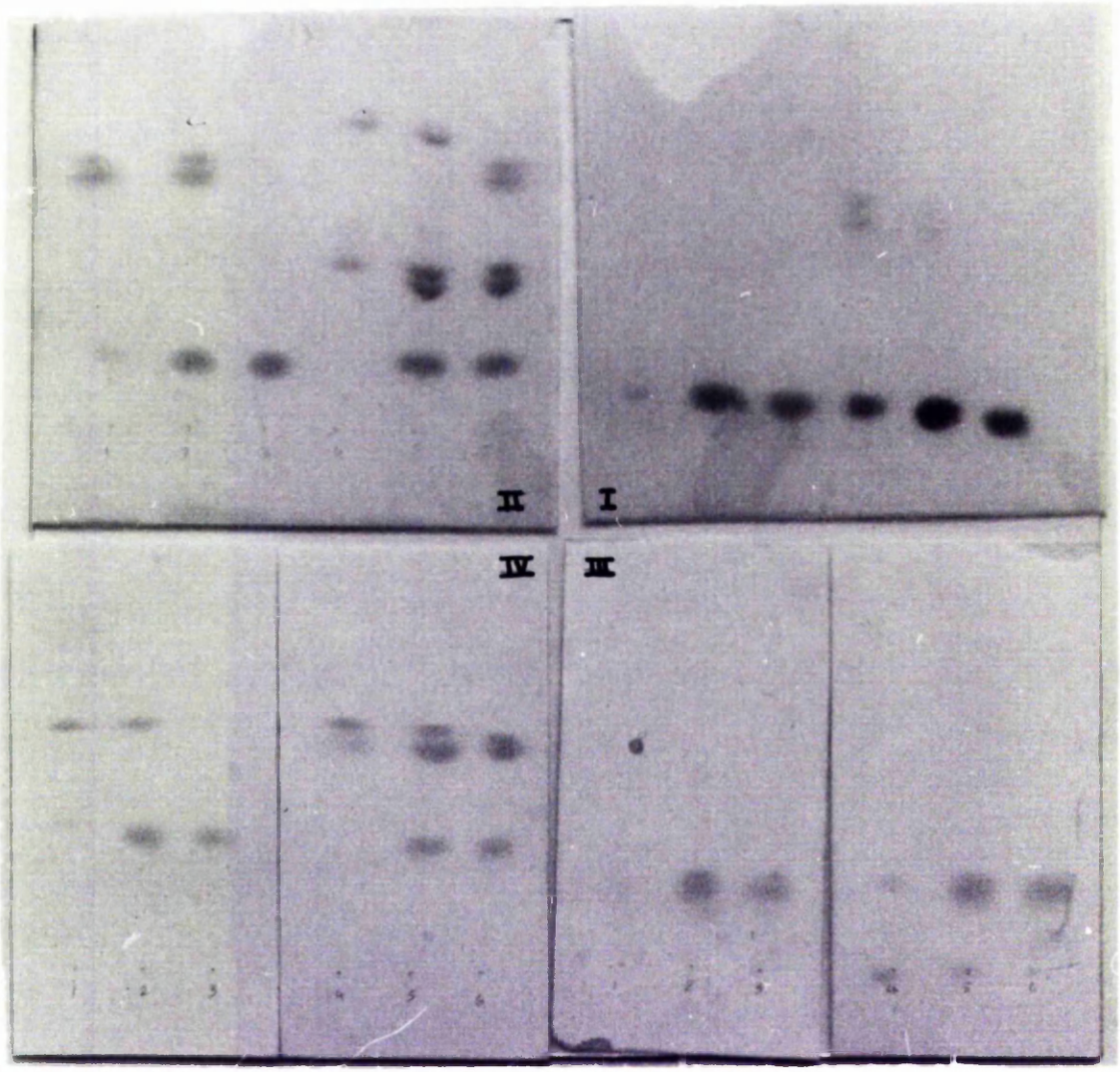


Fig. 30 B.

The N-acetylamines did not clearly separate from bulk of the amino acids (Fig. 30 I and II). For a better resolution and to increase the mobility of the compounds a buffer of lower pH was employed. At pH 2.0 all the three classes of compounds were separated (Fig. 31). The basic amino acids, lysine, arginine and histidine ran ahead of the monoamines, which in turn were separated from a broad band of neutral amino acids. At the trailing edge of this band ran Dopa and 5-HT. Finally the N-acetylated mono-amines were well separated from the amino acids, and ran as a slow moving compact band, without separating from each other. Cellulose sheets were found to be superior to silica gel.

3.1.3 Elution of the amino compounds from cellulose sheets

Various eluents such as H_2O, different proportions of, acetone/0.1N HCl, 0.1N HCl, and non-polar solvents like benzene and ethyl acetate were used to elute amines and amino acids from cellulose sheets. The most effective elution of all the three classes of compounds was achieved by acetone/0.1N HCl (9:1 v/v) Fig. 32. The recoveries of catecholamines and tyrosine from the cellulose sheets after electrophoresis were determined spectrophotometrically by the absorbance of the compounds at 280 nm, and those of amino acids by reacting with ninhydrin reagent (Moore & Stein, 1948). In Table 6 are given the recoveries of the representative compounds. In most cases the recovery was greater than 80% but in a few cases it was much lower, e.g. the very basic amino acid arginine.

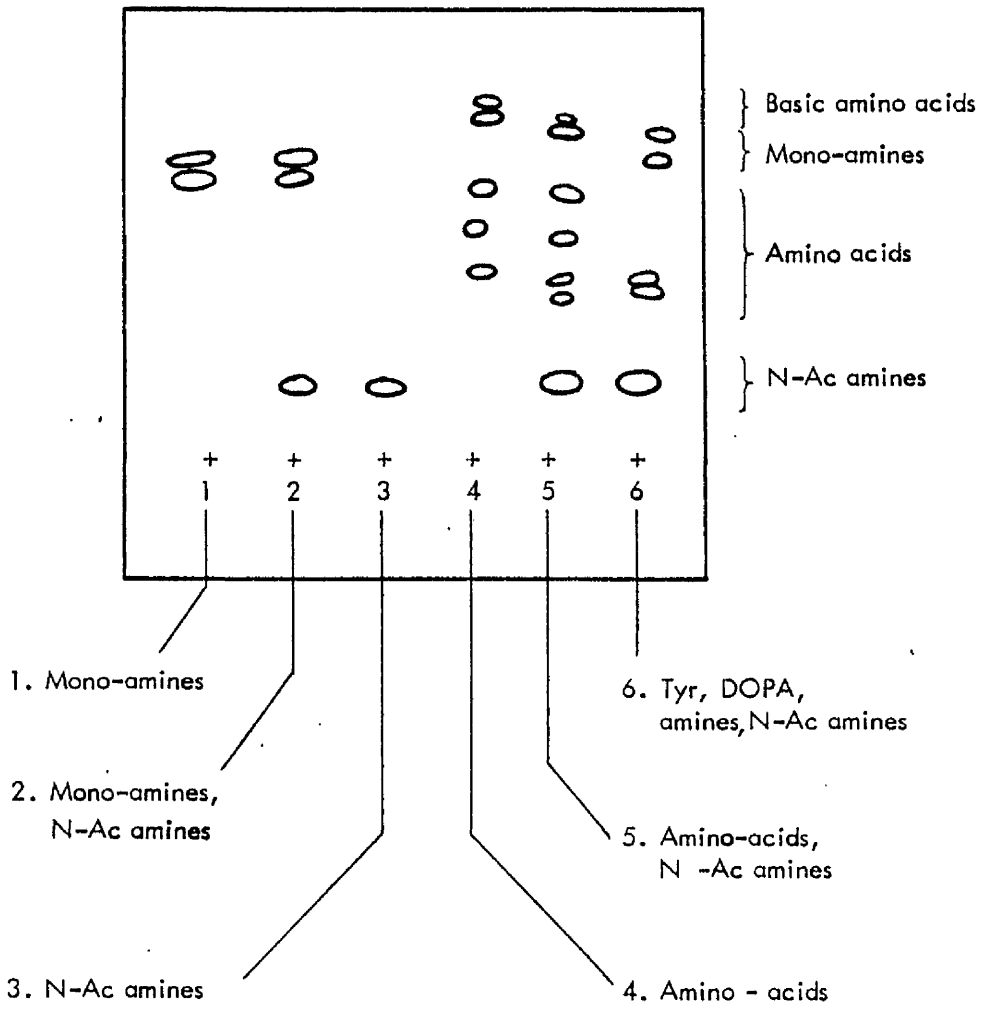


Figure 31. Electrophoretic separation of standard amines, N-acetylamines and amino acids on plastic backed cellulose sheets at pH 2.0, using 0.75 M formic acid buffer, by a 15 minute run with a potential difference of 400 v and 0.8 mA/cm current.

Fig. 32 - Extraction of Dans-derivatives of N-acetylamines (1), amino acids (2) and Dans-amines alongwith basic amino acids (3) from cellulose sheets after electrophoresis (details in Fig. 31). Unstained area of cellulose sheets corresponding to stained standard mixture was scraped into small tubes and extracted twice with the respective extracting agent. The contents in each vial were freeze dried and the residue redissolved in acetone. The mixture was subjected to two dimensional chromatography on polyamide sheets.

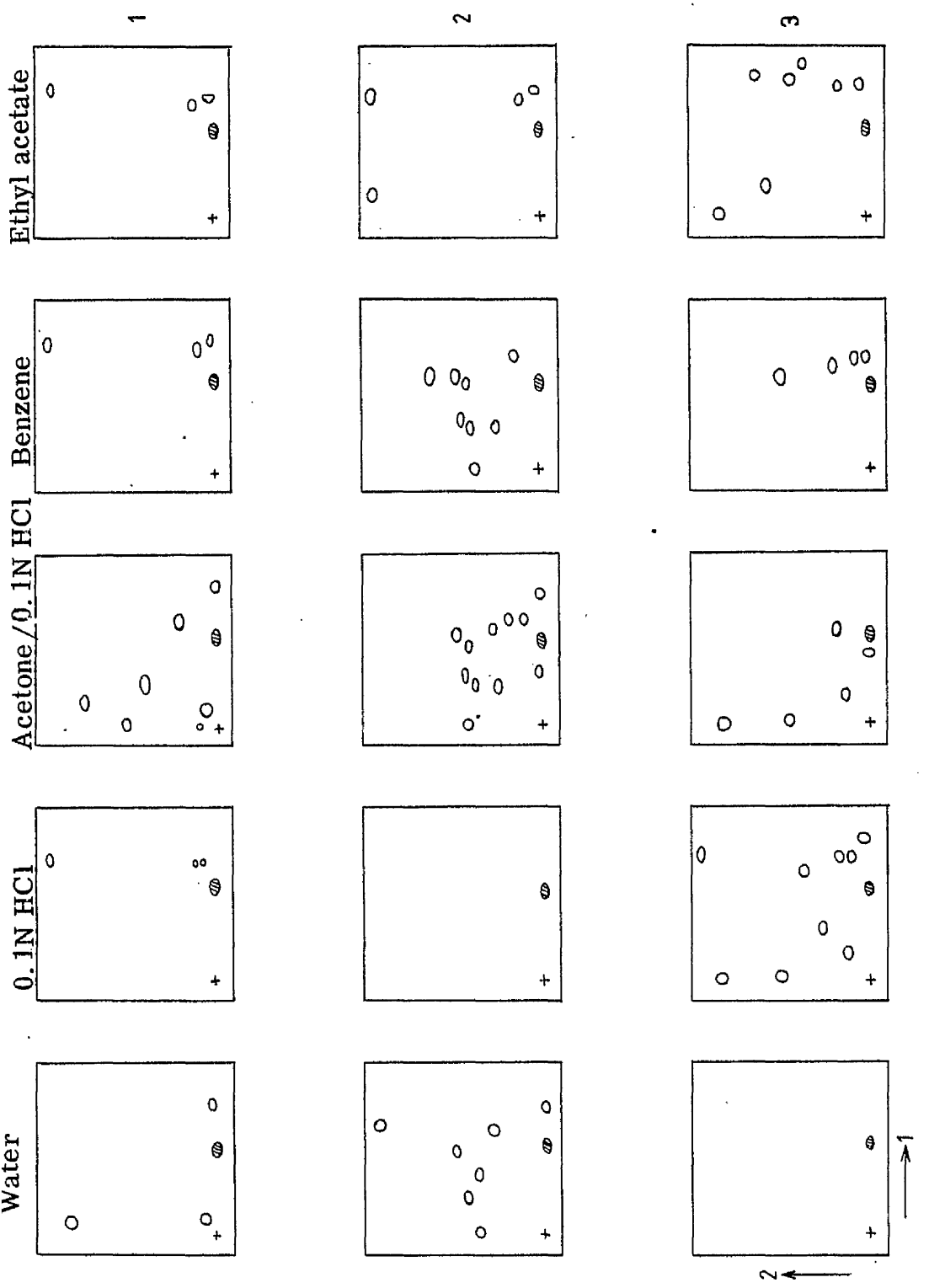


Figure 32.

Table 6. Recovery of amines and amino acids from cellulose sheet by elution with acetone /0.1NHCL (9:1 v/v)

Compound	% Recovery
Octopamine	92.7
N-acetyloctopamine	95.6
Tyrosine	84.4
Leucine	92.5
Methionine	83.0
Glutamic acid	84.9
Proline	90.7
Arginine	30.0

Known amounts of amines and amino acids were applied to cellulose sheets, and eluted with acetone /0.1N HCl (9:1 v/v).

The recoveries of octopamine, N-acetyloctopamine and tyrosine were determined spectrophotometrically at 280 nm. The amino acids were estimated by ninhydrin reagent.

3.1.4 Separation of Dans-derivatives by thin-layer chromatography

After the mixture of amino compounds had been partially resolved by electrophoresis, they were eluted, dansylated and further separated by chromatography on polyamide sheets. The two stages of the process are shown in Fig. 33. This allowed an almost complete and unambiguous identification of the complex mixtures.

3.1.5 Resolution of the Dans-amino acids by multiple development on polyamide layers

As mentioned in Section 3.1.1 single development of chromatograms is sufficient to clearly separate the various dansyl derivatives of mono-amines, but the amino acids show some overlaps. To ensure the complete separation from each other of the dansylated derivatives, four solvent mixtures were used in two-dimensional chromatography on polyamide layers (Fig. 34). After the usual two-dimensional development in 3% formic acid and Toluene/acetic acid (9:1 v/v) (Osborne, 1973)), the plate was again developed in the second direction in toluene/ethyl acetate/acetic acid (9:5:1 v/v/v). This solvent allowed the separation from iso-leucine of a component of the haemolymph and nervous tissue of the locust, identified as putrescine (Gould & Cottrell, 1974 and also see Section 3.3.7.1), and also ensured the separation of Dans-NH_2 from Dans-alanine. Finally to ensure a complete separation of the derivatives of aspartate and glutamate, a line was scored across as shown in Fig. 34c and the lower part developed in butyl acetate/methanol/acetic acid, 30:20:1 v/v/v (Brown and Perham, 1973). The various stages of the multiple development are shown in Fig. 34, A-C for standard amino acids and for haemolymph amino compounds in Fig. 35. Finally Fig. 36 represents the cumulative chromatogram showing the resolution of the standard

Figure 33 - Diagrammatic representation of the two stage separation of the amino acids, amines and N-acetylated amines. In the centre is the separation of the mixture into four main groups by electrophoresis. The polarity of the potential difference and the line of application are shown. On either side are the chromatograms on polyamide layers of the dansyl derivatives of the four groups of compounds. Solid numbered arrows indicate the solvents and the direction of development. The solvents were: 1. 3% formic acid and 2. toluene/acetic acid(9:1 v/v). The identify of the derivatives is as follows:

A. 1. N-acetyltyramine, 2. N-acetyloctopamine, 3. dansyl hydroxide.

B. 1. tyrosine, 2. leucine, 3. phenylalanine, 4. tyrosine, 5. isoleucine, 6. proline, 7. valine, 8. alanine and ammonia, 9. glycine, 10. glutamate and aspartate, 11. dansyl hydroxide, 12. dihydroxyphenylalanine.

C. 1. tyramine, 2. octopamine, 3. dansyl hydroxide.

D. 1. lysine, 2. γ-aminobutyric acid, 3. dansyl hydroxide, 4. histidine and arginine.

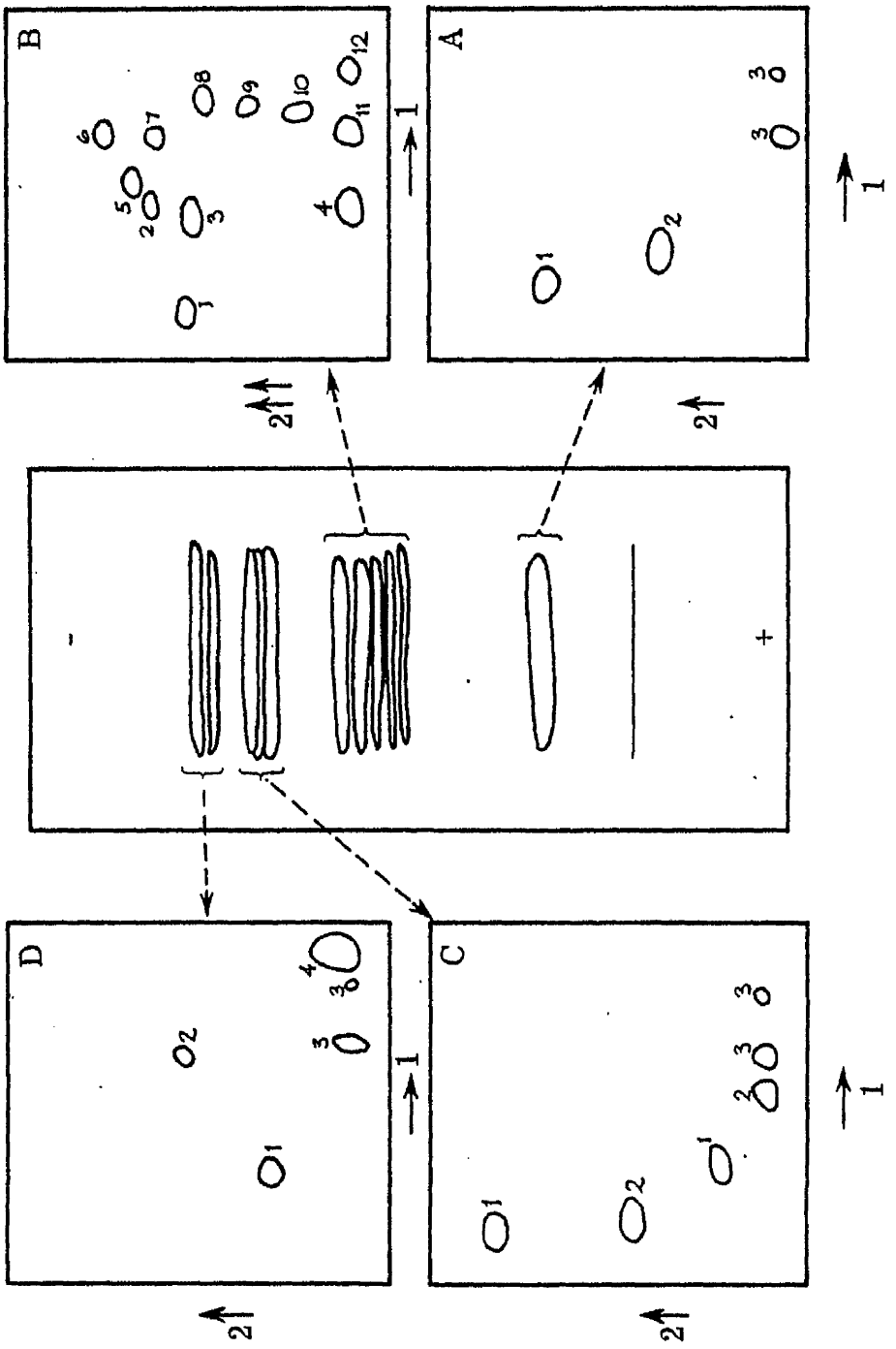


Figure 33.

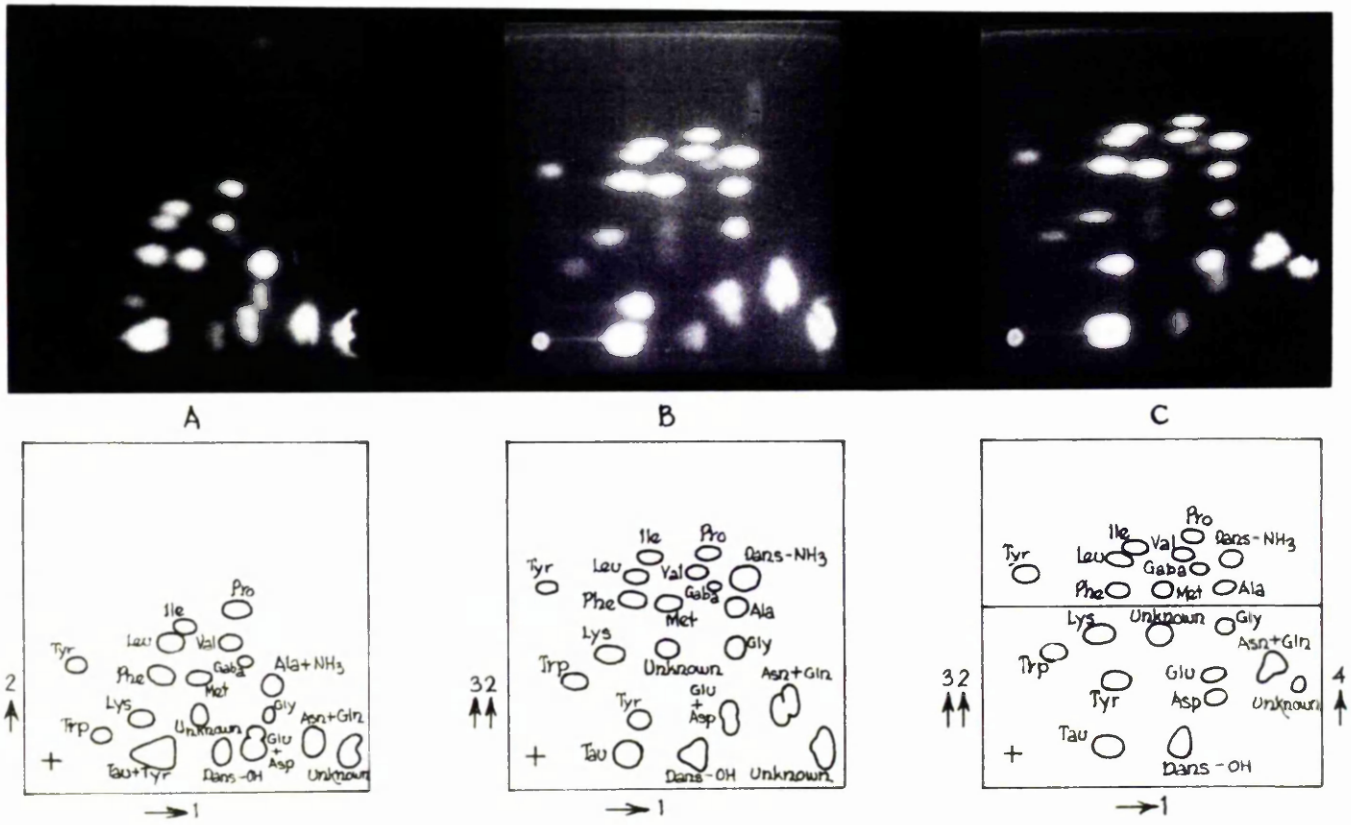


Figure 34. Photographs (top) and diagrams (bottom) show the separation of Dans-amino acids on polyamide sheets (5x5 cm). Solvents are: (1) 3% Formic acid, (2) Toluene/Acetic acid (9:1 v/v). (3) Toluene/Ethyl acetate/Acetic acid (9:5:1 v/v/v) and (4) Butyl acetate/Methanol/Acetic acid (30:20:1 v/v/v). The separation of mono-Dans-tyrosine from Dans-taurine and of Dans-NH_3 from Dans-alanine is achieved by the third solvent used in second dimension (B). The lower half of chromatogram is then run in the fourth solvent (C) to resolve Dans-glutamate from Dans-aspartate.

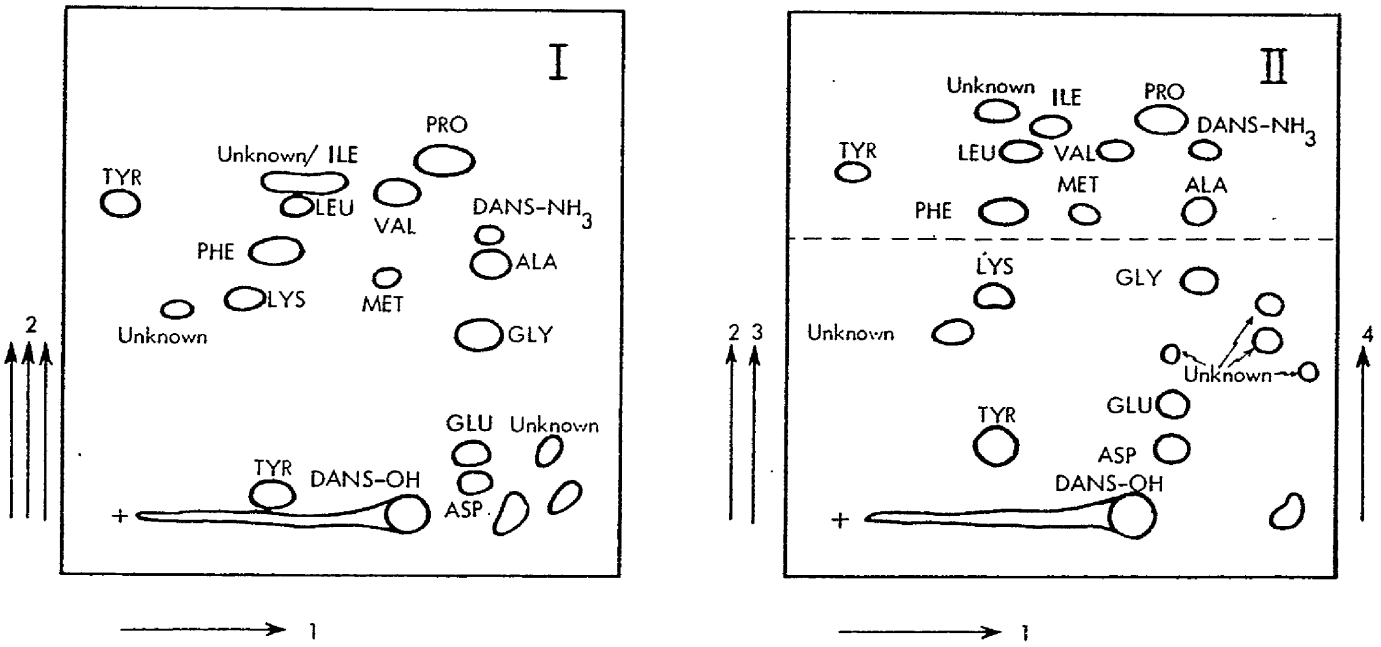


Figure 35. Bidimentional separation of Dans-derivatives of amino acids from haemolymph of Schiestocerca americana gregaria on polyamide Sheets (5x5 cm). The soluent systems used were; 1, 3% formic acid; 2, toluene/acetic acid (9:1 v/v); 3, toluene/ethylacetate/acetic acid (9:5:1 v/v/v); and 4, Butyl acetate/methanol/acetic acid (30:20:1 v/v/v).

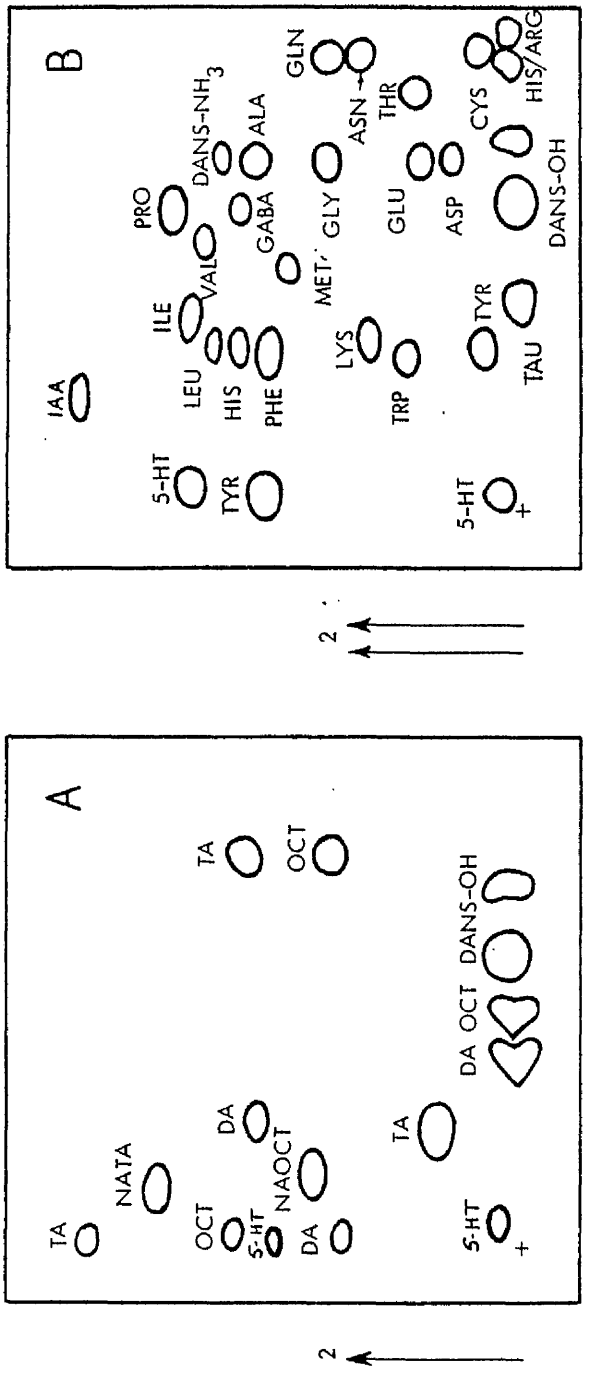


Figure 36. Bidimentional separation of Dans-amines + N-acetylamines (A) and Dans-amine Acids (B) on polyamide sheets (5x5 cm) using 3% formic acid as solvent in direction 1 and toluene/acetic acid (9:1 v/v) in direction 2.

dansyl-amino acids and dansyl amines along with the Dans-N-acetyl-mono-amines.

3.1.6 Yields of Dans- derivatives

Using mixtures of known amounts of amino acids and amines and reacting them with [^{3}H]-Dansyl-chloride of a known specific activity, an estimate was obtained of the extent of derivatization of different compounds. Recoveries from one experiment are shown in Table 7. There was a great deal of variation between different compounds, with amino acids generally giving the highest yield followed by the amines. These observations support the view that lower recovery of label from incomplete reaction of amines with Dans-Cl. The two N-acetylamines which were tested reacted poorly with Dans-Cl. This may have been due to the fact that the reaction was with the ring hydroxyl groups and not with amine groups which are masked with the acetyl group.

3.1.7 Amount of the nervous tissue required for the estimation of amino compounds

Table 8 represents the radioactivity recovered from the polyamide layers using different quantities of nervous tissue. In the analysis of the amino acids the incorporation of the label in the form of [^{3}H]-Dans-Cl was adequate with material from as little as 0.5mg of tissue. In the case of the amines and their N-acetylated derivatives larger quantities of tissue (5-10 mg) were required to ensure accuracy of estimation.

Table 7. Yields of dansyl derivatives of different compounds.

COMPOUND	YIELD (%)
Proline	24
Valine	23
Alanine	16
γ-aminobutyric acid	17
Glycine	24
Glutamate	17
Aspartate	10
Isoleucine	25
Leucine	23
Phenylalanine	19
Tyrosine	40*
Octopamine	15*
Dopamine	4*
Tyramine	29*
N-acetyl octopamine	11
N-acetyl tryamine	2

The figures are the means of duplicate samples from one experiment. The yields were calculated by scintillation counting of the derivatives after elution from the chromatograms.

* Figures for the amines and tyrosine represent the upper limit of the yield, as they represent the combined radioactivity of all the derivatives formed from each compound.

Table 8. Total incorporation of label into identified dansyl derivatives in extracts from different amounts of nervous tissue

Compound	Total radioactivity (dpm x 10^{-3}) recovered from the following amounts of tissue		
	0.5mg	1.0mg	2.0mg
Proline	170.3	343.0	565.9
Valine	3.3	6.6	28.5
γ-amino butyric acid	10.9	21.2	60.2
Alanine	6.9	13.5	36.9
Glutamate	5.7	11.3	23.6
Octopamine	1.4	8.8	60.6
Dopamine	2.2	7.2	31.4
Tyramine	0.7	1.7	2.7
N-acetyl octopamine	0.1	0.2	0.4
N-acetyl tyramine	0.2	0.6	0.7

Different volumes of extracts of 8 thoracic ganglia were reacted with ^{3}H-dansyl chloride, and the derivatives isolated, eluted and counted.

3.1.8 Quantitative accuracy of dansylation procedure

Quantitative estimates of amino compounds using only [^{3}H]-Dans-Cl reported by Osborne (1973); Osborne and Neuhoff (1974); Robertson and Osborne (1979); Meyer et al (1980), in my hands proved to be imprecise and inconsistent (Table 8). By using the double isotope method, however, a high degree of accuracy and precision was achieved. In Table 9 are given the quantitative recoveries of the amino acids mixed with known amount of [^{14}C]-labelled amino acids as internal standards, and taking a portion of the mixture for further dansylation and microchromatography. The results obtained accurately reflect the known concentrations of the amino acids. It shows that mixing of internal radiolabelled standards prior to dansylation or other treatment of the tissue can account for any losses during the handling of the sample and accurate estimates can be made without correction to determine recoveries. It is evident from Table 10 that by double isotope procedure accurate quantitative estimate could be made as shown by the recovery of the standard amino acids added to the brain homogenate and haemolymph of locust. In Table 11 are given the mean values and standard deviations of the concentrations of amino acid in different samples of haemolymph from the same locust. In only a few cases did the range of the standard deviation exceed 10% of the mean of the three samples. These results exemplify the precision of the method.

Table 9. Recovery of standard amino acids with the double isotope dansylation procedure

Amino acid	A		B		
	% recovery		% recovery		% Mean ± S.D.
	1	2	1	2	
Alanine	98	108	114	99	104.8 ± 6.6
Glycine	96	97	102	98	98.3 ± 2.3
Leucine	100	101	100	104	101.7 ± 1.7
Phenylalanine	98	102	108	104	103 ± 3.6
Proline	94	96	106	100	99.0 ± 4.6
Valine	99	98	101	97	98.8 ± 1.5

A known amount of [^{14}C]-amino acids was added to 5 nmole solution of standard amino acids. After mixing the contents were divided into two equal portions (1 and 2) and reacted separately with equal volume of [^{3}H]-Dans-Cl. From the ratio of $^{3}H/^{14}C$ the amount of each amino acid recovered was worked out with reference to standard curves. A and B are values of separate experiments.

Table 10. Recovery of added amino acids to haemolymph and nervous tissue of locust, Schistocerca americana gregaria.

Amino Acids	Expected values	Observed values	% Rec.	P
Nervous tissue	n = 4	n = 4		
Alanine	36.37 ± 2.46	36.20 ± 6.31	99.5	0.9605
Aspartate	14.98 ± 2.08	14.24 ± 2.85	95.1	0.6865
Glutamate	14.04 ± 1.90	12.44 ± 2.08	88.7	0.3383
Haemolymph	n = 3	n = 4		
Glycine	5.23 ± 0.23	5.30 ± 0.78	101.3	0.8941
Iso-leucine	0.71 ± 0.03	0.74 ± 0.03	103.5	0.3910
Leucine	0.80 ± 0.02	0.80 ± 0.04	100	0.9220
Phenylalanine	1.28 ± 0.07	1.27 ± 0.27	99.2	0.9302
Proline	3.97 ± 0.25	3.88 ± 0.46	97.7	0.7726
Valine	1.09 ± 0.05	1.01 ± 0.02	92.6	0.0345

Deproteinized tissue extract and haemolymph samples were divided into two portions. To one portion a known amount of standard amino acids was added and both samples were analyzed for the presence of amino acids.

Table 11. Precision of the estimates of the concentrations of amino acids in locust haemolymph by the double isotope method

Amino-acid	Concentration (µmol/ml)	S.D./Mean (%)
Alanine	0.63 ± 0.04	6
Aspartate	0.38 ± 0.03	8
Glutamate	0.57 ± 0.03	5
Glycine	9.47 ± 0.38	4
Isoleucine	0.43 ± 0.05	11
Leucine	0.60 ± 0.03	5
Phenylalanine	1.62 ± 0.08	5
Proline	6.93 ± 0.41	6
Tyrosine	1.78 ± 0.18	10
Valine	1.31 ± 0.18	13

Concentrations are the means (± S.D.) of three samples of haemolymph of a single locust run in parallel. Internal standards were used, and the concentrations calculated from ^{3}H to ^{14}C ratio.

3.1.9 Extraction of amino compounds from the nervous tissue

In Table 12 are given the comparative yields of amino acids extracted from the thoracic ganglia of locusts employing different solvents. It is evident with all three procedures tried there were no significant differences in the yield of amino acids except for aspartic acid and glycine. Slightly higher values of aspartic acid were obtained by keeping the tissue frozen prior to homogenization (i.e. immediate freezing possibly prevents metabolism by the highly active aminotransferase, See section 4.5) while glycine values were found to be higher after extraction with 0.3M $HClO_4$.

Considering this, it was decided to homogenize the tissue in insect saline, kept at $0^{o}C$ which had the added advantage of the extraction of amino compounds in the same medium after in vitro incubations. The homogenates were kept in ice until further processed, a period of no more than 5 minutes.

The results in Table 13 show that homogenizing the tissue immediately after the dissection or keeping the tissue in ice-cold saline up to 30 minutes (the average time involved in the dissection of 5-6 locusts) did not significantly alter the concentration of most of the free amino acids. On the other hand leaving the tissue even at low temperature ($0^{o}C$) for longer periods caused a substantial decline in free amino acids. Further experiment showed that during this period of 90 minutes (time required to dissect 15 locusts), there was loss of the free amino acids to the medium ranging from 8% to as much as 48% (Table 14).

Table 12. Comparison of procedures of homogenization on the endogenous concentrations of free amino acids in the nervous tissue of Schistocerca americana gregaria

Amino Acid	Extracting agent		
	Insect saline	Insect saline (tissue previously) frozen	0.3M $HClO_4$
	n=4	n=4	n=4
Alanine	9.94 ± 1.79	11.03 ± 0.54	10.67 ± 1.76
Aspartic acid	6.37 ± 1.36	9.75 ± 1.09*	5.79 ± 0.70
Gaba	2.04 ± 0.25	2.89 ± 0.14	2.83 ± 0.38
Glutamate	8.22 ± 0.98	10.88 ± 1.40	8.60 ± 1.07
Glycine	4.94 ± 1.33	3.86 ± 0.54	8.66 ± 1.51*
Isoleucine	0.13 ± 0.03	0.11 ± 0.01	0.10 ± 0.05
Leucine	0.16 ± 0.05	0.19 ± 0.03	0.26 ± 0.10
Phenylalanine	0.15 ± 0.03	0.16 ± 0.02	0.16 ± 0.05
Proline	9.61 ± 1.60	12.38 ± 0.65	12.35 ± 0.46
Tyrosine	0.23 ± 0.05	0.23 ± 0.07	0.21 ± 0.09
Valine	0.95 ± 0.26	0.92 ± 0.13	0.93 ± 0.16

Values are expressed (µmol/g wet weight tissue) as mean ± S.D. The locust were immobilized by keeping at -20°C for 10 minutes before dissection. All the homogenates were made in ice. The significance of difference in values was checked by students t-test.

*Significantly different from the other two treatments.

Table 13. Changes in the endogenous levels of free amino acids after leaving the tissue in the saline at 0°C for various lengths of time

Amino Acid	Initial Concentration n=6	30 minutes n=4	90 minutes n=4
Alanine	11.60 ± 0.54	13.67 ± 2.46	9.60 ± 1.39
Aspartic Acid	6.17 ± 0.48	8.16 ± 2.08	3.52 ± 0.40
Gaba	2.89 ± 0.25	2.82 ± 0.43	1.57 ± 0.13
Glutamic acid	7.08 ± 1.20	7.22 ± 1.91	4.99 ± 0.26
Glycine	2.06 ± 0.43	1.82 ± 0.27	1.94 ± 0.50
Isoleucine	0.19 ± 0.03	0.14 ± 0.02	0.12 ± 0.03
Leucine	0.13 ± 0.05	0.11 ± 0.04	0.21 ± 0.05
Phenylalanine	0.55 ± 0.09	0.14 ± 0.06	0.13 ± 0.01
Proline	7.46 ± 2.67	8.26 ± 1.41	7.63 ± 0.38
Tyrosine	0.97 ± 0.40	0.61 ± 0.10	0.36 ± 0.08
Valine	0.89 ± 0.25	0.65 ± 0.19	0.56 ± 0.10

The values are presented as μmol/g wet weight tissue and are average ± S.D. of at least 4 separate estimates.

Table 14. Release of the amino acids to the medium after leaving the nervous tissue at 0°C for 90 minutes

Amino Acid	Total (in tissue and medium)	In tissue	% released to the medium
Alanine	13.95 ± 2.00	9.6 ± 1.39	31.1 ± 4.3
Aspartic acid	6.34 ± 0.95	3.52 ± 0.40	44.2 ± 3.8
Gaba	2.08 ± 0.28	1.57 ± 0.13	24.3 ± 4.6
Glutamic acid	7.74 ± 0.44	4.79 ± 0.26	38.0 ± 4.3
Glycine	3.73 ± 0.74	1.94 ± 0.50	48.1 ± 7.5
Isoleucine	0.13 ± 0.04	0.12 ± 0.03	8.2 ± 9.0
Leucine	0.23 ± 0.06	0.21 ± 0.05	5.2 ± 10.4
Phenylalanine	0.19 ± 0.05	0.13 ± 0.01	28.9 ± 17.4
Proline	11.81 ± 1.03	7.63 ± 0.38	35.3 ± 2.7
Tyrosine	0.49 ± 0.11	0.36 ± 0.08	25.5 ± 2.4
Valine	0.76 ± 0.17	0.56 ± 0.10	25.2 ± 6.9

The values are presented as μmol/g wet weight tissue and are average ± S.D. of at least 4 separate estimates

In reported experiments needing larger amounts of tissue, batches of 30-50 insects have often been dissected (Hiripi & S. Rozsa, 1973; J.F. Donnellan, personal communication). There is always a danger of the loss of the metabolites during the long storage period prior to experimentation. On the other hand, if the tissue is immediately frozen in liquid nitrogen, in dry ice, or kept at -20°C in deep freeze, subsequent thawing of the tissue results in neuronal disintegration (histological observations not shown here) which renders it unsuitable for metabolic studies.

Therefore, to minimize changes of amino acid concentrations, during the course of these investigations the dissections were carried out in small batches requiring no more than 30 minutes to accumulate sufficient tissue.

3.1.10 a) Body weight and volume of haemolymph of locust

In Table 15 are given the mean weights of the male and female adult locusts. The female locusts are bigger than the male locusts, weighing on average 3.56g and 2.19g/locust respectively. This means that although volume of haemolymph is 546µl for a female locts and 332 µl for a male locust the ratio of this volume to body weight is the same for both sexes (152-153 µl/g). These findings are comparable to those of Lee (1961) for Schistocerca (Table 15). The reported results for Locusta migratoria show higher values for volume of haemolymph than Schistocerca; nevertheless these are consistent within a species.

Table 15. The body weight and haemolymph volume of the adult locusts

	Sex	Body weight (g)	Volume of haemolymph µl	Ratio of haemolymph to body weight (µl/g)	Reference
Schistocerca americana gregaria	Male	2.189±3.06(5)	332±50(4)	152	Present study
	Female	3.559±0.476(5)	546±112(3)	153	
Schistocerca americana ganglia	Male	1.168±0.118(6)	165±20(6)	142	Lee (1961)
	Female	1.318±0.024(4)	195±6(4)	148	
Locusta migratoria	Male	1.705±0.298	359±151	211	Loughton and Tobe (1969)
	Female	2.521±0.690	595±338	236	
Locusta migratoria		1.256±0.483	253±18	201	Hill and Goldsworthy (1968)

Volume of haemolymph was estimated by radiolabelled inulin dilution technique as given in section 2.4.2. Values in the parenthesis are the number of estimations.

3.1.10 b) Protein content of the nervous tissue of locust

Due to the small amount of nervous tissue (~2mg) available from a single locust, and the difficulty in precisely weighing this amount of tissue, the wet weight of the tissue was determined indirectly by estimating its protein content.

The nervous tissue of locust contains 53 µg protein per mg of fresh weight (Table 16). Cerebral and metathoracic ganglia show a higher (but statistically non-significantly different) content of protein than meso-thoracic ganglia. This value is similar to those found by Clement (1979) for thoracic ganglia of locust (53µg/mg wet weight of tissue) and by Taylor and Newburgh (1979) in the nerve cord of Manduca sexta (54µg/mg fresh weight tissue)

3.1.11 Qualitative survey of amino compounds in the nervous tissue and haemolymph of locust

Thin layer chromatography on polyamide by the method previously described following the dansylation of extracts from the nervous tissue and haemolymph of locust resolved as many as 39 different derivatives (Fig. 37). Most of them were immediately identifiable, but some unknown spots also appeared on the microchromatograms. As many of these were always present in the known mixture of Dans-amino acids as well as in tissue extracts, they were taken to be by-products of the dansylation procedure.

Generally all the important amino acids previously reported to be present in insect CNS (Ray, 1964; Osborne and Neuhoff, 1974; Schlesinger, 1977) were found in the nervous tissue while some occurred in the haemolymph either in very low concentration or were undetectable e.g. Gaba could not be detected in the haemolymph.

Table 16 Determination of protein in the nervous tissue of locust in relation to fresh wet weight

Ganglia	No. of Ganglia	Weight of Ganglia (mg)	Total Protein (µg)	µg Protein/ mg fresh weight	Mean (± S.D.) µgProtein/ mg fresh weight
Cerebral	1	2.4	104	43.47	
			141	58.89	
	2	4.4	290	65.91	
			265	60.23	
	3	5.7	330	57.89	
			313	54.91	56.88±7.50
Meso-Thoracic	2	2.0	85	42.50	
			118	59.16	
	4	4.4	175	39.77	
			165	37.50	
	6	4.7	230	48.94	
			230	48.94	46.14±7.92
Meta-Thoracic	2	1.7	91	53.43	
	4	5.0	262	52.5	
			222	44.0	
	6	6.0	395	65.83	
			390	65.00	56.15±9.22
Combined Average					52.83±9.15

Fig. 37 - Autoradiographs of micochromatograms (top) and diagrams from them (bottom) showing the main amino compound present in the thoracic ganglia (A) and haemolymph (B) of locust Schistocerca americana gregaria.

1. starting point
2. Dans-OH*
3. Dans-taurine
4. Dans-OH
5. Dans-N-tyrosine
6. Dans-Cystein
7. Dans-Lysine
8. unknown
9. Dans tryptophan
10. unknown
11. Dans-bis tyrosine
12. Dans-bis-serotonine
13. unknown
14. Dans-putrescine
15. Dans-isoleucine
16. Dans-leucine
17. Dans-phenylalanine
18. Dans-methionine
19. unknown
20. unknown
21. Dans-proline
22. Dans-valine
23. Dans-Gaba
24. Dans-alanine
25. Dans-NH_2
26. unknown
27. Dans-glycine
28. unknown
29. unknown
30. Dans-putrescine
31. Dans-glutamine
32. Dans-arginine, lysine, histidine
33. Dans-cystine
34. unknown
35. Dans-OH
36. Dans-aspartic acid
37. Dans-glutamic acid
38. unknown
39 unknown

* Reaction of Dans-Cl with water. Details of chromatographic procedure in material and methods section

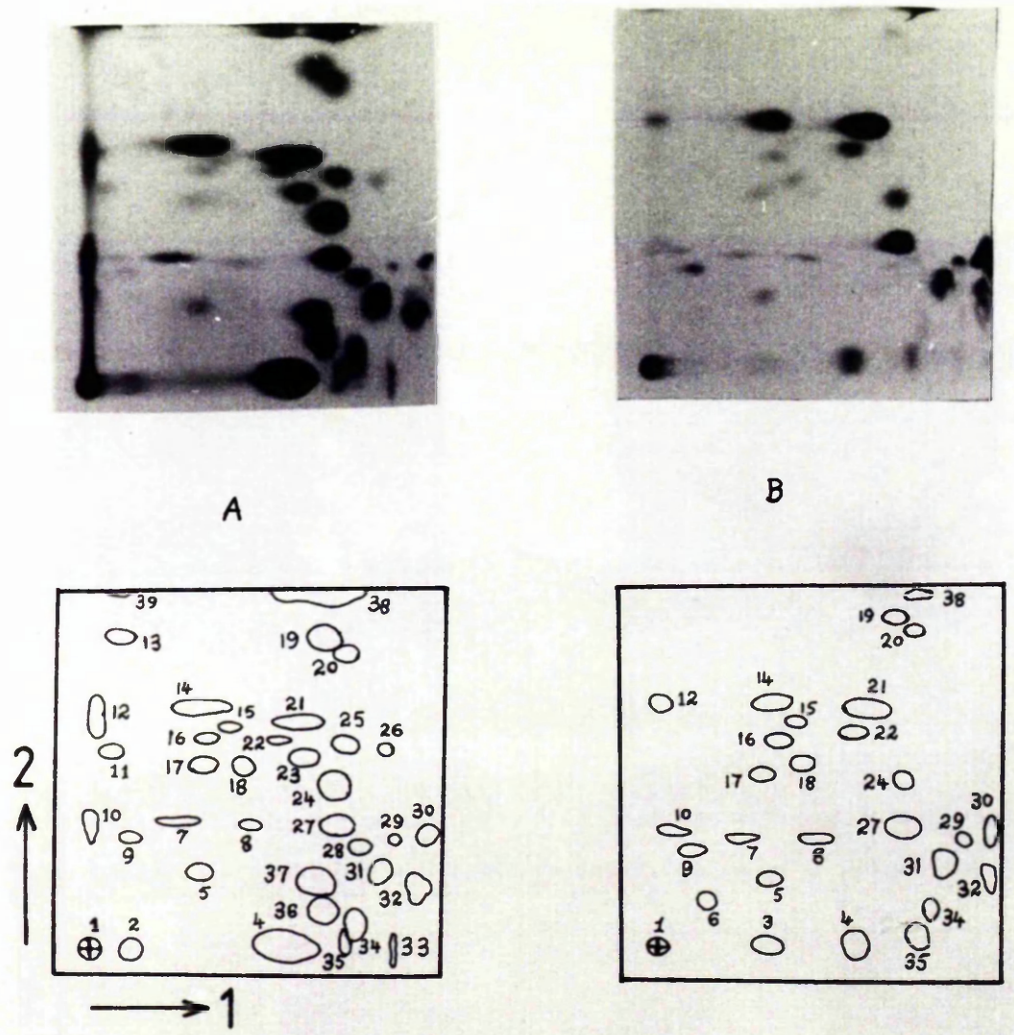


Figure 37.

Similarly aspartate and glutamate were virtually missing from haemolymph. These compounds were present in the nervous tissue in significant amounts, which is in keeping with these compounds having either a neurotransmitter function or an important role in the metabolism of the tissue.

The presence of taurine (for further confirmation of the identity see Section 3.2.7 and (Osborne, 1973 and Meyer et al., 1980) in the haemolymph was recorded whereas it was not detectable in the extracts of nervous tissue. Other differences observed included the greater quantity of alanine in the nervous tissue compared to the haemolymph. Derivatives of proline, glycine and putrescine were predominant in the samples of haemolymph and nervous tissue. The occurrence of an individual amino acid in such a high concentration could indicate the possibility of the substance having a functional role, particularly in metabolism. The other identifiable amino acids include, tyrosine, cystegine, lysine, tryptophan, iso-leucine, leucine, phenylalanine, methionine, valine, alanine, glutamine, arginine and histidine.

3.1.12 Quantitation of amines

Single lable dansylation (Section 3.1.7) showed that nearly all the important amino acids and biogenic amines i.e. tyramine, dopamine, octopamine and their N-acetyl-derivatives, NATA and NAOA could be detected (Table 17). Quantitation of the amines by the use of single radiolabel ([^{3}H]-Dans-Cl) was too inaccurate to yield consistent results in the present study. Those shown in Figure 38 are representative curves (from a number of estimates) of [^{3}H]-Dans-DA, OA, TA and Nor-valine (used as internal standard to determine the

Table 17. Incorporation of radioactivity into different compounds in extract from thoracic ganglia of locust (Schistocerca americana gregaria) by reaction with [^{3}H]-Dans-Cl

Compound	Radioactivity (dpm) associated with compounds extracted from different quantities of nervous tissue		
	0.5mg	1mg	2mg
Proline	170334	342995	565909
Valine	3320	6641	28450
Gaba	10881	21211	60138
Alanine	6899	13468	36846
Glycine	4235	28908	47930
Glutamic acid/ Aspartic acid	5692	11257	23585
Octopamine	2387	8887	60590
Dopamine	2121	7192	31393
Tyramine	709	1728	2690
N-acetyltyramine	333	625	800
N-acetyloctopamine	240	248	466

The amino compounds, after extraction from the tissue, were reacted with [^{3}H]-Dans-Cl (specific activity; 500μCi/ml). Identical volumes of the derivatives were chromatographed on polyamide sheets (5x5cm), and their position revealed by examination in U.V. light. Unlabelled carriers were used to make visible the Dans-amine and Dans-N-acetylamine. The fluorescent spots were cut out and the radioactivity determined by scintillation spectrometry.

Fig. 38 - Standard curves for [^{3}H]-Dans-amines. 10, 100, 500 and 1000 p moles in 4µl of 50mM $NAHCO_3$ of individual amines were reacted with 4µl of [^{3}H]-Dans-Cl (Specific activity 500 µCi/ml). 1 µl of reaction mixture was resolved on polyamide sheet alongwith unlabelled Dans-amines to locate the derivatives. Various spots were visualized in U.V. light and cut out. Radioactivity associated with derivative was measured by liquid scintillation spectrometry. Nor-valine was included as internal standard to work out the recovery

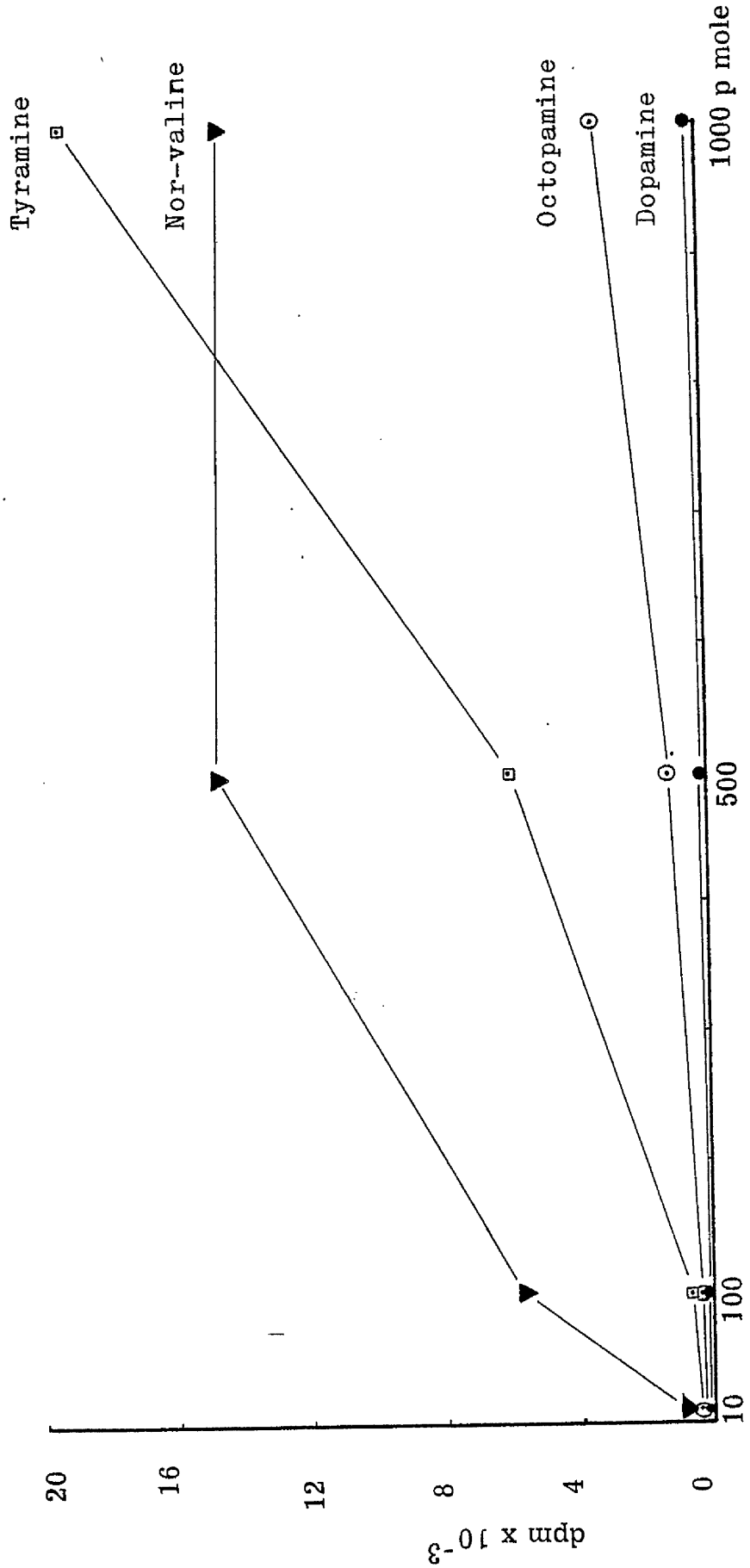


Figure 38. Standard curves of amines after reaction with [^{3}H]-Dans-Cl.

recovery, Seiler, 1970; Osborne, 1973). It is evident from these figures that it was difficult to get a linear relationship between the concentration of amines and their dansyl derivatives. Therefore, it was decided to apply the double isotope technique of Brown and Perham (1973) in all quantitative work. Due to the dearth of commercially available [^{14}C]-amines, particularly [^{14}C]-OA, and [^{14}C]-N-acetylamines, the attempts to quantify the amines and their N-acetyl-derivatives with this procedure was abandoned. Nevertheless, the single label dansylation using [^{14}C]-Dans-Cl followed by autoradiography was sufficient to reveal qualitatively (and semi-quantitatively) any excessive accumulation or release of these compounds into the incubation media in different experimental circumstances.

3.1.13 Quantitation of amino acids

Results obtained with double isotope procedure for the concentrations of free amino acids in the haemolymph and nervous tissue are presented in Table 18. The high standard deviation shown for some amino acids was due to individual variation among locusts and not to the imprecision of the method. Wyatt et al. (1973) and Stevens (1961) found a great variability in the haemolymph of cockroaches obtained from the same colony. This is a further argument for working as much as possible with the tissue from one locust and against pooling tissue from various insects.

Glycine and proline (10.94 and 9.54 µmol/ml respectively) were the amino acids found in highest concentration in the haemolymph. At the other end of the concentration range were glutamate and aspartate (0.49 and 0.72 µmol/ml). Gaba could not be detected in the haemolymph.

Table 18. Concentrations of free amino acids in adult locust *Schistocerca americana gregaria*

Amino Acid	Haemolymph (µmol/ml)	Nervous tissue	
		Cerebral ganglia (µmol/g wet weight)	Thoracic ganglia (µmol/g wet weight)
	n=5-11	n=6	n=9
Alanine	1.42±0.62	7.81±0.29	11.03±0.50
Aspartic acid	0.72±0.50	3.93±0.47	5.17±0.73
Gaba	-	1.03±0.30	2.89±0.15
Glutamic acid	0.49±0.14	4.66±0.82	6.83±1.25
Glycine	10.94±2.19	2.57±0.38	3.86±0.43
Isoleucine	1.16±0.41	1.37±0.07	1.90±0.63
Leucine	1.09±0.26	0.23±0.03	1.24±0.51
Phenylalanine	1.37±0.80	0.16±0.03	0.55±0.09
Proline	9.45±1.93	9.34±0.53	7.46±2.67
Tyrosine	1.63±0.26	0.41±0.04	0.97±0.40
Valine	1.95±0.79	1.86±0.14	1.89±0.58

The other amino acids estimated were valine (1.95µmol/ml), alanine (1.42µmol/ml), leucine (1.09µmol/ml), isoleucine (1.16µmol/ml), phenylalanine (1.37µmol/ml) and tyrosine (1.63µmol/ml).

Proline was found in significantly higher concentration than other amino acids both in cerebral and thoracic ganglia. This was in accordance with findings for many insects by other investigators (Cockroach, Ray, 1964; Werman, 1972; Osborne, 1973 and Locust, Schlesinger, 1977). A considerable difference was observed between haemolymph and nervous tissue in the concentrations of glycine, phenylalanine, glutamate, aspartate and alanine (Table 18). Glycine occurs at a higher concentration (10.94 µmol/ml in haemolymph), whereas a lower concentration of 3.68µmol/g was found in the thoracic ganglia and 2.57µmol/g tissue in cerebral ganglia. Alanine, aspartate and glutamate were found in the nervous tissue in about 10 times the concentration present in the haemolymph. In the case of phenylalanine the concentration in the tissue was lower than that in the haemolymph. It was 1.37µmol/ml in haemolymph and 0.55 and 0.16µmol/g tissue in thoracic and cerebral ganglia respectively.

3.1.14 Total free amino acids in thoracic ganglia (plus media) after incubations in various media

In an attempt to optimize the incubation conditions the effect of suspending the nervous tissue in a variety of media was investigated. The total concentration of free amino acids at the end of 60 minutes in different media is given in Table 19 and the net change as compared to the normal concentrations is presented in Table 20. The main features can be described as follows:-

Table 19. Total free amino acids in, and released from, the thoracic ganglia of Schistocerca americana gregaria after a 60 minute incubation in various media

Amino acid	Insect Saline n=3	Saline + 10mM KCN n=3	Saline + 10mM glucose n=4	Iso-osmolal saline + 10mM glucose n=4	Iso-osmolal saline; ganglia pre-incubated n=3	Concentration in freshly dissected tissue n=9
Alanine	23.58±2.02*	24.87±3.02***	34.83±0.96*	32.96±4.88***	11.23±0.78	11.03±0.50
Aspartic acid	3.93±0.48^{+++}	2.08±0.16^{+}	6.54±0.64^{+++}	5.94±0.23	5.26±0.61	5.17±0.73
Gaba	2.74±0.17	3.53±0.07***	4.86±0.97^{++}	3.06±0.57	3.76±0.33^{++}	2.89±0.15
Glutamic acid	6.77±0.39	4.40±0.48^{++}	7.51±0.45	7.72±0.50	4.48±0.50^{++}	6.83±1.25
Glycine	3.75±0.16	3.51±0.51	5.49±0.67^{++}	5.95±0.82^{++}	24.80±2.51*	3.86±0.43
Iso-leucine	1.12±0.02^{+++}	0.80±0.09^{++}	0.67±0.14^{++}	0.94±0.05^{+++}	2.47±0.67	1.90±0.63
Leucine	0.88±0.17	0.93±0.13	0.75±0.21	1.11±0.15	2.07±0.54	1.24±0.51
Phenylalanine	0.59±0.01	0.62±0.03	0.67±0.21	0.36±0.03^{++}	2.43±0.06*	0.55±0.09
Proline	2.45±0.58^{++}	7.14±0.76	5.07±0.68	4.32±0.96	8.86±0.11	7.46±2.67
Tyrosine	0.96±0.13	0.58±0.07	1.35±0.12	1.36±0.22	7.87±0.73*	0.97±0.40
Valine	1.31±0.12	1.05±0.17^{+++}	1.50±0.10	1.50±0.25	5.14±0.28**	1.89±0.58

Amino acid concentrations were estimated by the double isotope dansylation method, and are presented (Mean ± S.D.) as µmol/g wet weight nervous tissue. The incubations were carried out at 37°C in fully oxygenated media. The composition of various media is given in section 2.4.5 . Level of Significant difference from normal concentration; P = *<0.0005, **<0.001 ***<0.005; $^{+}$<0.01, $^{++}$<0.05, $^{+++}$<0.1.

Table 20. Net change in free amino acids in the thoracic ganglia of Schistocerca americana gregaria after a 60 minute incubation in various media.

Amino acid	Insect Saline n=3	Saline + 10mM KCN n=3	Saline + 10mM glucose n=4	Iso-osmolal saline + 10mM glucose n=4	Iso-osmolal saline; ganglia pre-incubated n=3	Concentration in freshly dissected tissue n=9
Alanine	+12.55 (114)	+13.84 (125)	+23.8 (216)	+21.93 (199)	+0.2 (1)	11.03±0.50
Aspartic acid	-1.24 (24)	-3.09 (60)	+1.37 (26)	+0.77 (15)	+0.09 (2)	5.17±0.73
Gaba	-0.15 (5)	+0.64 (22)	+1.97 (68)	+0.17 (6)	+0.87 (30)	2.89±0.15
Glutamic acid	-0.06 (1)	-2.43 (36)	+0.68 (10)	+0.89 (13)	-2.35 (34)	6.83±1.25
Glycine	-0.11 (3)	-0.35 (9)	+1.63 (42)	+2.09 (54)	+20.94 (543)	3.86±0.43
Iso-leucine	-0.78 (41)	-1.1 (58)	-1.23 (65)	-0.96 (51)	+0.57 (30)	1.90±0.63
Leucine	-0.36 (29)	-0.31 (25)	-0.49 (40)	-0.13 (10)	+0.83 (67)	1.24±0.51
Phenylalanine	+0.04 (7)	+0.07 (13)	+0.12 (22)	-0.19 (35)	+1.88 (342)	0.55±0.09
Proline	-5.01 (67)	-0.32 (4)	-2.39 (32)	-3.14 (42)	+1.4 (19)	7.46±2.67
Tyrosine	-0.01 (1)	-0.39 (40)	+0.38 (39)	+0.39 (40)	+6.90 (711)	0.97±0.40
Valine	-0.58 (31)	-0.84 (44)	-0.39 (21)	-0.39 (21)	+3.25 (172)	1.89±0.58

Values are expressed as µmol/g wet weight tissue. The number in parenthesis give the % change as compared to endogeneous concentrations.

3.1.14.a Incubation in saline without glucose

The incubation of thoracic ganglia in simple saline produced a slight (non-significant) decline in the concentration of Gaba, glutamate, glycine, leucine, phenylalanine, tyrosine and valine. Alanine on the other hand was found in markedly higher amounts (increase of 114%) as compared to aspartic acid, iso-leucine and proline which showed a decrease of -24%, -41% and -67% respectively. This is explicable by the possible involvement of these amino acids in general and energy metabolism (Collett, 1976). Blocking the oxidative metabolism by the addition of KCN (an electron transport chain inhibitor) did not have any significant effect except that the amount of proline remained high. This gave a further hint that proline may serve as substrate under aerobic conditions. Aspartate and iso-leucine again showed a decline along with valine (-44%) suggesting no involvement with aerobic metabolism. A slight fall in the amount of glutamic acid with about an equal increase in Gaba was observed. This would be consistent with the one being precursor of the other. Even with the aerobic metabolism inhibited the amount of alanine was still more than that found in freshly dissected tissue. This suggested its accumulation is an accompanyment of anaerobic metabolism inevitable with intact ganglia even under optimal conditions in vitro.

3.1.14.b Incubation in saline containing glucose

Addition of glucose to the medium had its greatest effect on the amount of alanine which rose 3 fold in the presence of sugar compared to only 2 fold in its absence. The presence of glucose also either prevented the decline found for some amino acids, or actually

increased their amount. Only iso-leucine declined significantly.

Incubation of the nervous tissue in iso-osmolal saline containing glucose showed comparable results to the one with insect saline and glucose and no further significant changes in total amounts were observed.

A substantial change was noted in the concentration (in and released from the tissue) of Gaba, glutamate, glycine, phenylalanine, tyrosine and valine when the tissue was pre-incubated in saline containing amino acids in concentrations equivalent to the average found in the insect haemolymph, and subsequently incubated in iso-osmolal saline and glucose. Glutamate was the only one showing a lower level than normally occurring in the tissue. The high values of valine (+ 172%), phenylalanine (+ 342%), glycine (+ 543%) and tyrosine (+ 711%) suggested the possibility of an active uptake mechanism for these amino acids. The obvious question at that point was whether it was due to the overall metabolism of these amino acid or was simply a reflection of high uptake from medium during pre-incubation. This was investigated by analyzing the nervous tissue immediately after the 30 minutes' incubation in saline containing amino acids (Table 21). No difference was observed in the concentration of Gaba, iso-leucine and leucine; aspartic acid was depleted and the rest of the amino acids showed elevated concentrations which were considerably higher than those present in the incubation medium. It can again be emphasized that the rise in glycine, phenylalanine, tyrosine and valine against the concentration gradient was probably due to active transport into the ganglia.

Table 21 Free amino acid concentrations in the thoracic ganglia of Schistocerca americana gregaria incubated in various media.

Amino acid	30 minute incubation in iso-osmolal saline + amino acids n=6	Total at the end of 60 minute incubation in iso-osmolal saline, ganglia pre-incubated n=3	Concentration in freshly dissected tissue n=9	Haemolymph µmol/ml n=5-11	Concentration of amino acid used for incubation µmol/ml
Alanine	18.19±3.63**	11.23±0.78	11.03±0.50	1.42±0.62	1.50
Aspartic acid	1.90±0.26*	5.26±0.61	5.17±0.73	0.72±0.50	1.00
Gaba	3.12±0.63	3.76±0.33**	2.89±0.15	-	-
Glutamic acid	4.50±0.35**	4.48±0.50**	6.83±1.25	0.49±0.14	0.50
Glycine	15.97±2.11*	24.80±2.51*	3.86±0.43	10.94±2.19	10.00
Iso-leucine	1.77±0.28	2.47±0.67	1.90±0.63	1.16±0.41	1.00
Leucine	1.32±0.23	2.07±0.54	1.24±0.51	1.09±0.26	1.00
Phenylalanine	1.95±0.23*	2.43±0.06*	0.55±0.09	1.37±0.80	1.50
Proline	12.09±1.88+	8.86±0.11	7.46±2.67	9.45±1.93	10.00
Tyrosine	5.76±0.56*	7.87±0.73*	0.97±0.40	1.63±0.26	1.50
Valine	3.63±0.50**	5.14±0.28*	1.89±0.58	1.95±0.79	2.00

The incubations were carried out at 37°C. in fully oxygenated iso-osmolal saline + 10mM glucose and containing the concentration of amino acids shown in column 5 of the table. The concentrations are given as µmol/g wet weight nervous tissue. The amino acid concentration total (i.e. both in tissue and medium) after a 60 minute incubation (column 2), normal concentration found in tissue (column 3) and haemolymph (column 4) are presented for comparison. Significance of difference from normal concentration - P = *<0.01, **<0.05, +<0.1.

3.1.15 Efflux of amino acids into the incubation media

3.1.15.a Saline without glucose

The efflux of amino acids from thoracic ganglia *in vitro* to the surrounding medium was investigated. It was observed that a greater proportion of each of those amino acids not considered possible transmitters (from previous work with insect and other nervous tissues) was released to the medium. The greater part of proline, alanine, iso-leucine and phenylalanine were found in the bathing medium whereas aspartate, glutamate and Gaba were retained to a great extent (Fig. 39). The retention of these compounds is consistent with them having specific, and energy-requiring retention mechanisms which in turn is often associated with the importance as neurotransmitters or their precursors. Further, the effect of blocking aerobic metabolism of the ganglia by the addition of 10mM KCN on the release of amino acids was investigated. In all cases except alanine the efflux increased. The most significant increases were observed with glutamate, aspartate and Gaba. The rest of the amino acids showed a non-significant change. This, too, is further evidence of specific uptake or retention mechanisms which allow accumulation against a concentration gradient.

3.1.15.b Saline containing glucose

Further the effect of provision of glucose as an energy source and of keeping the osmolarity of the saline near to that of haemolymph was studied. The maximum O_2 consumption *in vitro* of the thoracic ganglia of locust requires 10mM glucose (Clement and Strang, 1978). Therefore, the tissue was incubated in a saline

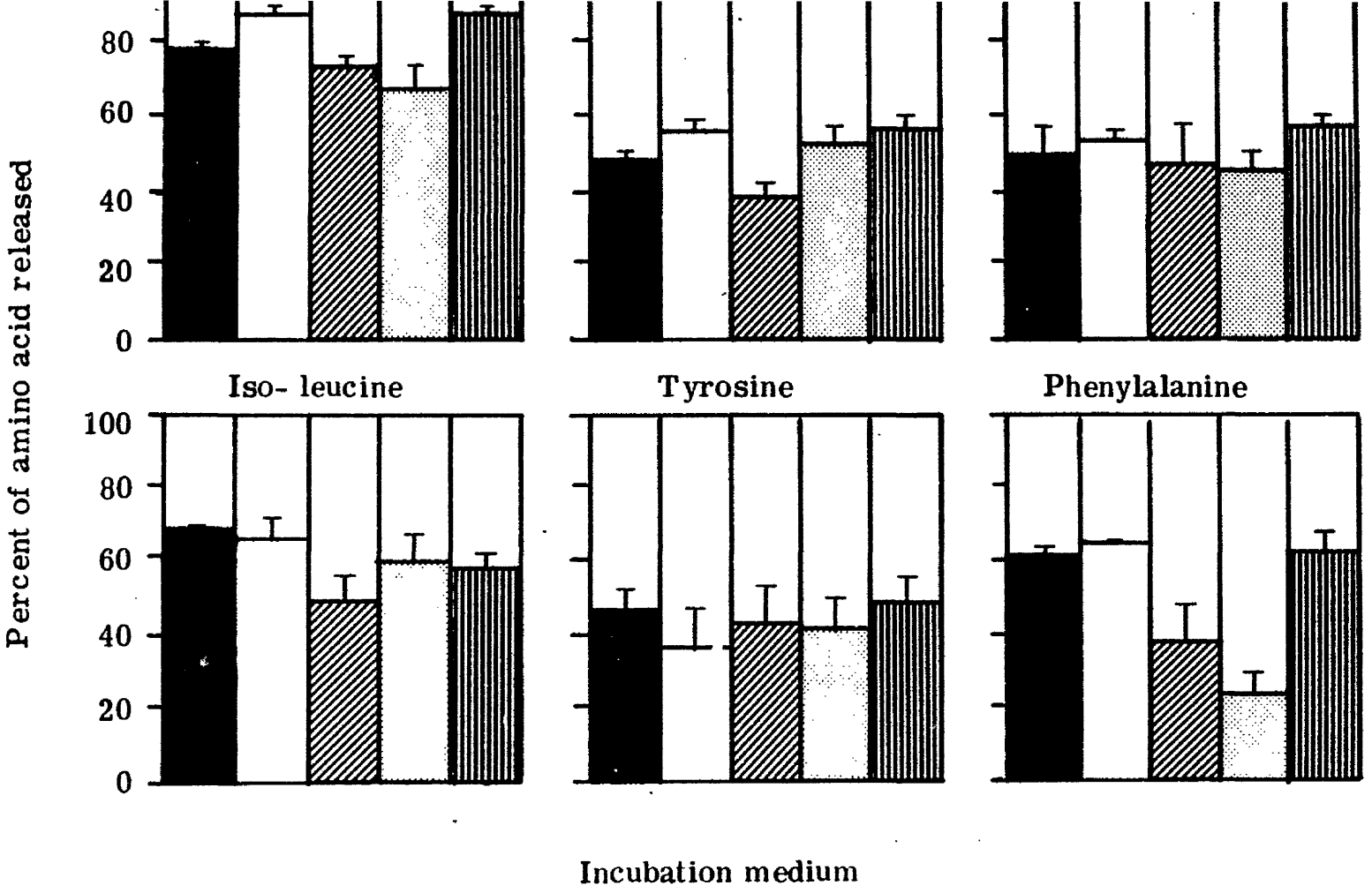


Figure 39. Efflux of amino acids from thoracic ganglia of Schistocerca americana gregaria to the fully oxygenated media after a 60 minute incubation at 37°c. Incubation media were; ■ insect saline; □ insect saline + 10 mM KCN; ▨ insect saline + 10 mM glucose; ▭ iso-osmolal saline + 10 mM glucose; and ▥ iso-osmolal saline + 10 mM glucose after the ganglia had previously been incubated for 30 minutes in iso-osmolal saline + amino acids.

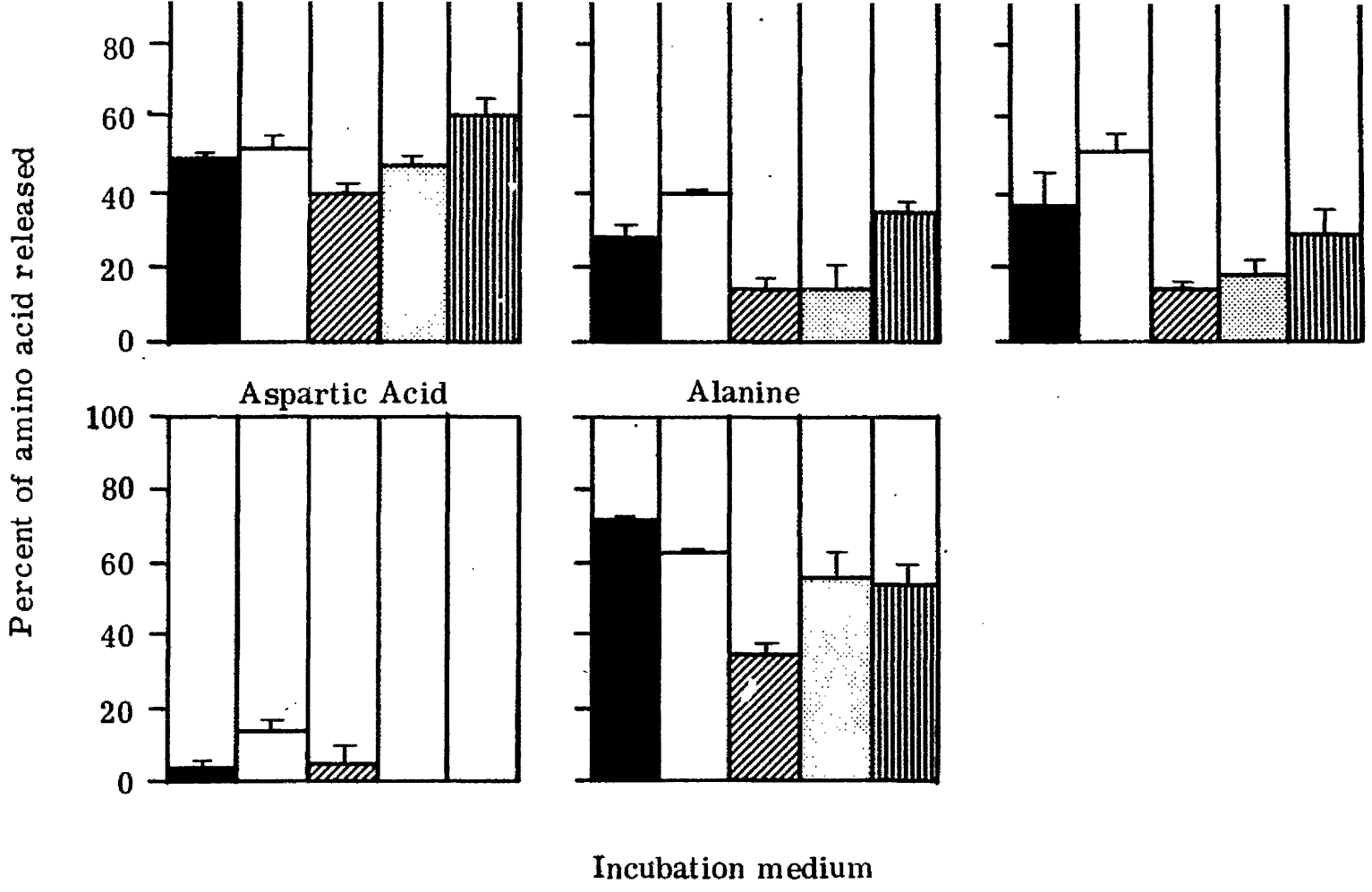


Figure 39. Efflux of amino acids from thoracic ganglia of Schistocerca americana gregaria to the fully oxygenated media after a 60 minute incubation at 37°c. Incubation media were; ■ insect saline; □ insect saline + 10 mM KCN; ▨ insect saline + 10 mM glucose; ▭ iso-osmolal saline + 10 mM glucose; and ▥ iso-osmolal saline + 10 mM glucose after the ganglia had previously been incubated for 30 minutes in iso-osmolal saline + amino acids.

containing 10mM glucose and 100mM sucrose. The purpose of the sucrose is to make it approximately iso-osmolal with average haemolymph. It was noted that in nearly all the cases the percentage of the amino acids retained increased. Greatest increase in retention was observed with glutamate, followed by Gaba and aspartate. Moreover, in iso-osmolal saline nearly all the aspartate was retained by the tissue.

To minimize the variation in the concentration of amino acids in the tissue from various insects, the effect of pre-equilibration in standard concentrations of amino acids was studied. Subsequent incubation in iso-osmolal saline showed that in all the amino acids studied, except aspartic acid, a greater outward flux was observed. The efflux was as much as 90% in proline and about 60% in valine, glycine, alanine, leucine, iso-leucine, tyrosine and phenyl-alanine. The increase in Gaba and glutamate was nearly twice that in the medium containing glucose only. This big rise in efflux from the tissue could be attributed to the accumulation of various amino acids in intracellular spaces and other cells and subsequent diffusion to medium devoid of any amino acids.

3.1.16 Rate of the release of amino acids from isolated ganglia

The rate of the release of amino acids was followed by taking samples from the incubation medium at various times and analyzing by dansylation. The nervous tissue were incubated in the medium with a) 10mM glucose; b) iso-osmolal saline containing 10mM glucose. In addition the effect of pre-incubation of nervous tissue in saline with amino acid on the rate of release investigated.

It was observed that there was an initial surge of the amino acids for 15 minutes and after that for periods up to 60 minutes the amount of amino acids in the medium did not change much (Fig. 40) except with the pre-incubated ganglia. In this case a rise in the levels of most of amino acids was found till 45 minutes after which either it levelled off (i.e. leucine, glycine, phenylalanine, tyrosine) or declined (proline, valine, iso-leucine and alanine).

The release of Gaba was unchanged in the pre-incubated ganglia, from 15 to 30 minutes, then it started increasing. Noteworthy was the fact that the concentration of aspartate and glutamate hardly changed in the medium from 15-60 minutes.

The efflux of amino acids at shorter intervals of 5 minutes up to 20 minutes was studied in iso-osmolal saline. It was observed that the initial surge was in fact in the first 5 minutes after which in many cases e.g. phenylalanine, leucine, iso-leucine and aspartate there was hardly any increase (Fig. 41). There was slow increase in glutamate which could be due to the synthetic processes going on. The increase in efflux was more pronounced in proline, glycine and alanine. The tyrosine release was also observed to increase over the period of study. The only peculiar observation was that of Gaba where an initial increase was followed by a decrease and finally a levelling off at 15-20 minutes. This decline could be due to the reuptake (Cutler and Young, 1979).

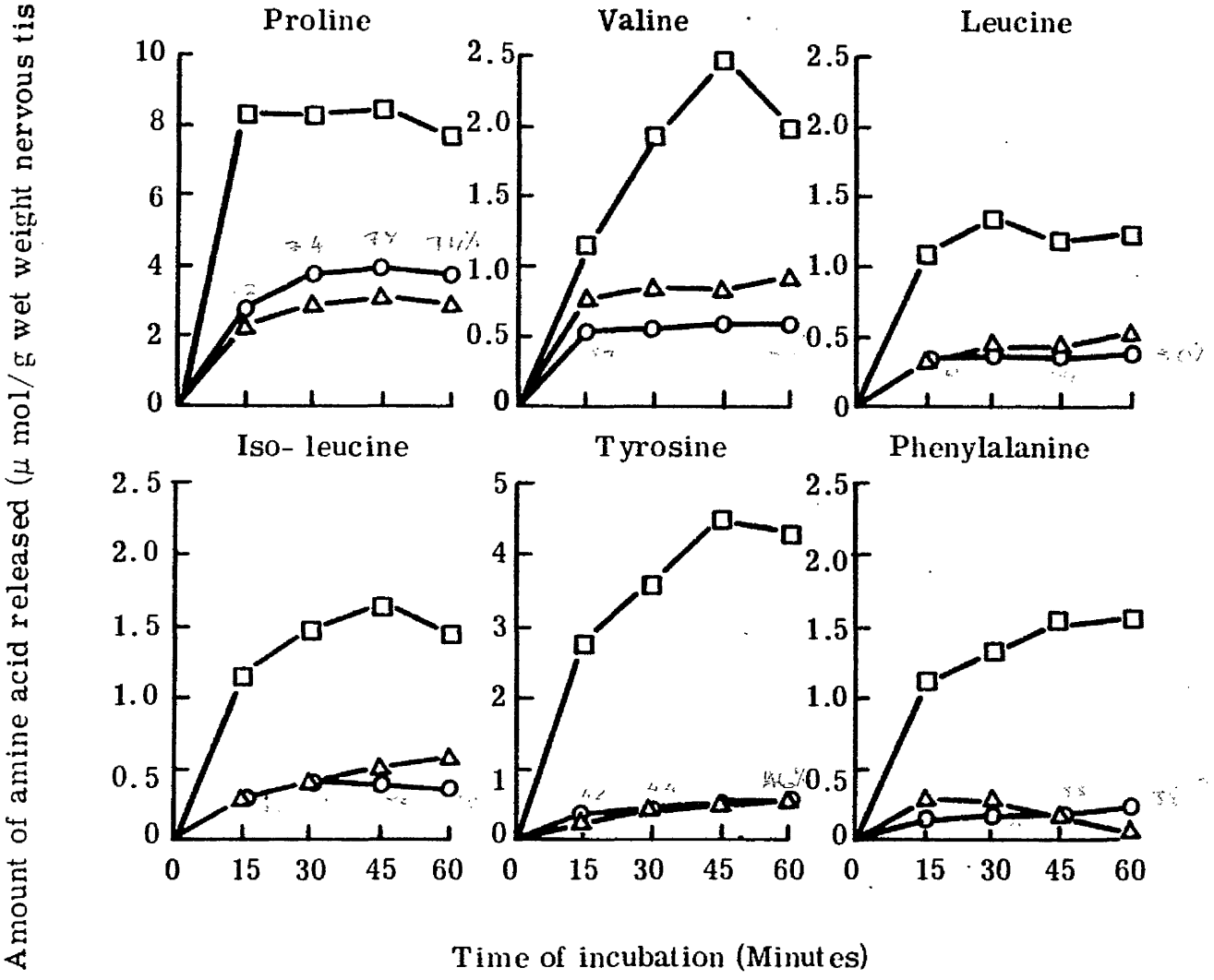


Figure 40. Release of amino acids from thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated media at 37°c. The media used were; o—o saline + 10 mm glucose; Δ—Δ iso-osmolal saline + 10 mM glucose; and □—□ iso-osmolal saline + 10 mM glucose after the ganglia were previously incubated for 30 minutes in iso-osmolal saline + amino acids. Each point is the average of 3 or 4 separate estimates.

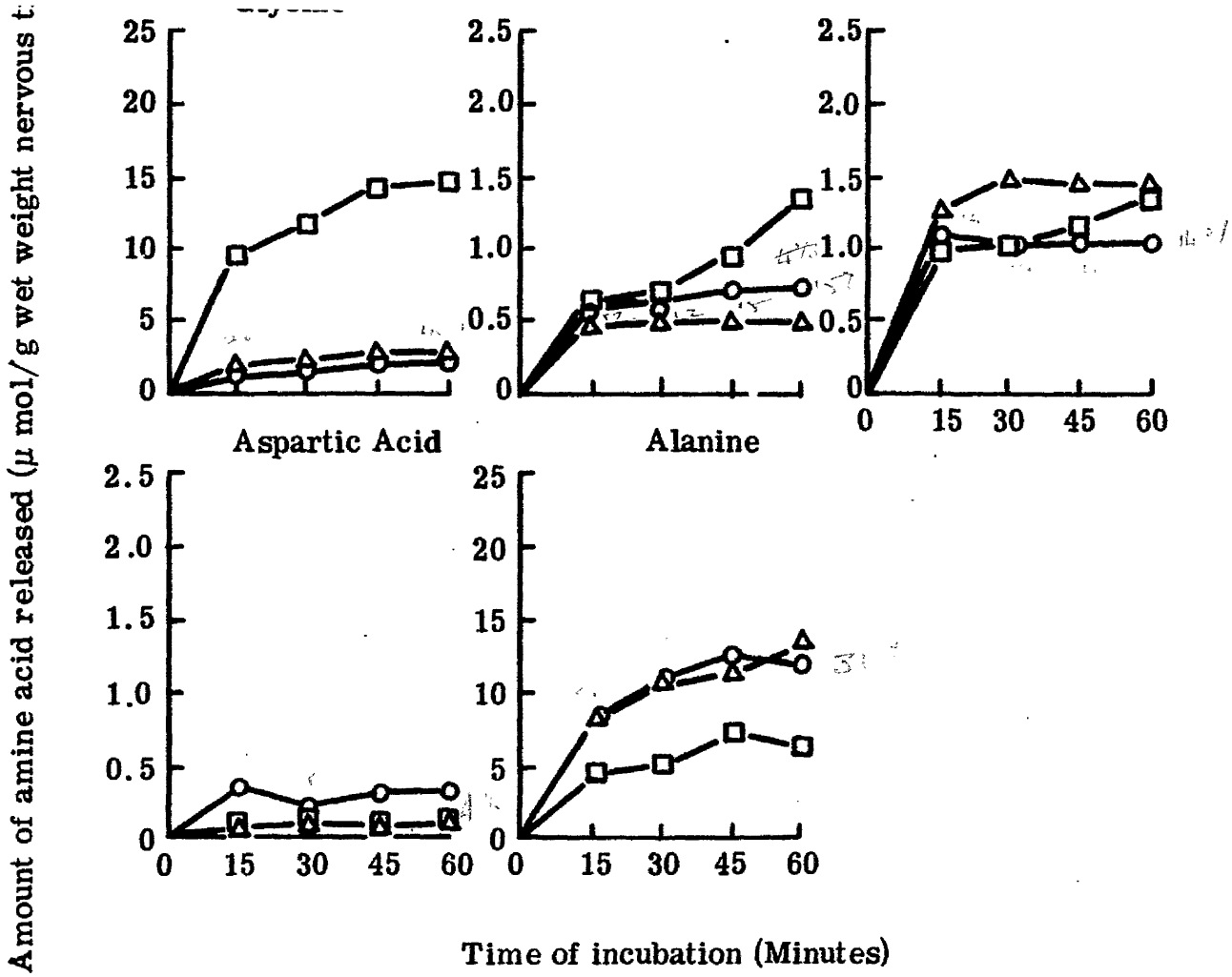


Figure 40. Release of amino acids from thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated media at 37°c. The media used were; o—o saline + 10 mm glucose; Δ—Δ iso-osmolal saline + 10 mM glucose; and □—□ iso-osmolal saline + 10 mM glucose after the ganglia were previously incubated for 30 minutes in iso-osmolal saline + amino acids. Each point is the average of 3 or 4 separate estimates.

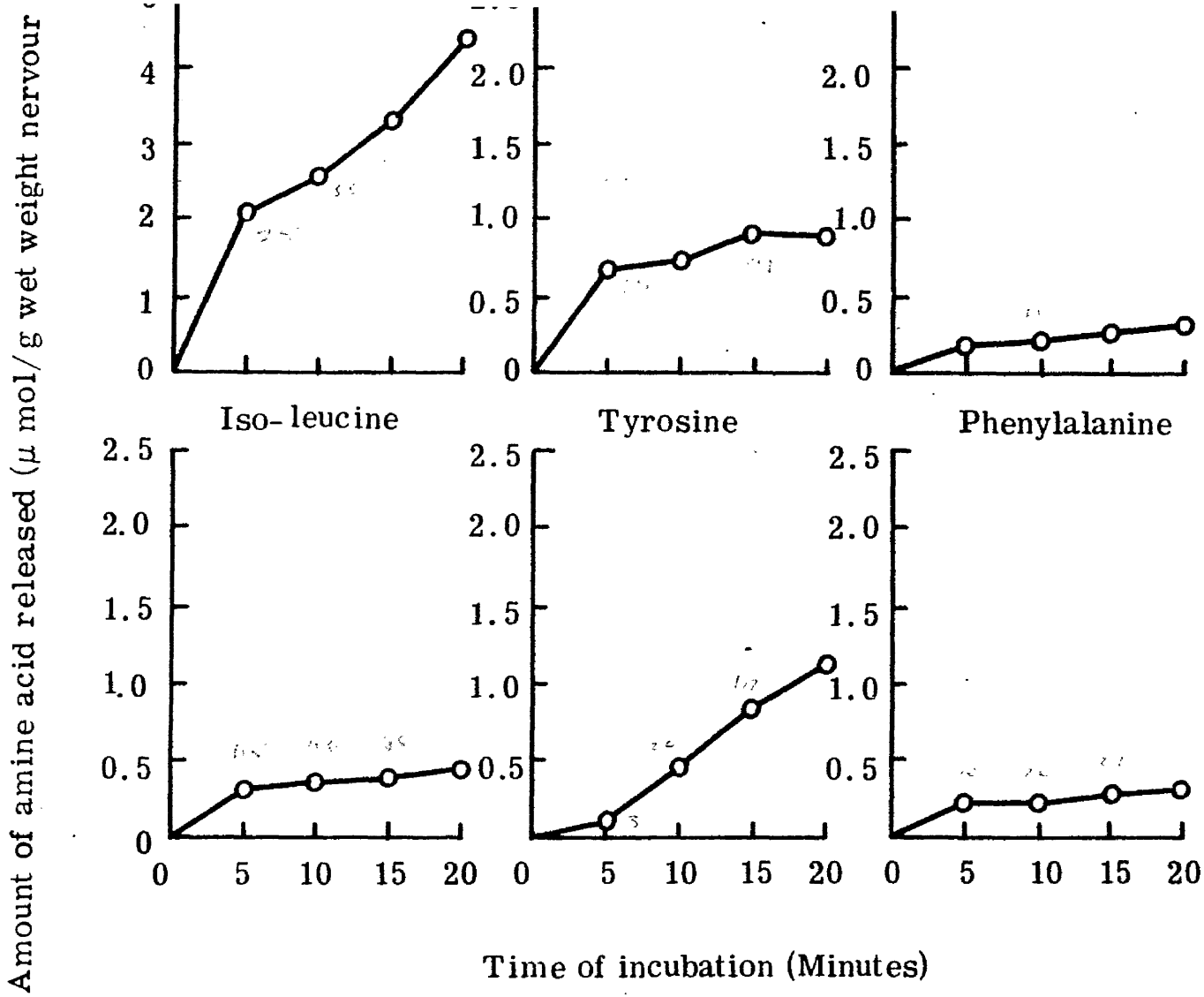


Figure 41. Release of amino acids from the thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated iso-osmolal saline + 10 mM glucost at 37° c. Each point is the average of 3 separate estimates.

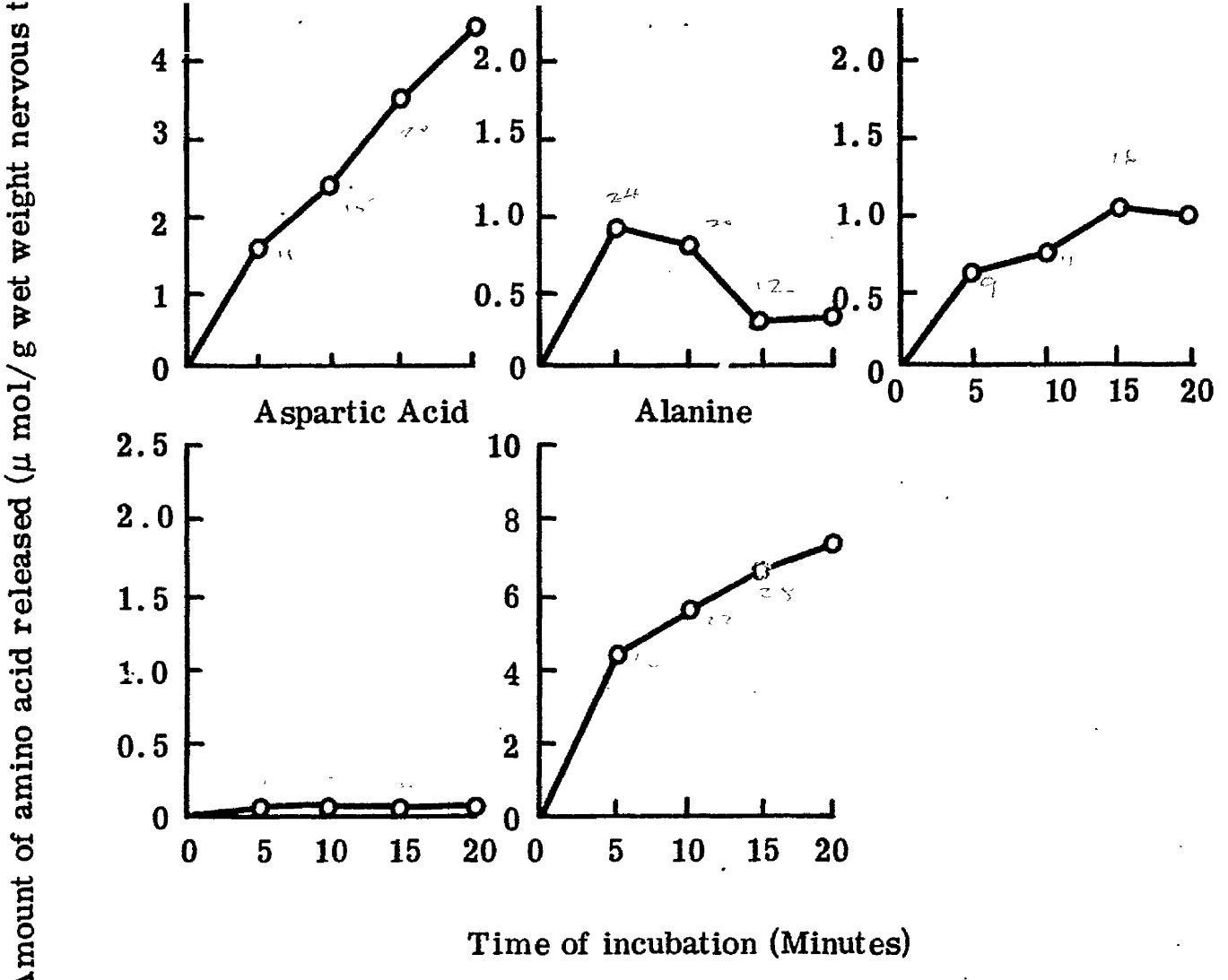


Figure 41. Release of amino acids from the thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated iso-osmolal saline + 10 mM glucost at 37° c. Each point is the average of 3 separate estimates.

3.1.17 Effect of temperature on the release of amino acids

Incubation of the thoracic ganglia at different temperatures (37°C, mammalian body temperature; 32°C, the temperature at which the locusts are kept; and 21°C, as the room temperature), revealed a positive temperature dependence of efflux (Fig. 42). The only exceptions were proline and alanine where release was higher at 32°C than at 37°C. The retention of aspartate was greater at high temperature. In all cases at 21°C there was initial surge of release followed by levelling off, whereas both at 32°C and 37°C more amino acids were released with the passage of time. In short, at room temperature the efflux of amino acids is at a lower rate than when the temperature exceeds 30°C. But at the higher temperatures i.e. 32°C and 37°C, the difference in efflux was not pronounced. Because in natural conditions the insects often sustain a body temperature between 30-40°C particularly during flight (Church, 1960) and also in order that the results could be compared with those previously obtained for locust nervous tissue, further metabolic studies were carried out at 37°C.

This set of experiments was of preliminary nature, and carried out to determine the optimum conditions for in vitro incubations of nervous tissue, which would imitate most closely to the situation in living insects. Nevertheless, they suggested some interesting points in the metabolism of certain amino acids. For instance, the increase in the amount of free alanine under all conditions of in vitro incubations, the decline in aspartate and the block of proline metabolism with KCN, raised questions as to the importance of these amino acids in the overall function and the response of the

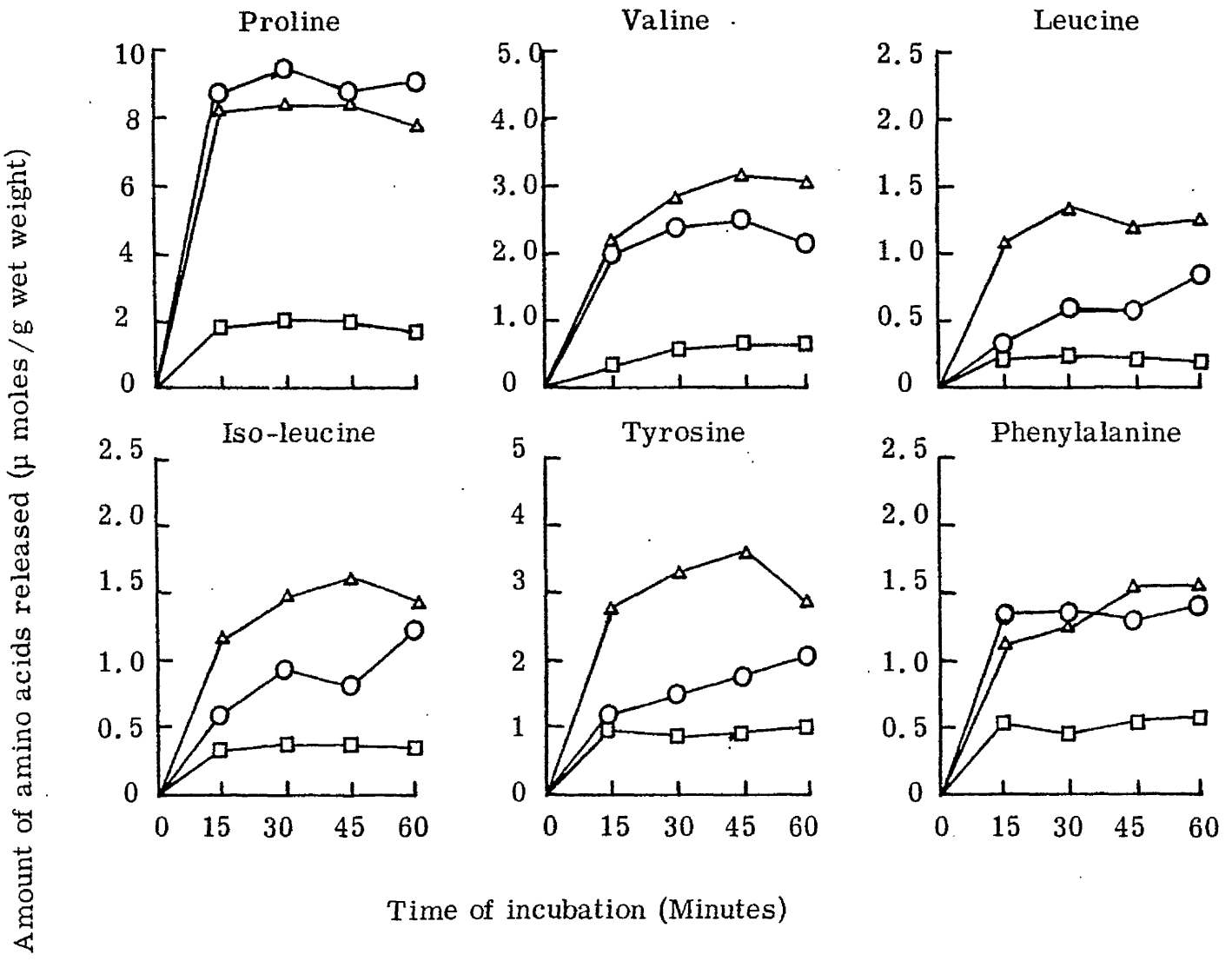


Figure 42 Release of amino acids from the thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated iso-osmolal saline containing 10mM glucose at 21°C, □ ; 32°C, ○ ; and 37°C △ . Each point is the average of 4 separate estimations.

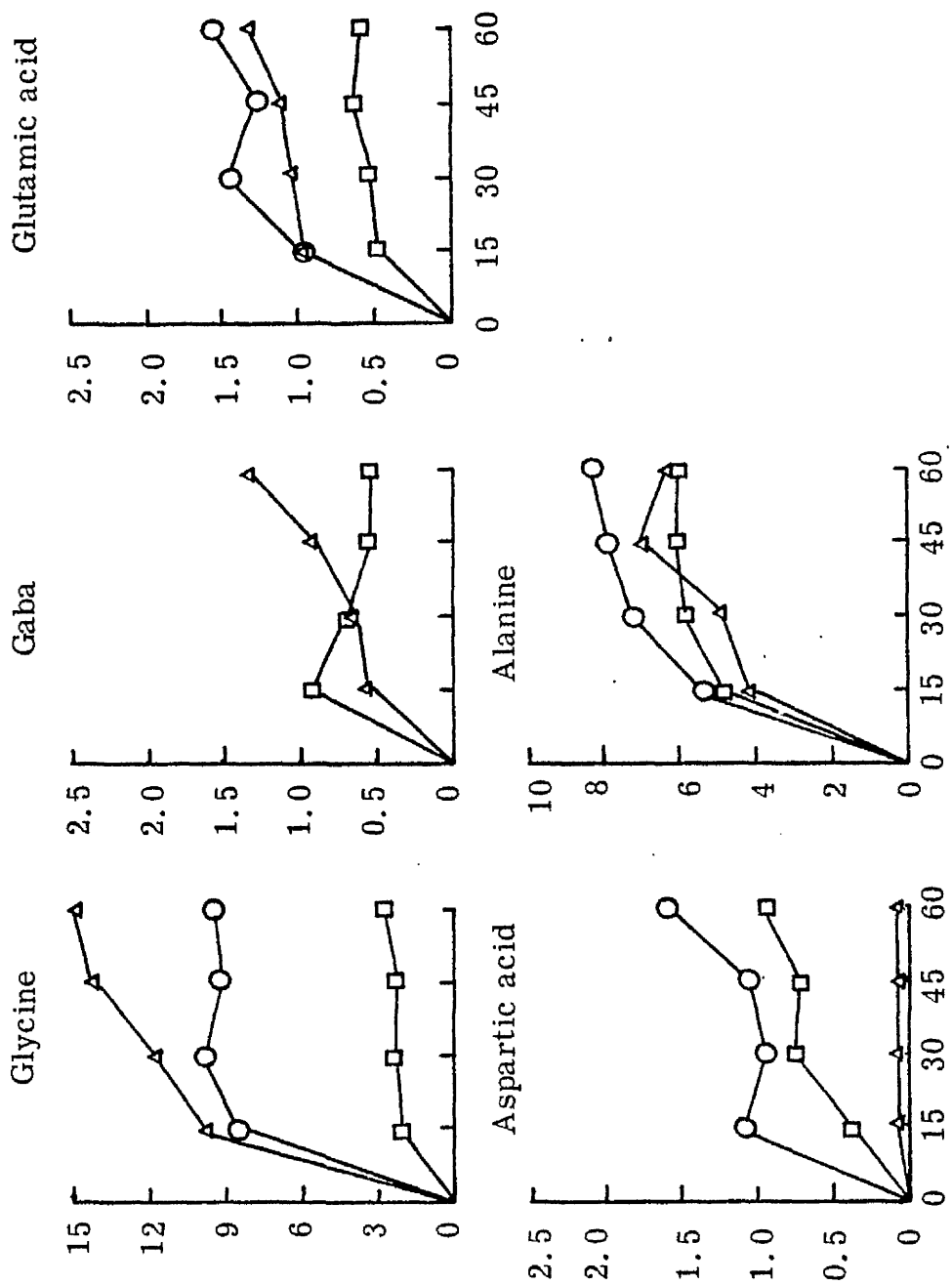


Figure 42 Release of amino acids from the thoracic ganglia of Schistocerca americana gregaria suspended in fully oxygenated iso-osmolal saline containing 10mM glucose at 21°C, □ ; 32°C O ; and 37°C Δ . Each point is the average of 4 separate estimations.

nervous tissue to extreme circumstances of aerobic and anaerobic metabolism. These points were, therefore, followed up in more detail (Section 3, part 3).

PART TWO

STUDIES ON THE ACTION OF INSECTICIDES, PHYSIOLOGICAL STRESS AND RELEVANT NEUROPHARMACOLOGY.

3.2.1 Effect of insecticidal stress on intact insects

a. Locusts

All the locusts which received the injections of insecticides showed some sort of paralytic effect. DDT at 100µg/locust was insufficient to cause any immediate symptoms of poisoning. After 30 minutes the locusts recovered from an initial effect and seemed quite normal even after 24 hours at room temperature. In consequence a higher dose of 1mg/locust was applied. The other insecticides (in doses of more than double the $L.D_{50}$, Chapman, 1976) were applied at 100µg/locust except methyl parathion which was injected at 300µg/insect. The insects receiving 10µl DMSO as control revived within 15-30 minutes, in the test insects receiving the toxic compounds typical symptoms of poisoning leading to stress syndrome (Heslop & Ray, 1959) could be observed. These insects showed hyperactivity with trembling and violent body movement and vigorous rhythmic movements associated with respiration (Harvey & Brown, 1951). In a short time they lost their righting reflex.

After 4-6 hours the haemolymph was collected. In addition, the thoracic ganglia were dissected out. Both tissues were analyzed for the presence of amino acids and other amino compounds. The qualitative analysis of nervous tissue did not reveal anything unusual in detectable quantities in the profile of free amino compounds. In the haemolymph, on the other hand, a substance accumulated which was hardly detected in unstressed locusts (Fig. 43). This substance appeared in the blood of locusts which received the insecticides γ-BHC, dieldrin, DDT, Nicotine, Fenvalerate, Malathion and Methyl-parathion. As this substance accumulated in response

Fig. 43 - Autoradiographs of the microchromatographs of [^{14}C]-Dans-derivatives from extracts of locust nervous tissue and haemolymph. The tissue was dissected 6 hours after the injection of insecticides dissolved in 10µl DMSO. Chromatography of the derivatives was carried out exactly as described before (Section 2.4.9.3). Direction and order of the solvent development is indicated by the numbered arrows. Autoradiographs were developed after 60 hours exposure.

A = control locusts receiving 10µl DMSO only; B = 100µl BHC;

C = 1mg DDT; D = 100 µg Dieldrin; E = 100µg Nicotine;

F = 100µg Fenvalerate; G = 100µg Chlordimeform;

H = 300µg Malathione and I = 100 µg Methyl parathion.

The arrow indicates the appearance and accumulation of an unidentified substance.

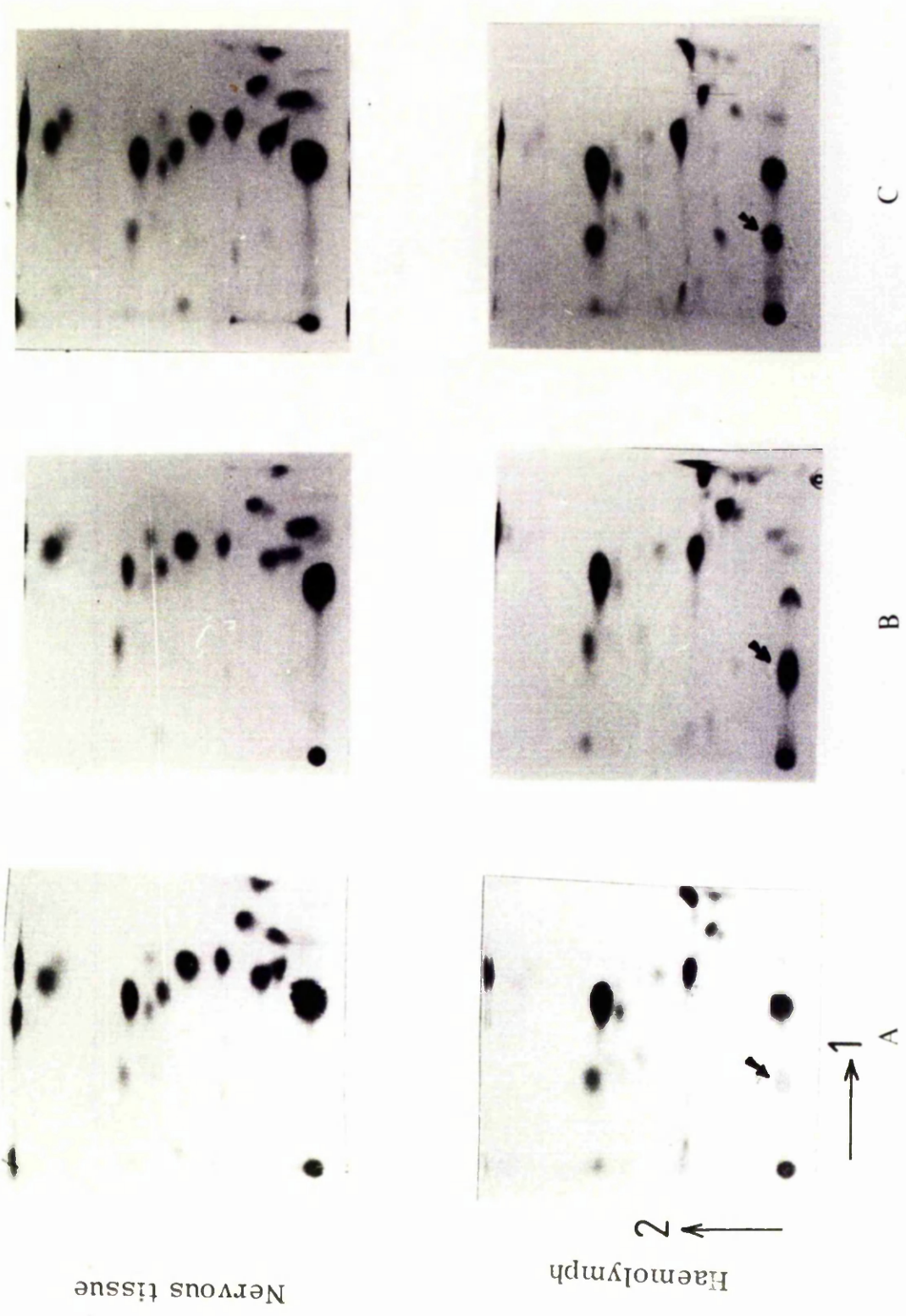


Figure 43

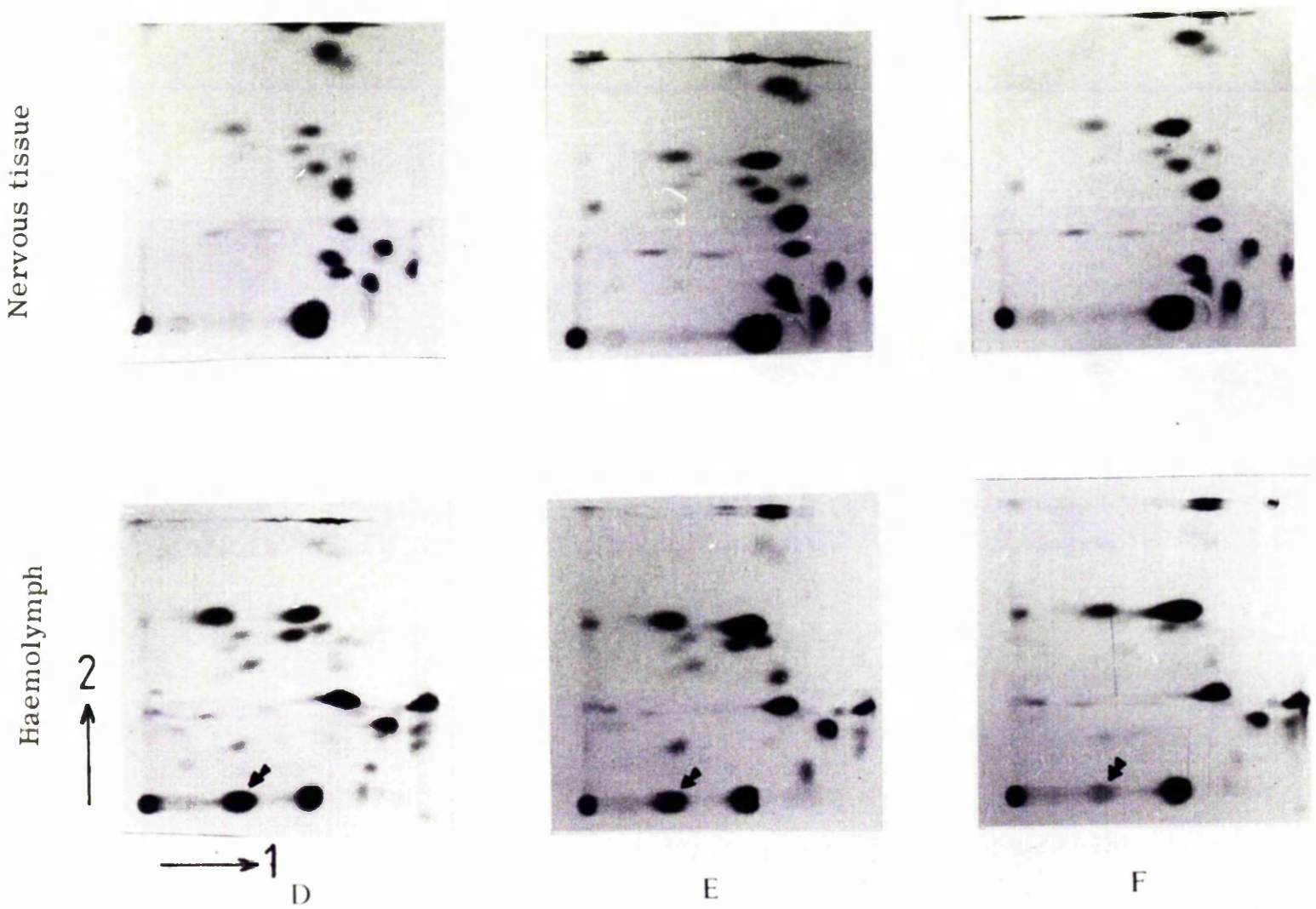


Figure 43

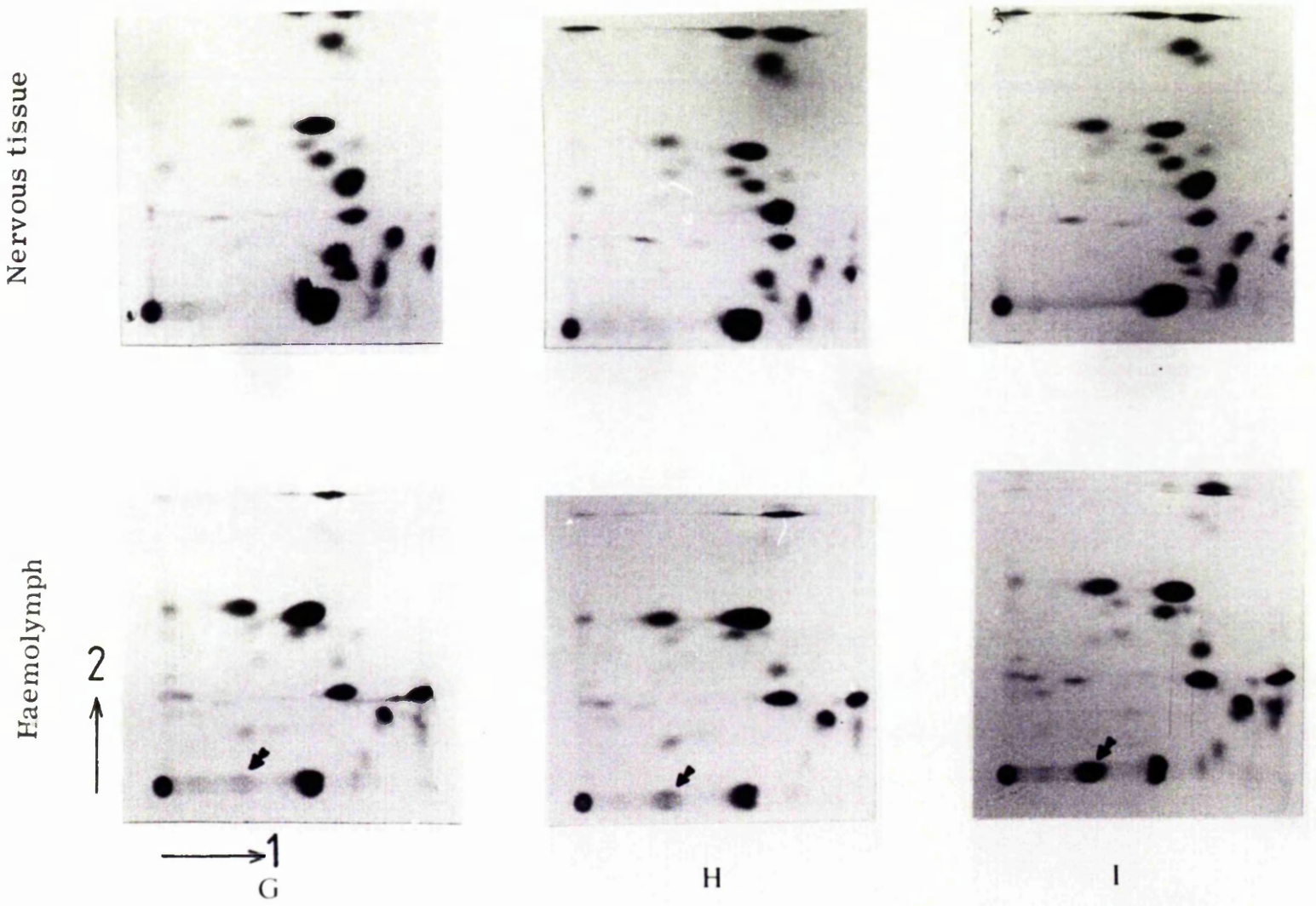


Figure 43

to all the insecticides studied except chlordimeform. To explore the possibility of this occuring in other insects, the study was extended to another insect (cockroach).

b. Cockroaches

A similar study carried out with American cockroaches revealed that this substance was also present in their haemolymph under the same conditions (Fig. 44). There was a large accumulation of this compound in the blood after the injection of γ-BHC and DDT. Again there was no evidence of this compound in substantial amounts in the nervous tissue.

3.2.1.2 Effect of stress due to enforced activity

The subjection of the insects to bodily stress by enforcing continuous activity showed that the same substance accumulates in the haemolymph (Fig. 45).

There was a clear difference in the response of locusts and cockroaches to bodily stress. An activity of 8 hours was sufficient to cause the accumulation of this compound in the cockroach while the locusts had to be subjected to stress for 24 hours before this substance could be detected in their haemolymph.

3.2.2 Change in the volume of haemolymph due to stress

There was the possibility that increased concentration of the unknown substance shown by these essentially qualitative results may be simply a reflection of the reduction of the haemolymph volume due to the dessication which is known to accompany lengthy periods of stress (Heslop & Ray, 1959). In agreement with the results of Cook et al (1969) this could be ruled out because the volume of the haemolymph 6 hours after the application of insecticides shows no significant change (Fig. 46).

Fig. 44 - Autoradiographs of the microchromatographs of [^{14}C]-Dans-derivatives from extracts of cockroach nervous tissue and haemolymph. The nervous tissue was dissected and haemolymph collected 4 hours after the injection of insecticide dissolved in 10µl DMSO. Chromatographic conditions are those described for in Fig. 43.

A; control cockroaches receiving 10µl DMSO only;

B; 100µg γ-BHC; and C; 1mg DDT.

The arrow indicates the accumulation of an unknown substance

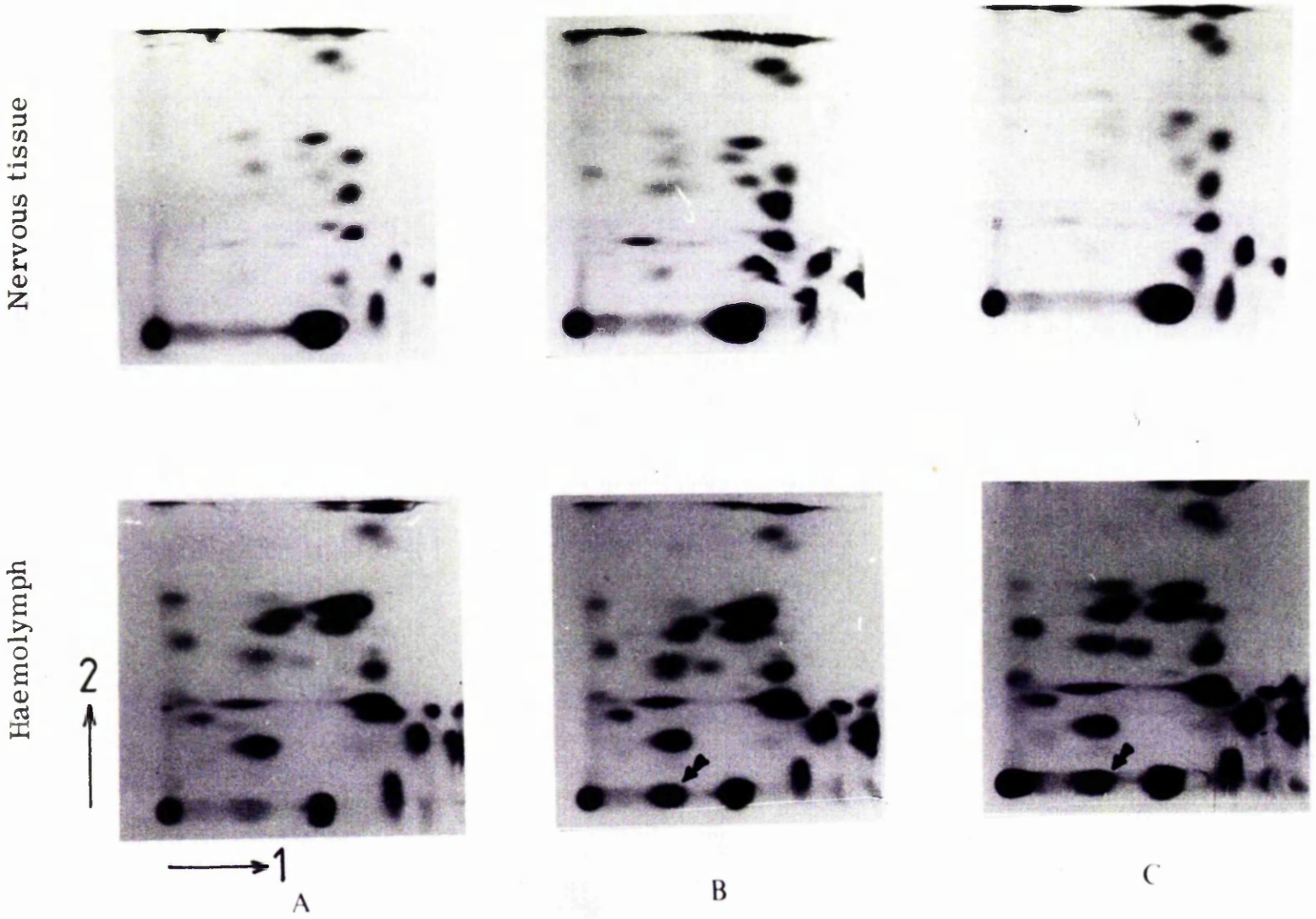


Figure 44

Fig. 45 - Autoradiograph showing the accumulation of an unknown substance (indicated by arrow), in the haemolymph of; A. locust subjected to 24 hours enforced walking activity; and, B. cockroach which had been walking continuously for 8 hours.

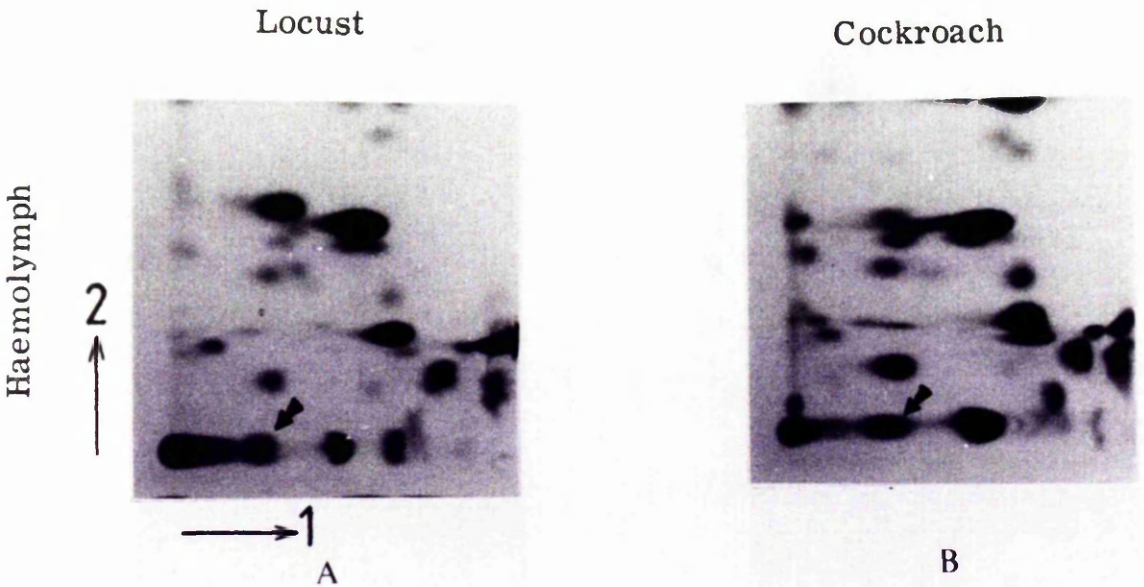


Figure 45

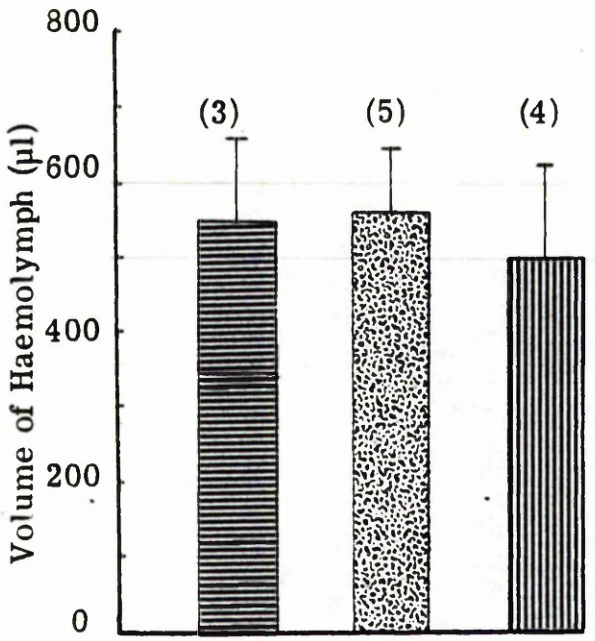


Figure 46 Volume of haemolymph of female locusts as estimated by $[^3H]$-Inulin dilution technique.
▤ = Normal locust, ▩ = Control locust (6 hours after the treatment with DMSO) and ▥ = Paralyzed locust (6 hours after treatment with γ-BHC).
Figures in parenthesis are the number of locusts analyzed.
Lines associated with each bar represent the standard deviation from the mean.

In addition, the other amino acids did not show any such noticeable increase in concentration as is evident from Fig. 43. If there had been a reduction of haemolymph volume it would have been expected that many other amino acids had shown a clear increase in concentration.

3.2.3 Application of insecticides to the isolated ganglia

Following the reports that stress causes the production and release of neurotoxic factors from the nervous tissue of insects (Sternburg, 1963), the isolated thoracic ganglia were pre-incubated in iso-osmolal saline prior to incubation in saline containing 10^{-3}M DDT, Dieldrin, Malathion, Methyl-parathion, Y-BHC, Fenvalerate and Chlordimeform. Nicotine, an agent well known to cause excitation in insect CNS (Roeder & Roeder, 1939), was also applied in concentrations of 10^{-4}M and 10^{-6}M.

The analysis of the extract of the nervous tissue and incubation medium failed to show the presence of the substance (Fig. 47) found to accumulate in the haemolymph of poisoned insects.

3.2.4 Quantitative results of in vitro incubation of the nervous tissue

Table 22 shows the results obtained by incubation of the isolated thoracic ganglia of locust in saline containing various agents and insecticides. Alanine was the amino acid showing highest increase in concentration, in the tissue and medium, following all incubations. This increase ranged from 11.23 to 20.68 µmol/g wet weight in Fenvalerate and up to 54.97 µmol/g wet weight in the presence of 10^{-3}M methyl-parathion. Proline was the other amino acid which occurred in significantly higher concentrations

Fig. 47 - Autoradiographs of the microchromatograms of [^{14}C]-Dans-derivatives from nervous tissue extracts and incubation medium after the isolated nervous tissue was incubated for 30 minutes in fully oxygenated insect saline containing insecticides. Ethanol or acetone (2% v/v) was added where necessary to the media to ensure the solution of insecticides. The exposure time was 120 hours.

A. Saline containing 2% ethanol; B. 10^{-3}MDDT; C. 10^{-3}M dieldrin; D. saline containing 2% acetone; E. 10^{-3}M malathion; F. 10^{-3}M methylparathion; G. 10^{-3}M γ-BHC; 10^{-6}M nicotine; I. 10^{-4}M nicotine; J. 10^{-3}M fenvalerate; k. 10^{-3}M chlordimeform and L. tissue extract following electrical stimulation of the thoracic ganglia at 50 Hz, 5 volts for 30 minutes.

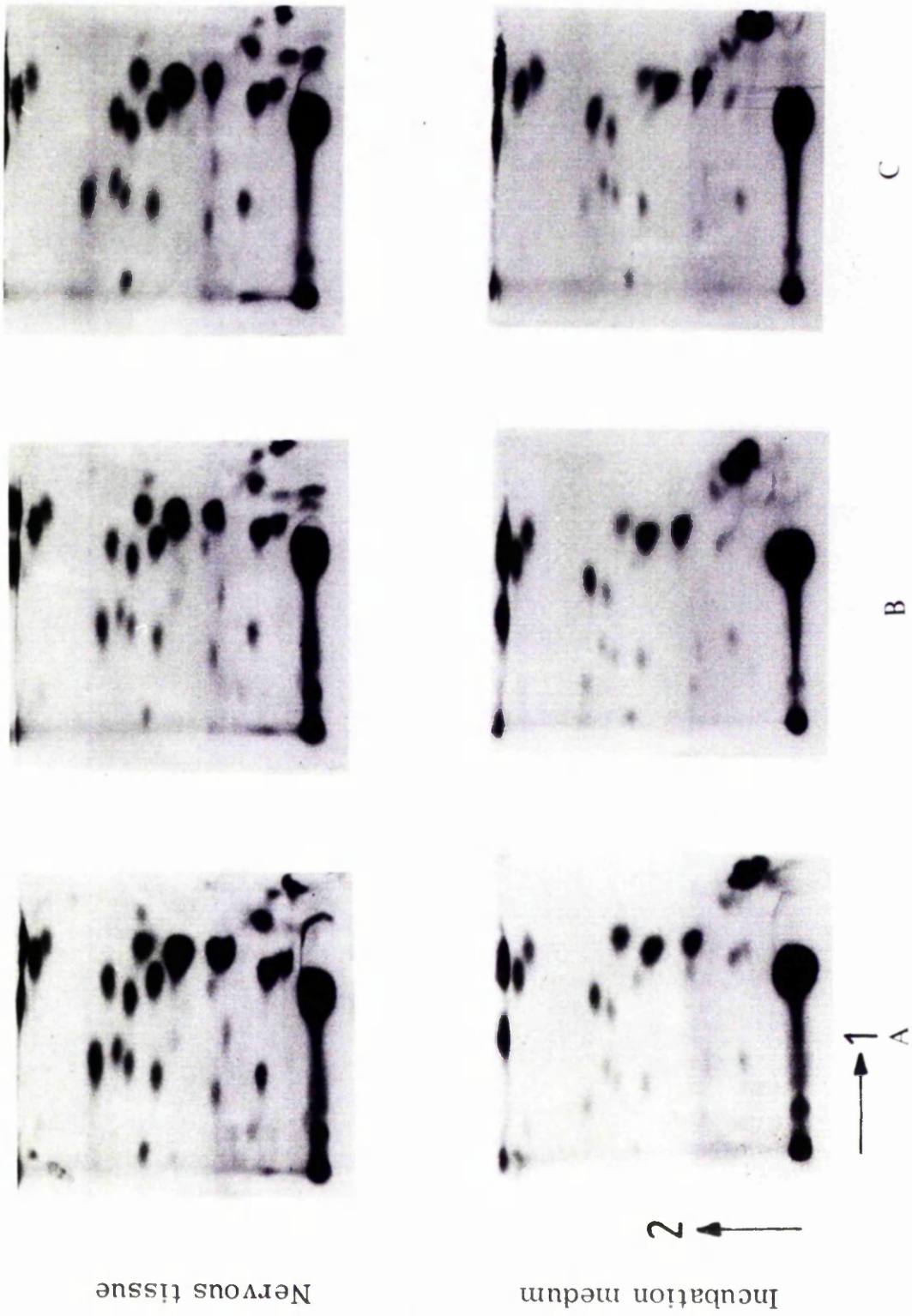


Figure 47

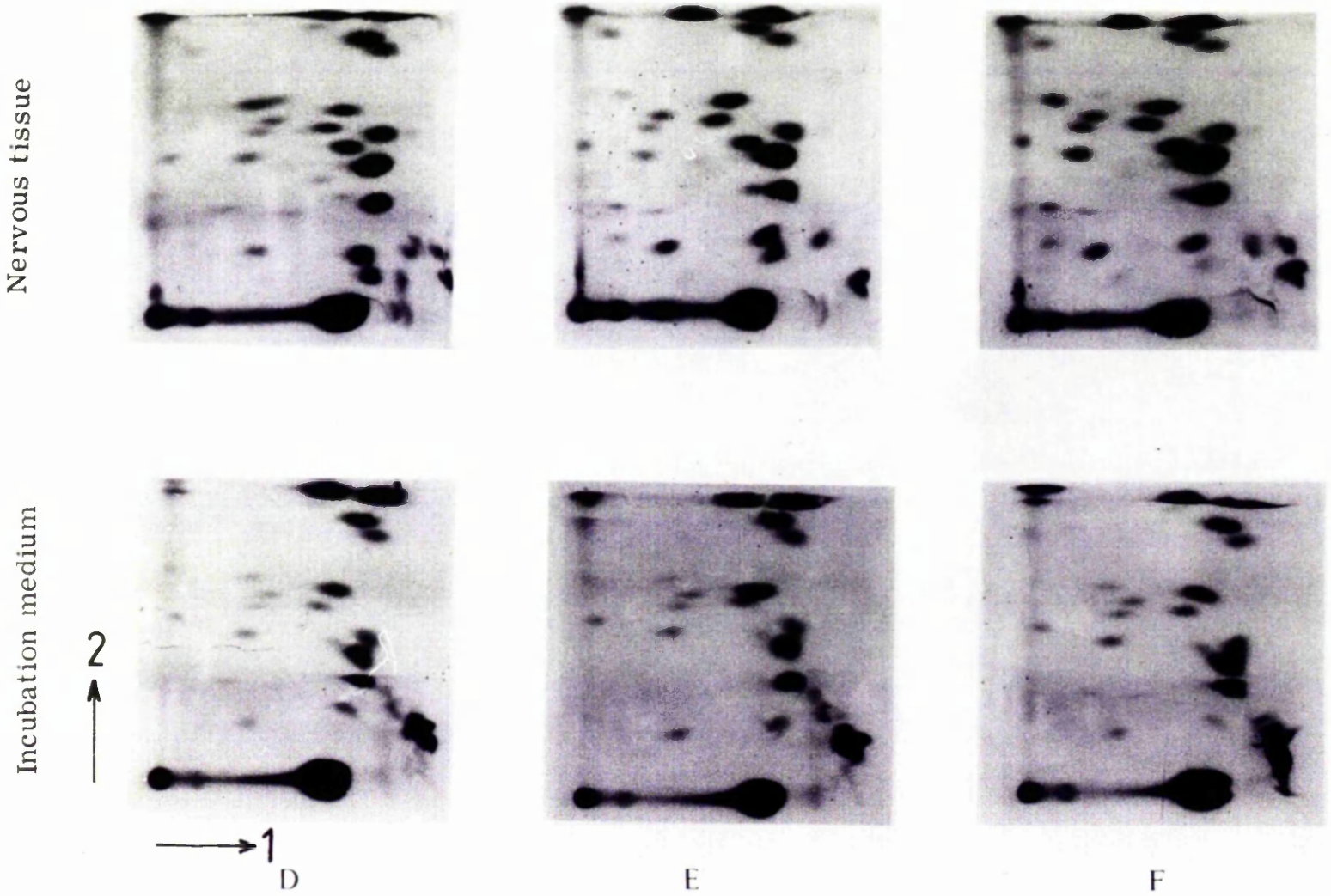


Figure 47

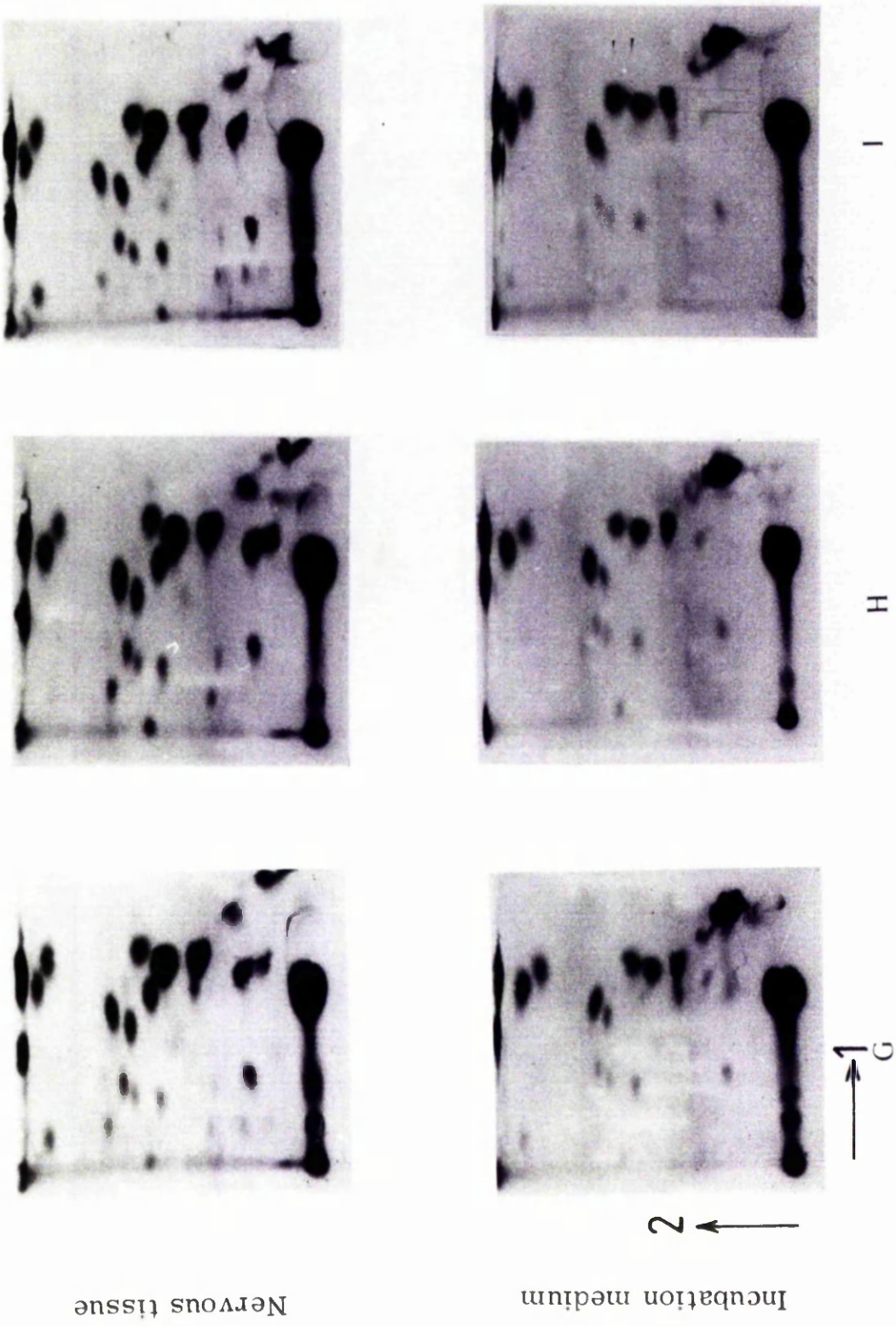


Figure 47

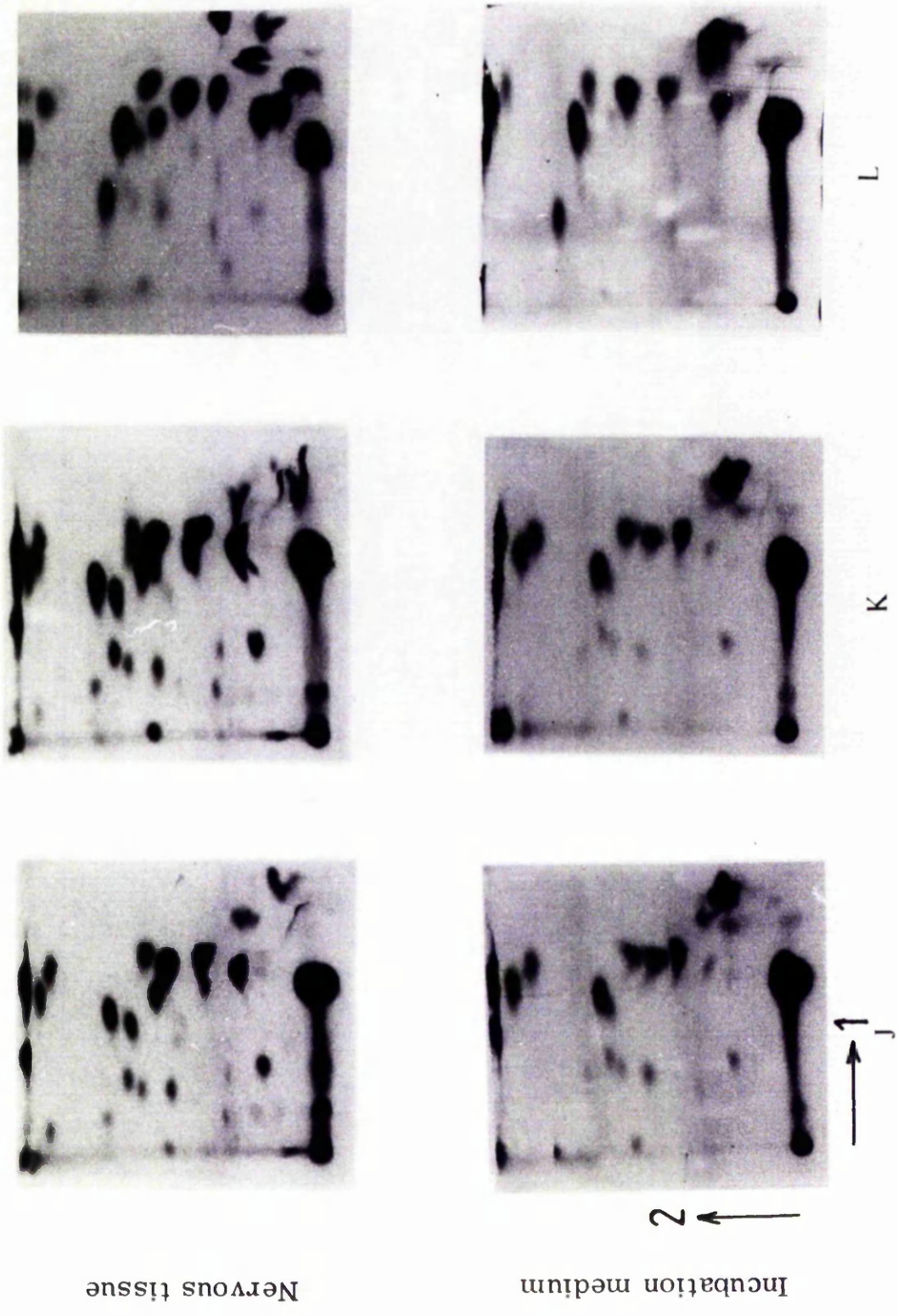


Figure 47

Table 22. Total free amino acids (μmole/g wet weight) in, and released from thoracic ganglia of Schistocerca americana gregaria suspended in media containing insecticides and under influence of electrical stimulation.

	Iso-osmolal saline	2% Acetone	2% Ethanol	10^{-3}M DDT	10^{-3}M Dieldrin	10^{-3}M Methyl Parathion	10^{-6}M Nicotine	10^{-3}M Lindane	10^{-4}M Nicotine	10^{-3}M Fanvalerate	10^{-3}M Malathion	10^{-3}M Chlordimeform	Elec. Stimulation
	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Alanine	11.23 ±0.78	30.93 ±2.49	41.30 ±1.15	45.11 ±7.96	45.68 ±2.88	54.97 ±5.56	28.09 ±0.97	34.04 ±0.75	22.60 ±2.58	20.68 ±5.27	39.32 ±1.71	27.47 ±3.31	21.38 ±2.50
Aspartic acid	5.26 ±0.61	3.79 ±0.75	3.80 ±0.20	4.04 ±1.06	4.79 ±0.92	1.69 ±0.23	2.95 ±0.51	3.23 ±0.07	3.50 ±0.24	4.06 ±1.52	5.50 ±0.38	8.51 ±1.70	10.96 ±2.07
Gaba	3.76 ±0.33	3.73 ±0.46	5.36 ±0.20	5.18 ±0.70	5.84 ±0.51	5.36 ±0.40	4.13 ±0.27	4.27 ±0.28	2.33 ±0.40	3.20 ±1.70	3.59 ±0.37	3.88 ±0.77	3.08 ±0.26
Glutamic acid	4.48 ±0.50	7.91 ±0.70	5.97 ±1.33	6.17 ±1.07	6.12 ±0.35	1.68 ±0.16	6.44 ±1.05	5.32 ±0.64	4.20 ±0.12	5.12 ±1.28	6.02 ±1.01	7.94 ±1.35	24.62 ±3.86
Glutamine	- -	1.35 ±0.25	2.30 ±0.60	1.02 ±0.04	2.13 ±0.70	0.67 ±0.06	2.79 ±0.49	1.25 ±0.07	1.91 ±0.56	1.41 ±0.57	2.37 ±0.57	0.72 ±0.16	7.43 ±3.22
Glycine	24.80 ±2.51	12.32 ±0.54	14.48 ±0.75	16.06 ±2.57	18.50 ±0.29	15.20 ±1.64	17.07 ±1.30	18.24 ±1.84	17.62 ±1.74	17.53 ±3.06	19.69 ±0.36	19.69 ±1.57	3.40 ±0.72
Iso-leucine	2.47 ±0.67	1.42 ±0.19	1.74 ±0.44	1.52 ±0.12	1.89 ±0.07	1.53 ±0.23	1.58 ±0.04	1.54 ±0.11	1.38 ±0.25	1.33 ±0.30	1.85 ±0.03	1.39 ±0.14	0.42 ±0.08
Leucine	2.07 ±0.54	1.36 ±0.29	1.47 ±0.07	1.51 ±0.44	2.31 ±0.06	1.27 ±0.25	1.20 ±0.20	1.19 ±0.16	1.40 ±0.15	1.42 ±0.29	1.91 ±0.15	1.31 ±0.24	0.21 ±0.05
Phenylalanine	2.43 ±0.06	2.46 ±0.25	2.47 ±0.31	2.54 ±0.16	3.20 ±0.41	2.12 ±0.70	4.91 ±0.89	3.36 ±0.30	2.32 ±0.08	2.16 ±0.57	2.32 ±0.23	1.64 ±0.30	0.18 ±0.05
Proline	8.86 ±0.11	9.27 ±1.02	7.86 ±1.70	8.22 ±1.15	11.46 ±0.78	11.72 ±1.36	14.34 ±0.42	12.49 ±1.17	7.98 ±0.40	10.30 ±2.29	16.83 ±0.88	17.37 ±1.14	13.27 ±1.58
Tyrosine	7.87 ±0.73	3.03 ±0.17	2.75 ±0.14	2.97 ±0.71	3.53 ±0.10	2.74 ±0.30	4.16 ±0.40	3.81 ±0.50	1.76 ±0.21	2.13 ±0.55	2.81 ±0.30	2.75 ±0.41	0.28 ±0.03
Valine	5.14 ±0.28	3.10 ±0.32	3.14 ±0.33	3.42 ±0.24	4.24 ±0.23	3.55 ±0.34	3.45 ±0.07	3.72 ±0.19	3.11 ±0.36	2.56 ±0.29	4.22 ±0.22	3.69 ±0.32	0.66 ±0.13

All the incubations were carried out at 37°C for 30 minutes in the presence of O_2 in iso-osmolal saline plus the component given above. The ganglia were pre-incubated for 30 minutes in iso-osmolal saline containing amino acids.

than non-treated tissue when the tissue was incubated with 10^{-6}M Nicotine, 10^{-3}M malathion and 10^{-3}M chlordimeform. Valine and glycine were found at slightly lower concentrations than in control incubations. The concentration of Gaba was higher after treatment with DDT, dieldrin, methyl-parathion, nicotine and γ-BHC treated tissue. Glutamate, except in the presence of 10^{-3}M methyl-parathion, was rather stable in its concentration. Aspartate decreased significantly with methyl-parathion and nicotine, whereas it was found at higher concentrations after incubation with chlordimeform.

The concentrations of iso-leucine, leucine, phenylalanine, tyrosine and glutamine were more or less the same in all the incubations.

3.2.5 Effect of electrical stimulation on the concentration of amino acids in the nervous tissue

Electrical stimulation of the isolated thoracic ganglia of locust failed to show any excessive accumulation of the unknown compound either in the nervous tissue or the medium (Fig. 47). The quantitative analysis revealed increased concentrations of alanine and glutamine, whereas the rest of the amino acids were present at the same concentrations as found in the nervous tissue of a normal locust.

3.2.6 Release of amino acids from isolated ganglia

a) Efflux of amino acids during the incubation with insecticides

Table 23 shows that Gaba, glutamate and aspartate were retained to a great extent in the nervous tissue incubated in iso-osmolal saline in the presence of glucose and insecticides. The release in all cases was less than 10-15% of the amount present in the

Table 23. Free amino acids (expressed as % of total) released from the thoracic ganglia of Schistocerca americana gregaria to the incubation media under the influence of insecticides and electrical stimulation.

	Iso-osmolal saline	2% Acetone	2% Ethanol	10^{-3}M DDT	10^{-3}M Dieldrin	10^{-3}M Methyl Parathion	10^{-6}M Nicotine	10^{-4}M Nicotine	10^{-3}M Lindane	10^{-3}M Fanvalerate	10^{-3}M Malathion	10^{-3}M Chlordi-meform	Elec. Stimul-ation
	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Alanine	55.27 ±5.19	46.74 ±2.80	32.40 ±0.91	31.43 ±1.56	37.94 ±2.31	39.27 ±2.72	33.04 ±1.59	35.62 ±2.46	22.69 ±4.13	37.27 ±6.05	27.00 ±5.59	21.81 ±2.07	27.90 ±7.29
Aspartic acid	0.73 ±0.21	2.19 ±0.60	6.81 ±1.76	9.82 ±1.38	6.60 ±1.61	6.05 ±4.16	6.10 ±1.10	5.07 ±3.07	8.96 ±0.36	3.34 ±2.99	14.51 ±7.25	1.97 ±0.72	28.01 ±9.60
Gaba	35.83 ±2.57	5.78 ±0.74	8.90 ±3.85	5.84 ±2.91	4.66 ±1.14	6.16 ±4.64	4.32 ±0.12	- -	4.51 ±0.67	- -	- -	- -	3.32 ±1.42
Glutamic acid	29.53 ±6.07	6.05 ±0.75	14.02 ±5.30	15.15 ±3.96	9.60 ±3.05	8.25 ±0.64	13.71 ±0.32	10.90 ±1.79	11.94 ±0.54	11.93 ±3.15	22.99 ±7.86	8.03 ±1.32	26.39 ±7.06
Glutamine	- -	80.09 ±3.98	84.84 ±4.24	73.08 ±5.99	82.51 ±3.38	69.25 ±6.96	86.45 ±1.73	80.03 ±7.45	66.49 ±1.46	79.17 ±19.02	96.31 ±0.94	86.32 ±1.05	47.25 ±3.95
Glycine	55.95 ±1.55	60.29 ±3.70	45.26 ±2.30	47.21 ±3.86	56.70 ±1.61	54.80 ±7.67	46.85 ±0.97	48.14 ±6.15	42.23 ±3.90	53.82 ±5.67	56.89 ±3.88	36.93 ±7.85	38.10 ±11.62
Iso-leucine	58.38 ±3.75	63.33 ±4.45	60.64 ±8.42	51.64 ±1.70	62.27 ±1.40	50.21 ±3.00	50.36 ±2.47	46.31 ±7.78	42.79 ±3.58	56.17 ±6.22	50.04 ±5.19	38.68 ±2.80	41.36 ±8.65
Leucine	59.60 ±2.31	64.06 ±5.13	53.35 ±10.72	52.65 ±4.20	64.82 ±0.73	58.34 ±11.44	63.46 ±4.65	52.41 ±3.10	51.25 ±4.87	60.43 ±2.52	50.59 ±6.60	54.42 ±3.87	44.44 ±11.11
Phenylalanine	62.72 ±6.07	76.20 ±2.99	59.17 ±6.92	58.21 ±4.18	65.17 ±3.93	58.83 ±4.13	83.68 ±4.69	61.86 ±9.18	53.33 ±2.99	68.12 ±1.76	57.32 ±7.21	50.63 ±11.14	36.34 ±17.76
Proline	87.77 ±1.01	74.44 ±1.50	66.59 ±4.88	69.59 ±3.38	75.85 ±1.92	72.63 ±3.48	62.52 ±1.66	68.19 ±4.38	61.61 ±3.18	66.44 ±4.87	66.40 ±7.12	49.91 ±6.25	39.75 ±5.46
Tyrosine	51.79 ±4.21	46.50 ±3.55	44.31 ±5.29	43.34 ±4.54	56.66 ±0.17	49.68 ±12.90	43.06 ±4.35	51.59 ±6.41	41.27 ±3.18	48.16 ±5.19	34.52 ±2.37	38.42 ±0.81	27.15 ±12.99
Valine	57.90 ±2.54	55.98 ±3.99	50.99 ±1.00	49.14 ±1.52	58.34 ±2.05	55.48 ±6.41	46.91 ±2.83	39.13 ±4.35	40.93 ±1.33	48.39 ±7.27	45.48 ±8.16	33.49 ±4.63	17.91 ±6.45

All the incubations were carried out at 37°C for 30 minutes in the presence of O_2 in iso-osmolal saline plus the component given above. The ganglia were pre-incubated for 30 minutes in iso-osmolal saline containing amino acids.

nervous tissue. These results are similar to that found in the preliminary tests discussed in the previous section. The diffusion of glutamine, proline and phenylalanine into the media was greater under the influence of insecticides. As much as 96% of the total glutamine was found in the medium in the presence of 10^{-3}M malathion. The release of phenylalanine was around 60% and that of proline was 60-70%.

Most of the other amino acids studied were found equally distributed between nervous tissue and media.

b. Efflux of amino acids during electrical stimulation

The most striking differences observed in the release of amino acids following electrical stimulation of the thoracic ganglia was a decline in the release of proline, valine and glutamine compared to incubation with insecticides (Table 23).

Glutamate and aspartate which were mostly retained in the presence of insecticides showed a 26% and 28% efflux after electrical stimulation. No release in Gaba was recorded under either circumstance.

In short, the different insecticides had a variable effect on the amount and release of amino acids in vitro. The pattern of release of amino acids was essentially same as seen in preliminary investigations (Section 3.1.15). In contrast to the effect of insecticides, the electrical stimulation resulted in the release of aspartate and glutamate which was otherwise always retained by the tissue.

3.2.7 Identification of the factor accumulating in the haemolymph under stressful conditions

Previous reports (summarized in Table 24) on the identity of neuroactive agent accumulating in the haemolymph of insects under stress have variously suggested it to be an aromatic amine and an ester (Hawkins and Sternburg, 1964), leucine or isoamylamine (Tashiro et al. 1972, 1975), and a catecholamine (Colhoun, 1960). In the present work the substance found to accumulate in the haemolymph under stress was different from these compounds. The evidence for its identity is discussed below.

a) Identification by Thin layer chromatography

The substance appearing in the blood of locusts and cockroaches subjected to stress by insecticides and enforced activity had the same chromatographic mobility on polyamide sheets as that of Dans-taurine relative to Dans-OH in the final development (Fig. 48). Note the movement of standard taurine and the unidentified substance (indicated by arrow). Moreover the hydrolysis of the extract of haemolymph, before or after dansylation, with 6N HCl at 100°C for 15 minutes did not have any effect upon the concentration of this spot, thereby suggesting that this substance was not a polymeric amino compound. The identity of this compound as taurine was further strengthened by subjecting the [^{3}H]-labelled Dans-derivatives to multiple thin layer chromatography (as detailed in Fig. 49), on polyamide sheets along with the derivative of authentic [^{14}C]-taurine. This was followed by elution from polyamide and further resolution of Dans-derivative on a silica gel sheet. The recovery of constant proportions of ^{3}H and ^{14}C in the same spot showed that this substance co-chromatographed with standard taurine under more

Table 24. Summary of data on the chemical identity of the factor accumulating in the blood of insects treated with various insecticides.

	Insect	Treatment	Factor	Reference
1.	Cockroach (*Periplaneta americana*)	DDT, TEPP Dieldrin	Catecholamine	Colhoun (1960)
2.	Cockroach (*Periplaneta americana*) Crayfish (*Orconectes virilus*) Crayfish (*O. propinguus*)	DDT, TEPP, TMPP and Elect. Stimulation	Aromatic amine	Hawkin & Sternburg (1964)
3.	Silkworm (*Bombyx mori*)	DDT	L-Leucine	Tashiro *et.al* (1972)
	Silkworm (*Bombyx mori*)	DDT	Iso-amylamine	Tashiro *et.al* (1975)
4.	Locust (*Schistocerca gregaria*) Cockroach (*Periplaneta americana*)	DDT, Dieldrin Lindane, Methylparathion, Nicotine, Fenvalerate, Malathion & Enforced walking	Taurine	Present author

Fig. 48 - Autoradiographs of microchromatograms showing the resolution of [^{14}C]-Dans-derivatives from haemolymph of locust 4 hours after injection with γ-BHC. A; the deproteinized extract was reacted with Dans-Cl and then hydrolyzed in 6N HCl at 100°C for 15 minutes prior to chromatography; B; the extract was hydrolyzed before reacting with Dans-Cl. D; the lower portion of the chromatograms was subjected to a final development in a third solvent (10% NH_3); C; for comparison standard amino acids including taurine were co-chromatographed in the same three solvents.

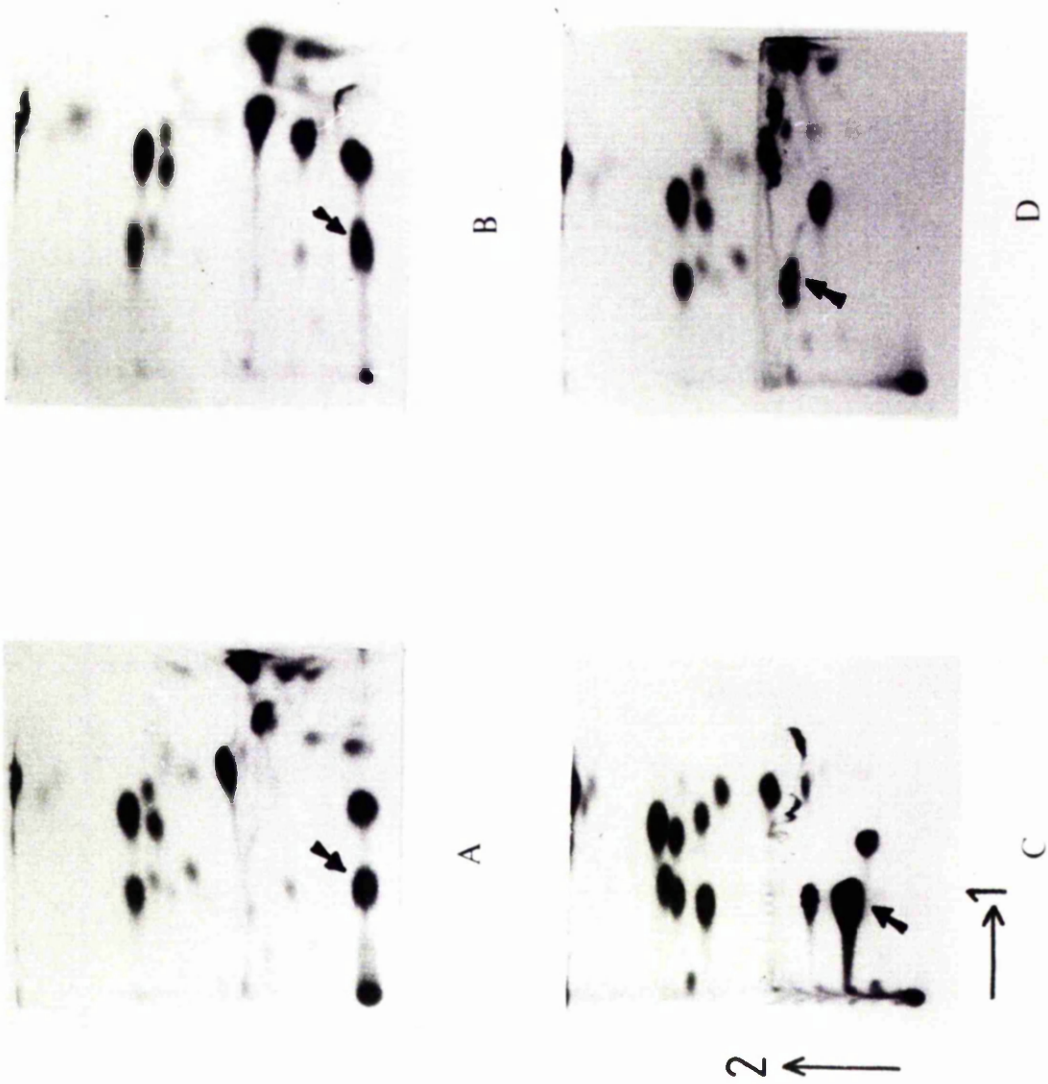


Figure 48

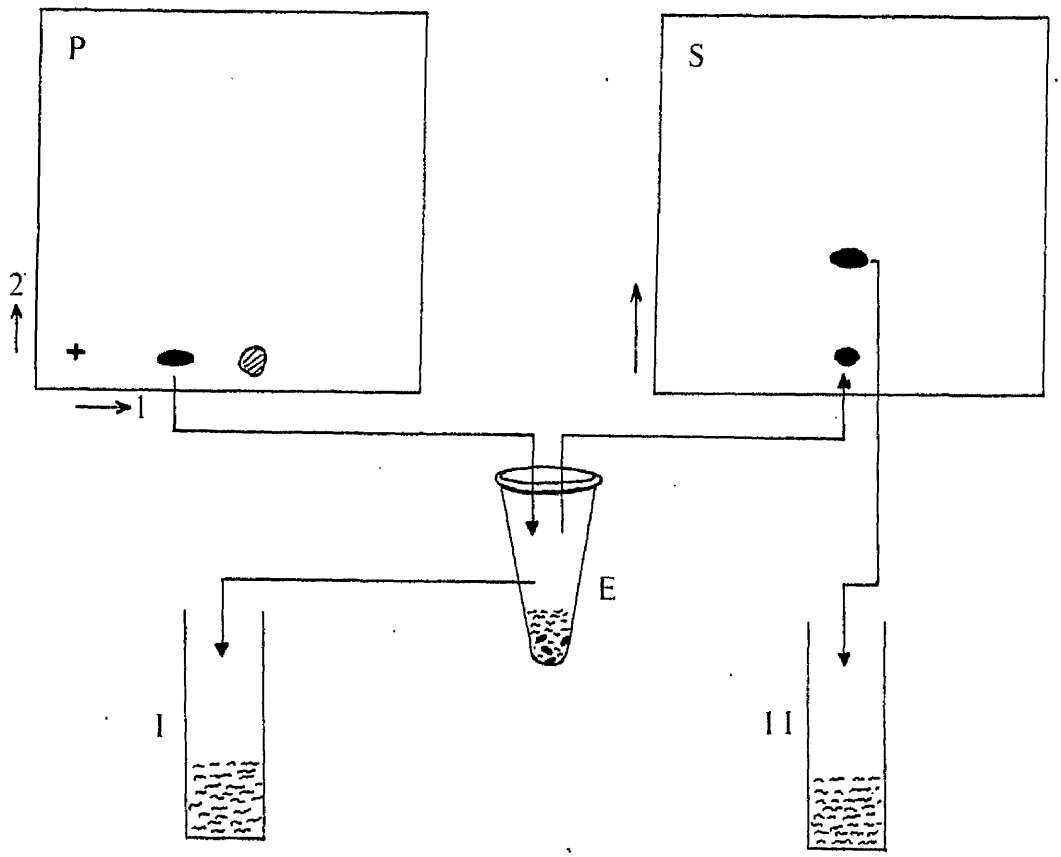


Figure 49 Scheme showing the procedure followed for co-chromatography of taurine with the unknown compound accumulating in the haemolymph of locust treated with γ-BHC.

Standard [^{14}C] -taurine was reacted with unlabelled Dans-Cl, and the extract of γ-BHC treated haemolymph was reacted with [^{3}H] -Dans-Cl. The derivatives were mixed and applied to a 5x5cm polyamide sheet (P), and subjected to bi-dimensional chromatography. The spot corresponding to Dans-taurine was cut out and eluted with 10% NH_3 (E). A portion of the eluent was directly transferred to scintillation vial (I) and the rest applied to a 5x5cm silica gel (S) and developed in chloroform/ methanol/acetic acid (15:4:1 v/v/v) (Seiler, 1970).

Cont .....

The UV-fluorescent spot on silica gel was scraped off into minivial (II) and radioactivity monitored by liquid scintillation counting. ^{3}H and ^{14}C were counted simultaneously with automatic correction for overlap. The results are presented in Table 25.

Table 25. Radioactivity associated with Dans-[^{14}C]-taurine and following multiple chromatography [^{3}H]-Dans derivative of unknown substance.

Sample		Radioactivity (dpm)		
		^{14}C	^{3}H	$^{14}C/^{3}H$
1	I	2093	988	2.12
	II	5108	2479	2.06
2	I	1624	597	2.72
	II	7022	2811	2.50

I and II represent the duplicate sample eluted from polyamide sheet and silica gel respectively as described in Fig. 49.

Sample 1 and 2 are from separate experiments.

than one set of chromatographic conditions (Table 25).

b) Identification by column chromatography

The identification of an amino compound whose concentration greatly increases was further confirmed by the ion-exchange column chromatography of the deproteinized haemolymph from stressed insects. It showed that there was a considerable increase in the concentration of taurine in the locusts treated with γ-BHC (Fig. 50). Quantitative results showed that taurine was the only amino acid increasing significantly in test insects. This increase was as much as 2-3 times the concentration of the control insects (Fig. 51). A small increase was also noticed in the concentration of alanine, leucine, arginine and threonine, whereas proline was found to be considerably reduced in concentration. The fall in proline and rise in alanine could be explained as being due to the oxidation of proline and concommitant formation of alanine (Section 4.5). A slight but insignificant increase was also observed in the concentration of taurine in the cerebral ganglia and thoracic ganglia which contrasted with the massive rise in the haemolymph (Fig. 52).

3.2.8 Neuropharmacological studies with isolated CNS

Before presenting the results of the electrical events recorded from the isolated nerve cord it would be worth pointing out that as the electrode was placed externally on the VNC, the sum of the spikes was recorded. The distance between the spiking neurons and electrode and the strength of electrical signals would determine the amplitude of the spikes which, therefore, would be different from one nerve preparation to another. However, once the electrode had been placed in its position, the resulting changes in

Fig. 50 - Resolution of free amino acids in the locust haemolymph by ion-exchange column chromatography. A; from haemolymph of a non treated locust;
B; from haemolymph of locust 6 hours after the injection of γ-BHC

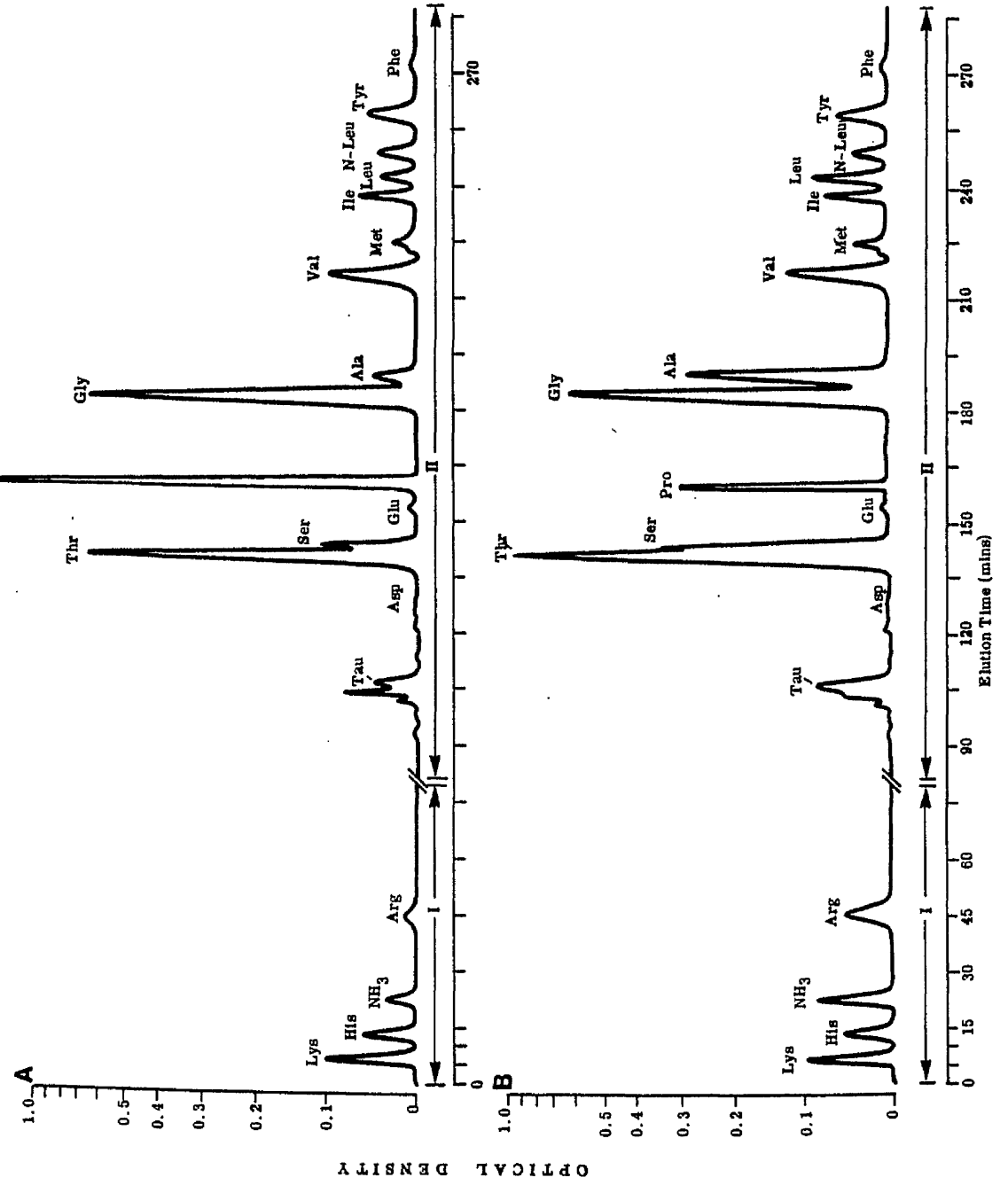

A
B
Lys
His
NH3
Arg
Tau
Asp
Thr
Ser
Glu
Pro
Gly
Ala
Val
Met
Ile
Leu
N-Leu
Tyr
Phe
I
II
OPTICAL DENSITY
Elution Time (mins)

Figure 50.

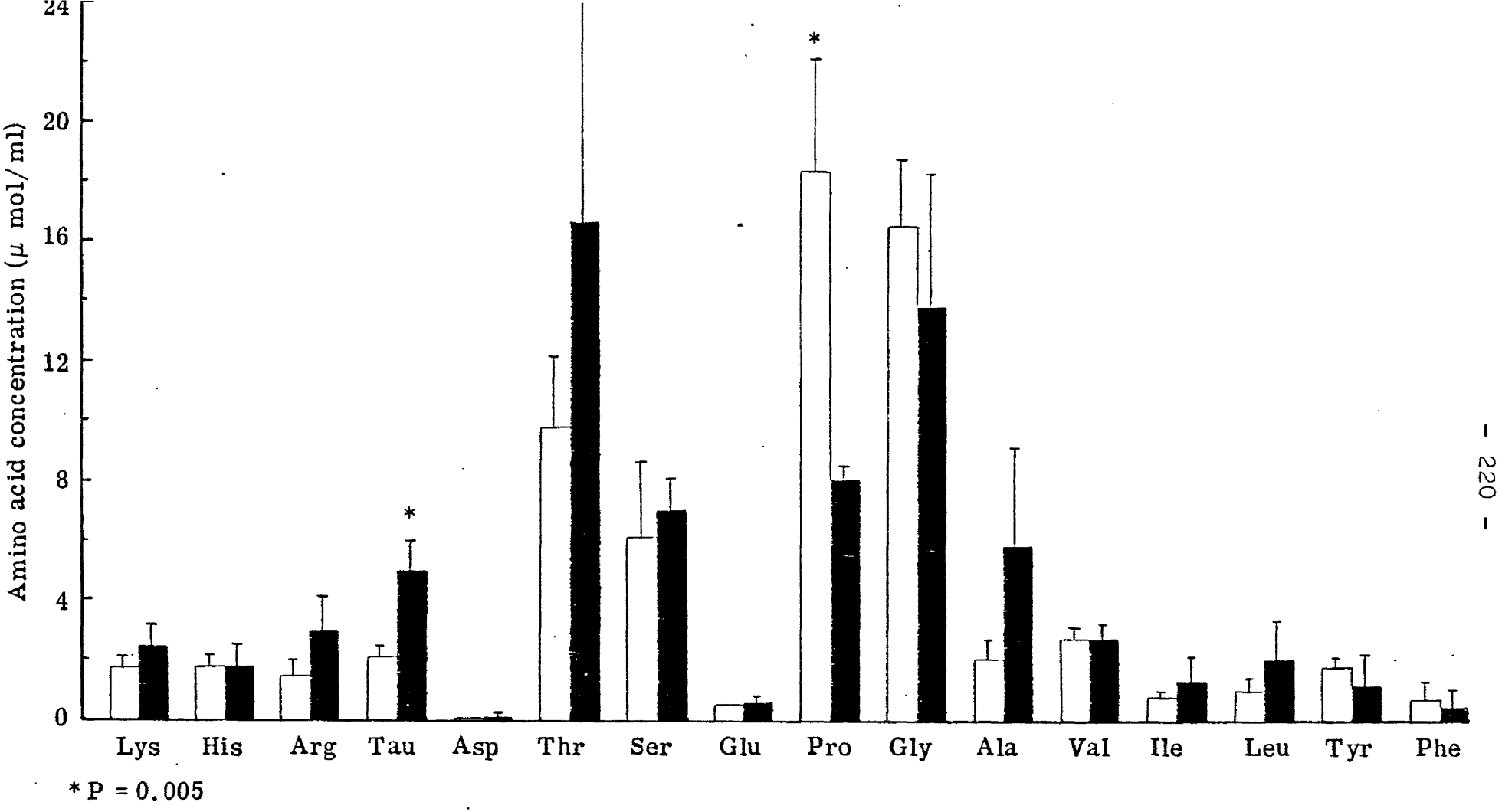


Fig.51 Concentration of amino acids in the haemolymph of locust (S. gregaria) 6 hours after the injection of γ-BHC (■). The controls (□) were injected with carrier solution only.

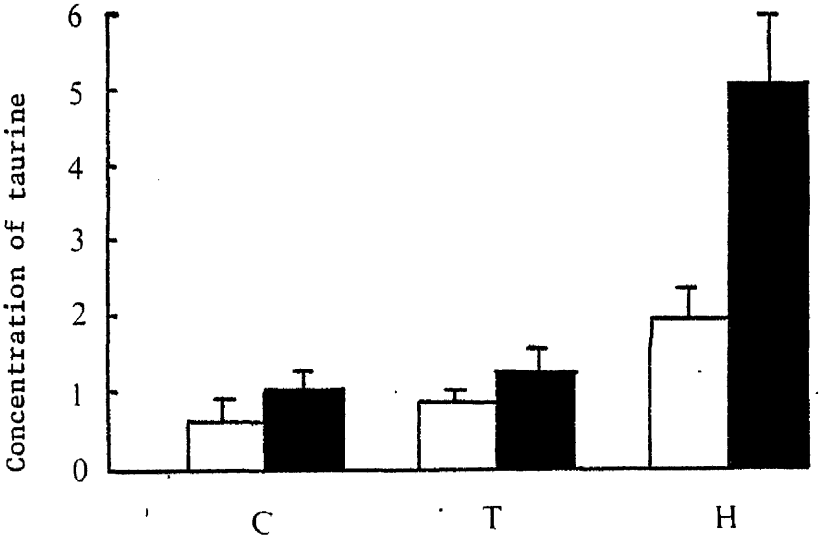


Fig. 52 - The change in the concentration of taurine in locust tissues due to insecticide treatment. The concentrations are expressed as μmol/g of tissue or μmol/ml of haemolymph. C = cerebral ganglia, T = thoracic ganglia and H = haemolymph. □ = concentration in normal locust; ■ = concentration in the locust 6 hours after the injection of γ-BHC. The vertical bars show the standard deviation of 4 different estimates.

the amplitude and frequency of firing would correspond to the actual experimental condition of that particular preparation e.g. the effect of various agents as manifested by increase or decrease in spontaneous activity.

a. Spontaneous nervous activity

As mentioned previously (Section 1.8.2) many reports have claimed that factors accumulating in the haemolymph of insects poisoned by insecticides show a stimulatory effect on the spontaneous nervous activity of the abdominal nerve cord of cockroach (Sternburg, 1959, 1963; Tashiro et al. 1972). The cockroach nerve cord has been reported to show activity 25 hours after its isolation. The locust seems less robust in this respect, vigorous spontaneous activity declining after 8 hours. Clement (1979) observed that activity could be recorded up to 6 hours in isolated thoracic ganglia of locust.

Figure 53 and Fig. 54 A,B, represent a typical record of the spontaneous activity of the isolated nerve cord of cockroach kept in iso-osmolal insect saline containing 10mM glucose as energy substrate. After the initial period of adjustment to the conditions in vitro the activity was quite stable and persistent over at least 2 hours, the maximum period needed to carry out these experiments. These results are in agreement with those of Clement (1979) with the locust CNS.

b. Nervous activity in response to poisoning of insects

As apparent from the figure 55, the isolated nerve cord removed from insects poisoned 5 hours previously with γ-BHC, compared to one from an unpoisoned insect showed a higher rate of firing, which was rather unstable over the period of recording

Fig. 53 - Spontaneous nervous activity recorded from isolated abdominal nerve cord of american cockroach placed in insect saline containing 10mM glucose. The traces represent the appearance of nervous activity after periods of; A = 30 minutes, B = 45 minutes, C = 60 minutes, D = 75 minutes, E = 90 minutes, F = 105 minutes and G = 120 minutes *in vitro*. For the amplitude of spikes in this and subsequent figures see the text.

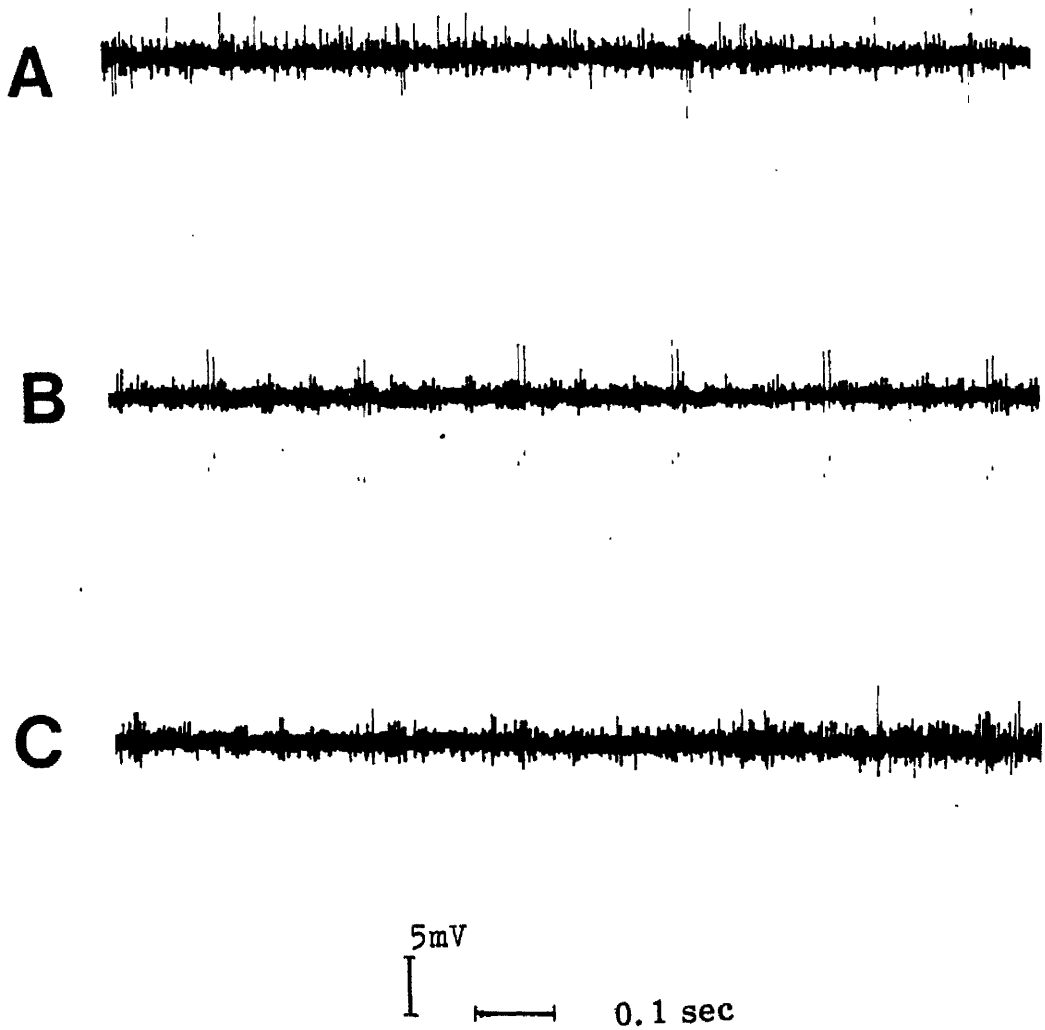


Fig. 53

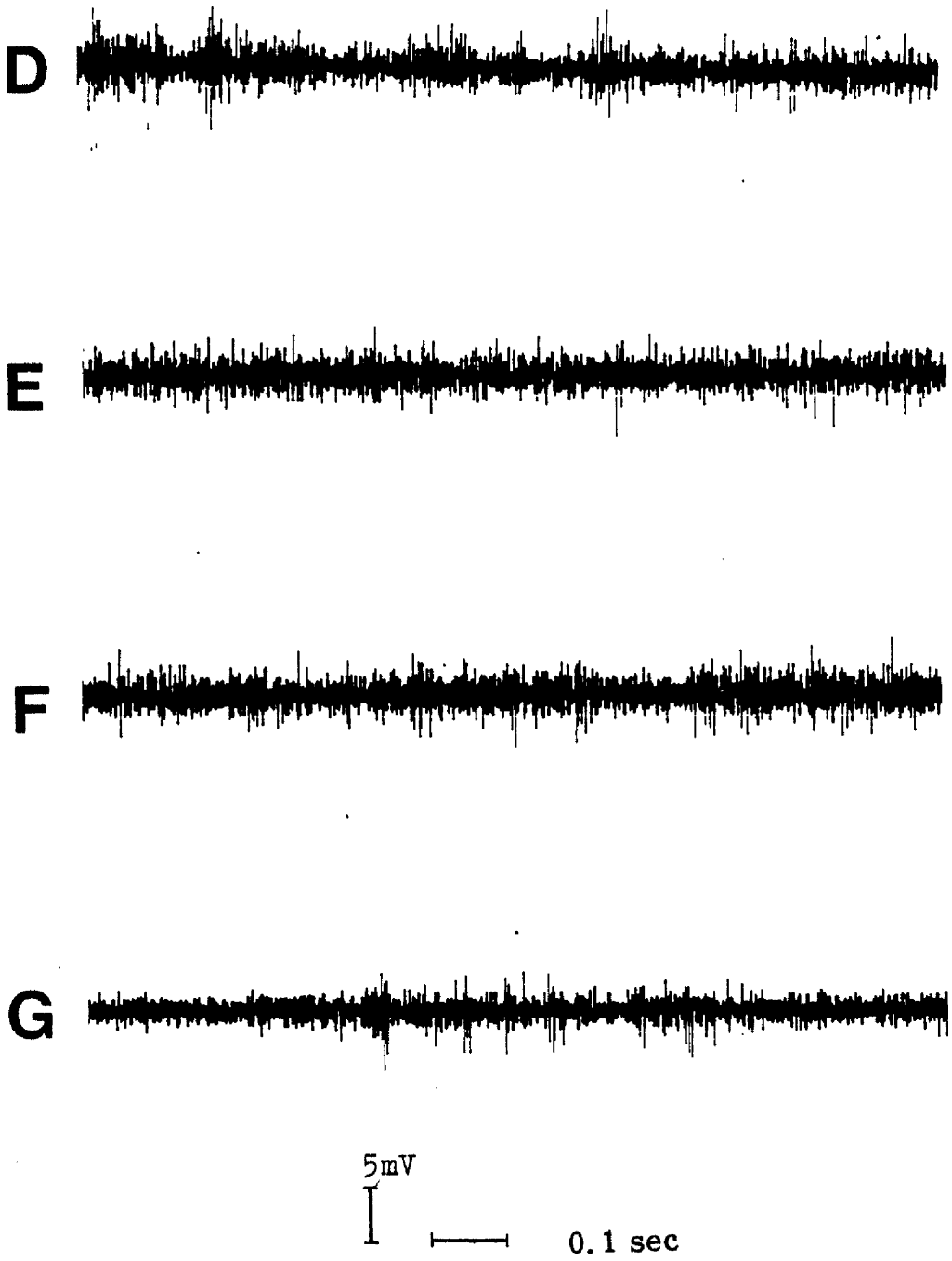


Fig. 53

Fig. 54 - Continuous record of the frequency of spontaneous nervous activity of the isolated nerve cord of normal cockroach (A and B) and nerve cord dissected from a poisoned cockroach (C). The detailed procedure of recording is given in Section 2.4.17.

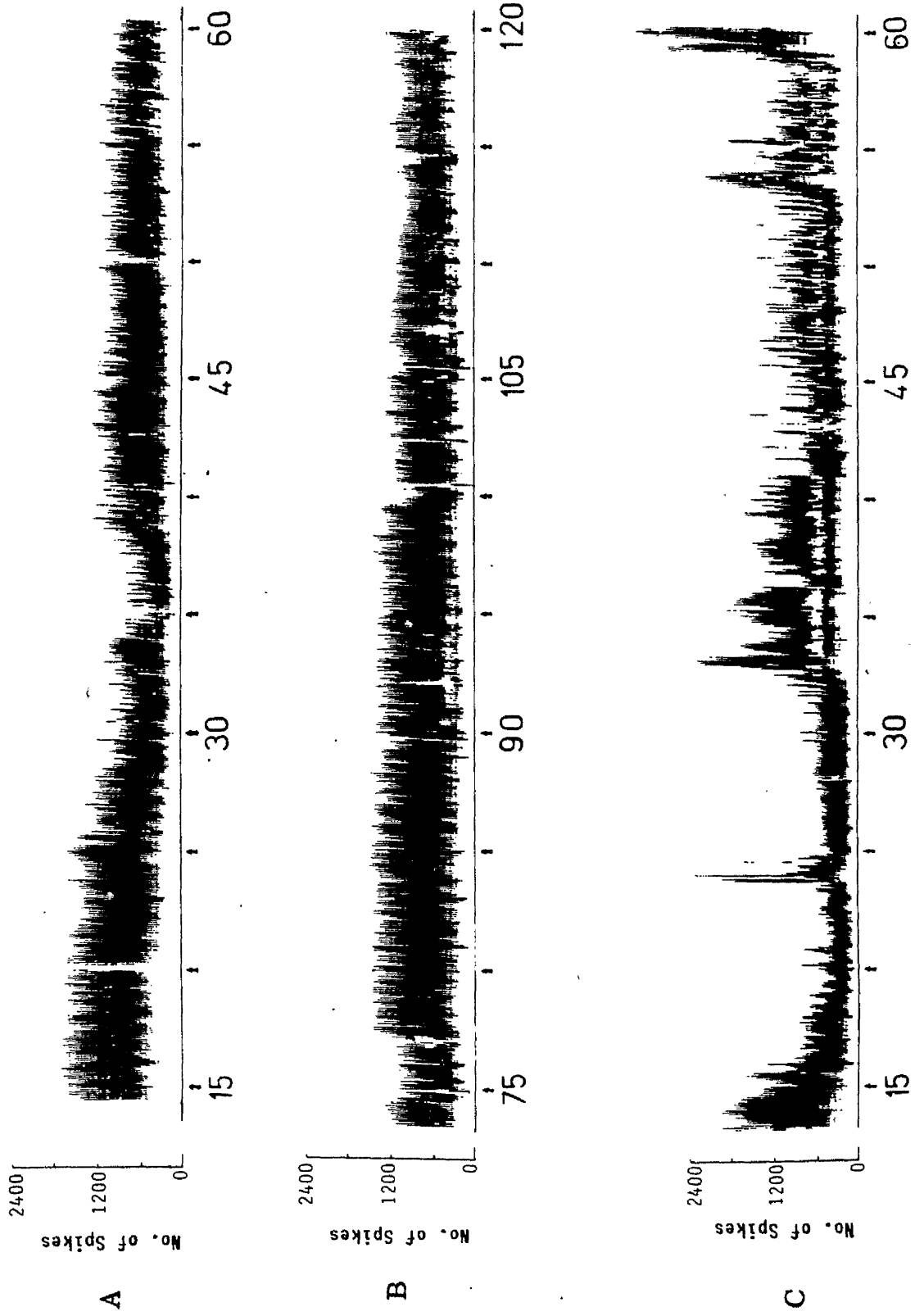

A
No. of Spikes
2400
1200
0
15
30
45
60
B
No. of Spikes
2400
1200
0
75
90
105
120
C
No. of Spikes
2400
1200
0
15
30
45
60

Figure 54

Fig. 55 - Spontaneous nervous activity recorded from isolated nerve cord of american cockroach poisoned with insecticide (γ-BHC). A = appearance of activity from a normal insect 30 minutes after dissection; B = activity of the cord 30 minutes, C = 35 minutes and D = 40 minutes after dissection from the insect which had received an injection of γ-BHC 5 hours previously.

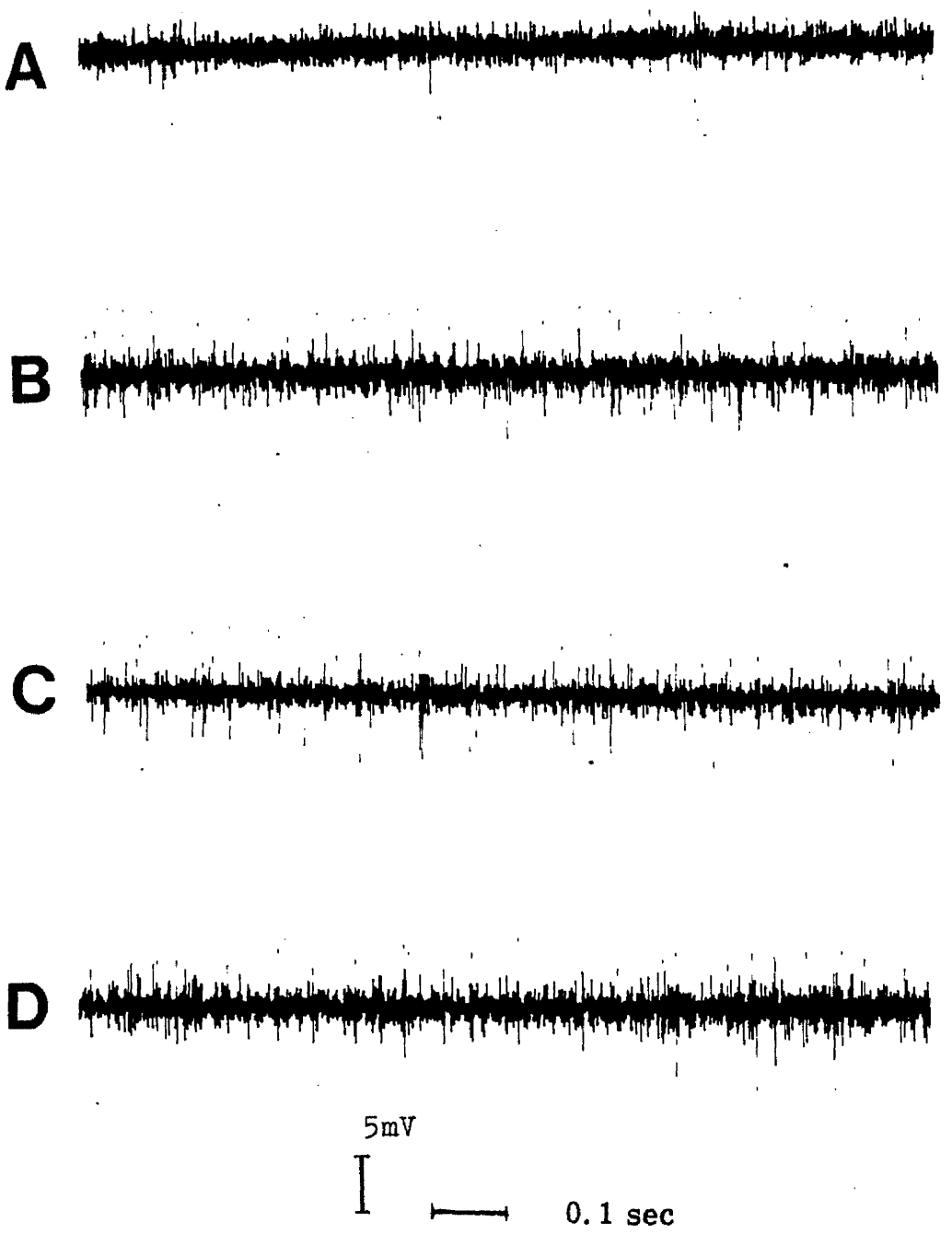


Fig. 55

(Fig. 54C). Most of the time the activity appeared in short bursts of enhanced neuronal firing.

c. Effect of nicotine on nervous activity

To verify that nerve cord preparations would respond adequately to the externally applied effectors, the cord was bathed with 10^{-3}M nicotine. The effect on spontaneous activity resulted in a sequence of events very characteristic of nicotine (Flattum & Sternburg, 1970). As shown in Fig. 56 and Fig. 57 there was immediate response and the rate of firing increased for 2-5 minutes before a complete block of nervous activity. When the nerve cord was washed with the saline the basal nervous activity returned. This proved that the ganglion was capable of response to the externally applied stimulus, rather than merely to uncontrollable endogenous conditions.

d. Effect of haemolymph from a poisoned insect on nervous activity

Perfusion of the isolated nerve cord with the haemolymph of the locust poisoned with γ-BHC resulted in a rather irregular but consistent, slight increase in the rate of firing (Fig. 58). Shankland and Kearns (1959) reported that their proposed "neurotoxin" was only effective when applied at 80 times dilution. Therefore, a range of dilutions of haemolymph in insect saline, (namely X400; X250 and X100) were applied as shown in Fig. 58. Contrary to the results of Shankland & Kearns (1959) it was observed that dilution of the haemolymph did not increase the potency of its effect, which varied directly with the concentration.

Fig. 56 - Response of the isolated nerve cord of american cockroach to perfusion with nicotine.
A = normal appearance of spontaneous activity, B = perfusion with 10^{-3}M nicotine showing excitation, C = block of the activity after 2 minutes, and D = washing the nerve cord with fresh saline reverts the normal activity.

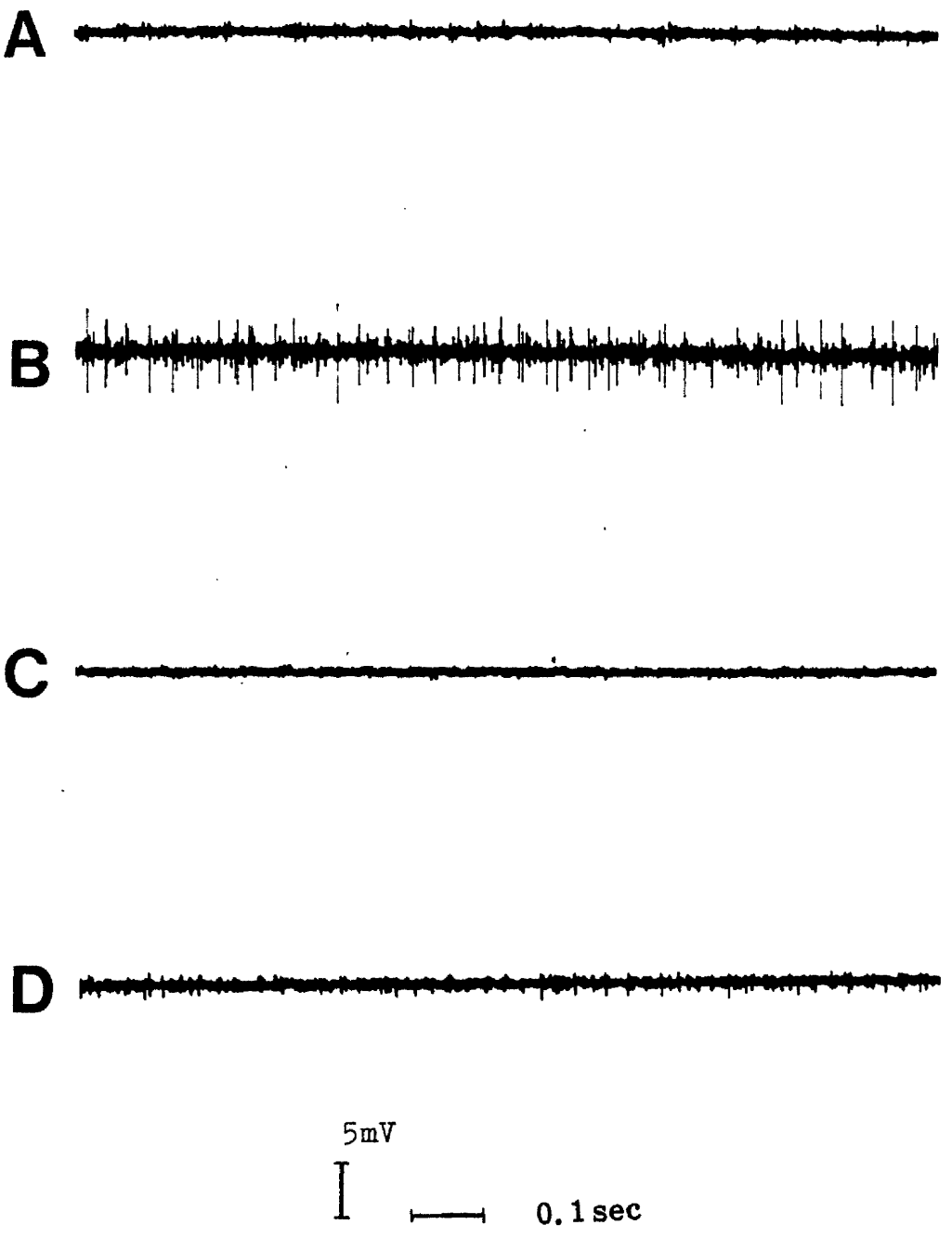


Fig. 56

Fig. 57 - Continuous record of the frequency of spiking in the isolated nerve cord of american cockroach showing the effect of 10^{-3}M nicotine on spontaneous nervous activity. T = time when test solution was applied and W = time when the nerve cord was washed with saline. Details of recording procedure are given in Section 2.4.17.

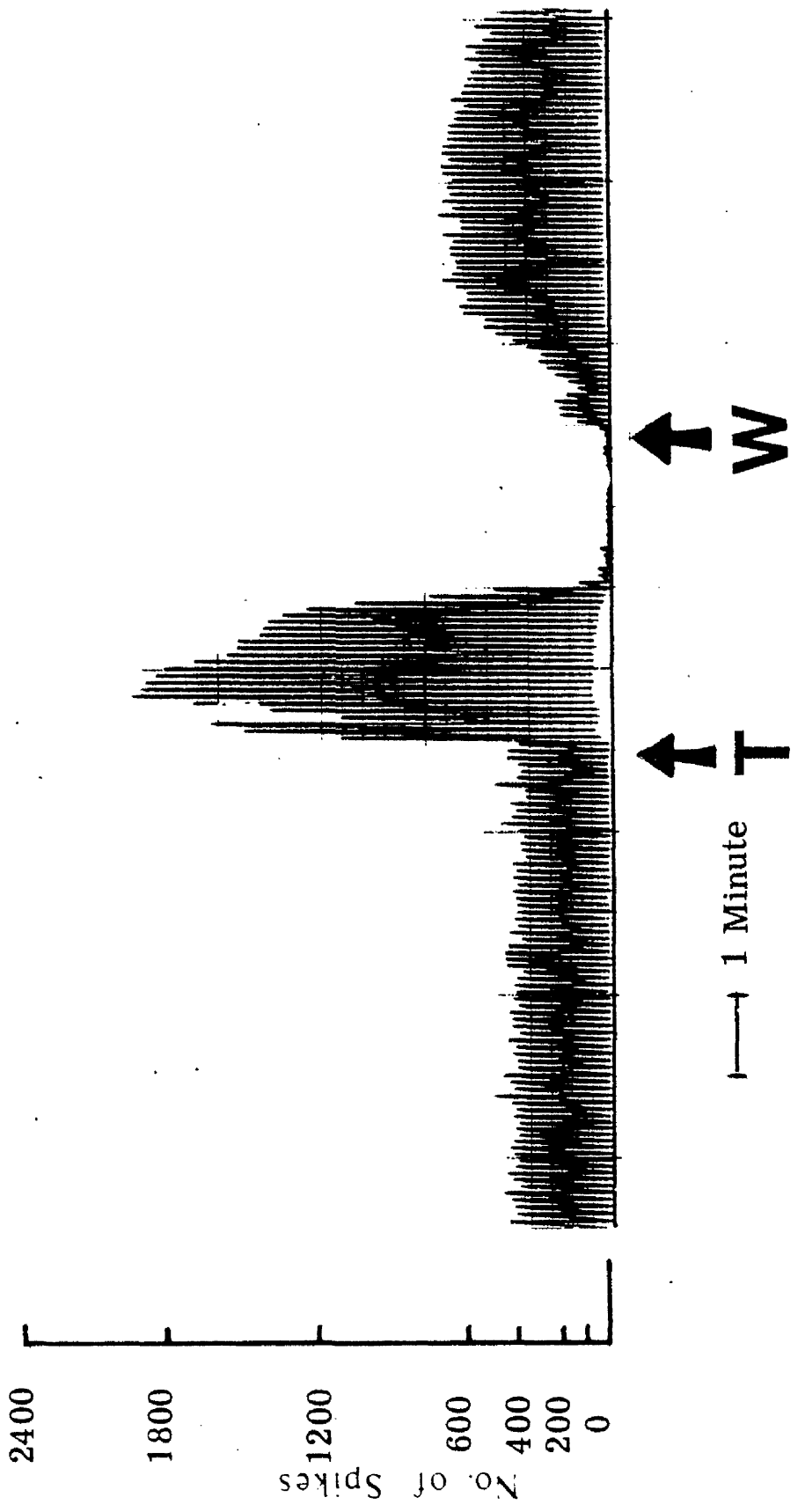


Figure 57

Fig. 58 - Response of the isolated nerve cord of american cockroach to perfusion with haemolymph of a locust paralyzed after treatment with γ-BHC.

A = appearance before perfusion with haemolymph.

B,C & D = effect of diluted haemolymph from paralyzed locust.

B - haemolymph diluted 400 times.

C - haemolymph diluted 250 times.

D - haemolymph diluted 100 times.

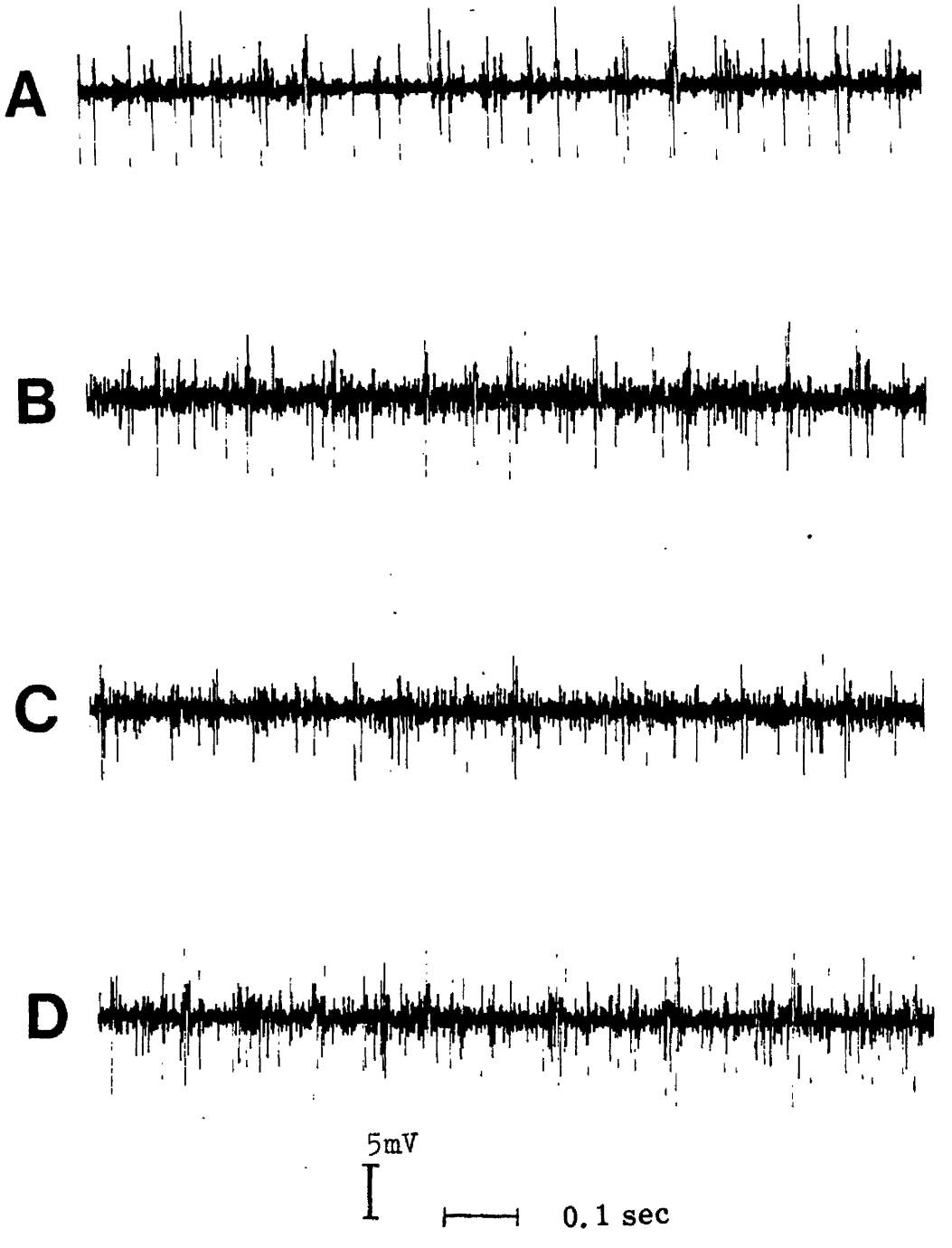


Fig. 58

e. Effect of taurine on the spontaneous nervous activity

As the taurine was the one amino compound definitely identified (Section 3.2.7) as increasing in concentration in stressed locusts and cockroaches, its effect was tested upon the spontaneous activity of isolated nerve cord.

The spontaneous nervous activity was considerably diminished when the cord was bathed in saline containing 10mM taurine (a concentration half that used by other workers, Gruener & Bryant, 1975; and Pelhate et al. 1978, in similar studies). The inhibitory effect of taurine was reversed by washing the cord (Fig. 59 & Fig. 60). Even perfusion of the isolated cord with a low concentration of taurine (e.g. $2x10^{-3}M$, that found in the haemolymph of normal locust) had a pronounced inhibitory effect. This inhibition increased with the concentration of taurine from $2x10^{-3}M$, through $5x10^{-3}M$ to $1x10^{-2}M$ (Fig. 61 and Fig. 62).

f. Effect of other amino acids on the spontaneous nervous activity

Tashiro and co-workers (1975) reported that leucine and isoamylamine (produced by the decarboxylation of leucine) at a concentration of $10^{-6}M$ and $10^{-5}M$ respectively, show an effect of excitation similar to that of the "neurotoxin" reported as present in the poisoned insects. They argued that these compounds could in fact be the "neurotoxins". Though the present study failed to record any production or accumulation of isoamylamine (Section 3.2.1), leucine was one of the amino acids found at a higher concentration in the haemolymph of poisoned insects (Section 3.2.7.b). The other amino acids found at a slightly higher levels

Fig. 59 - Response of the isolated nerve cord of american cockroach to perfusion with taurine.

A; normal appearance in insect saline;

B; after perfusing with 10^{-2}M taurine; and

C; washing the nerve cord with fresh insect saline.

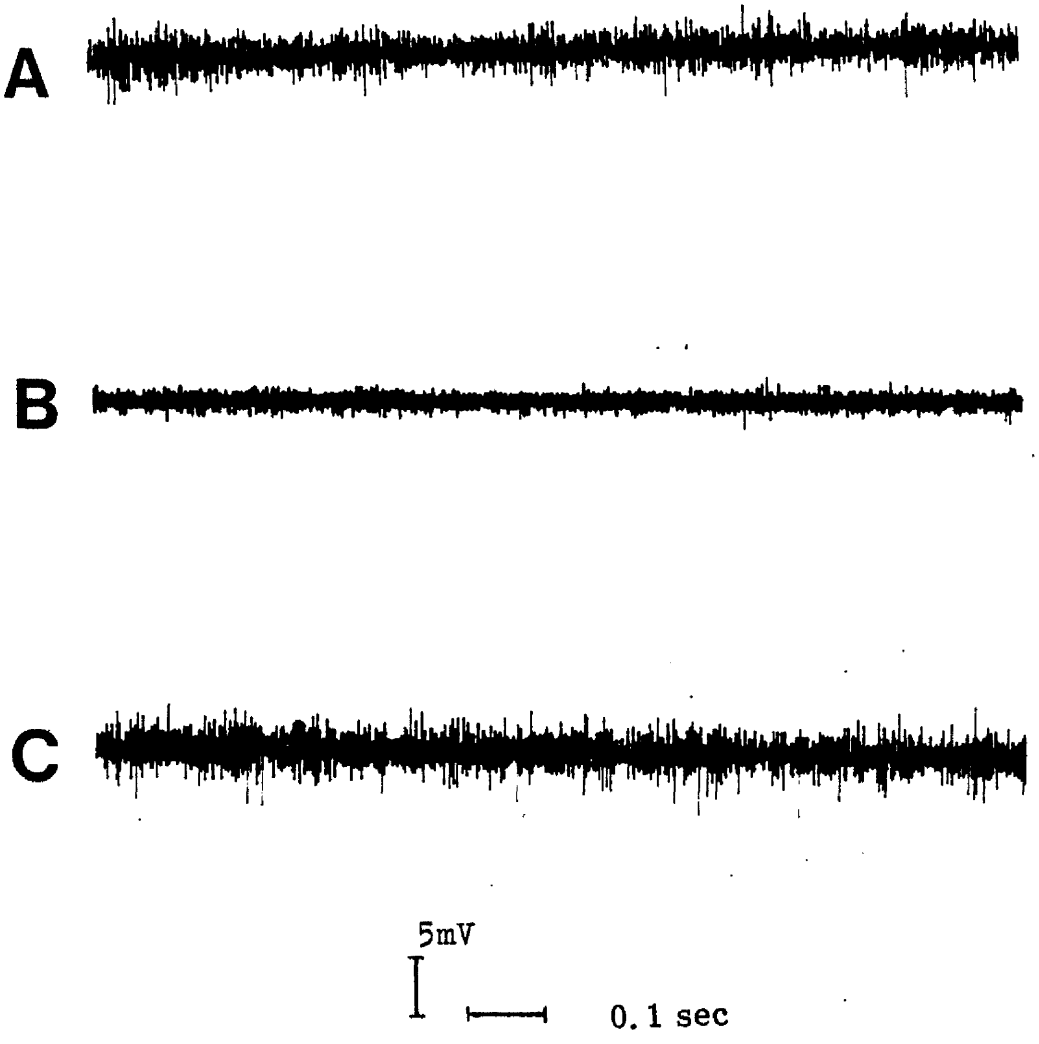


Fig. 59

Fig. 60 - Continuous record of the frequency of spiking in the isolated nerve cord of american cockroach showing the effect of 10^{-2}M taurine on spontaneous nervous activity. T = time of application of test solution and W = time when the nerve cord was washed with saline. The details of recording procedure are given in Section 2.4.17.

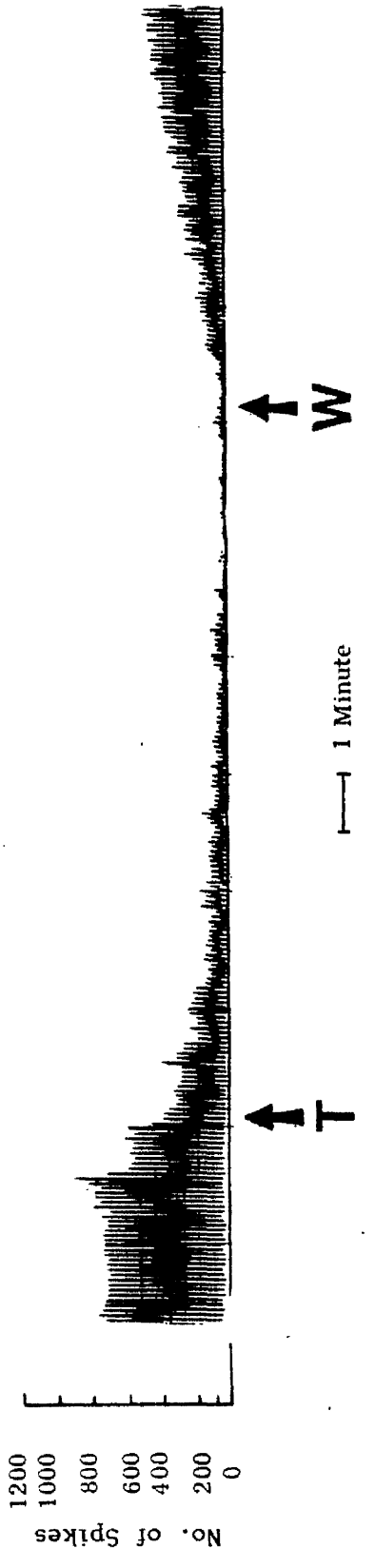


Figure 60

Fig. 61 - Response of the isolated nerve cord of american cockroach to perfusion with various concentrations of taurine. A; normal appearance in insect saline; B; perfused with 2 x 10^{-3}M taurine; C; perfused with 5 x 10^{-3}M taurine; and D; perfused with 1 x 10^{-2}M taurine.

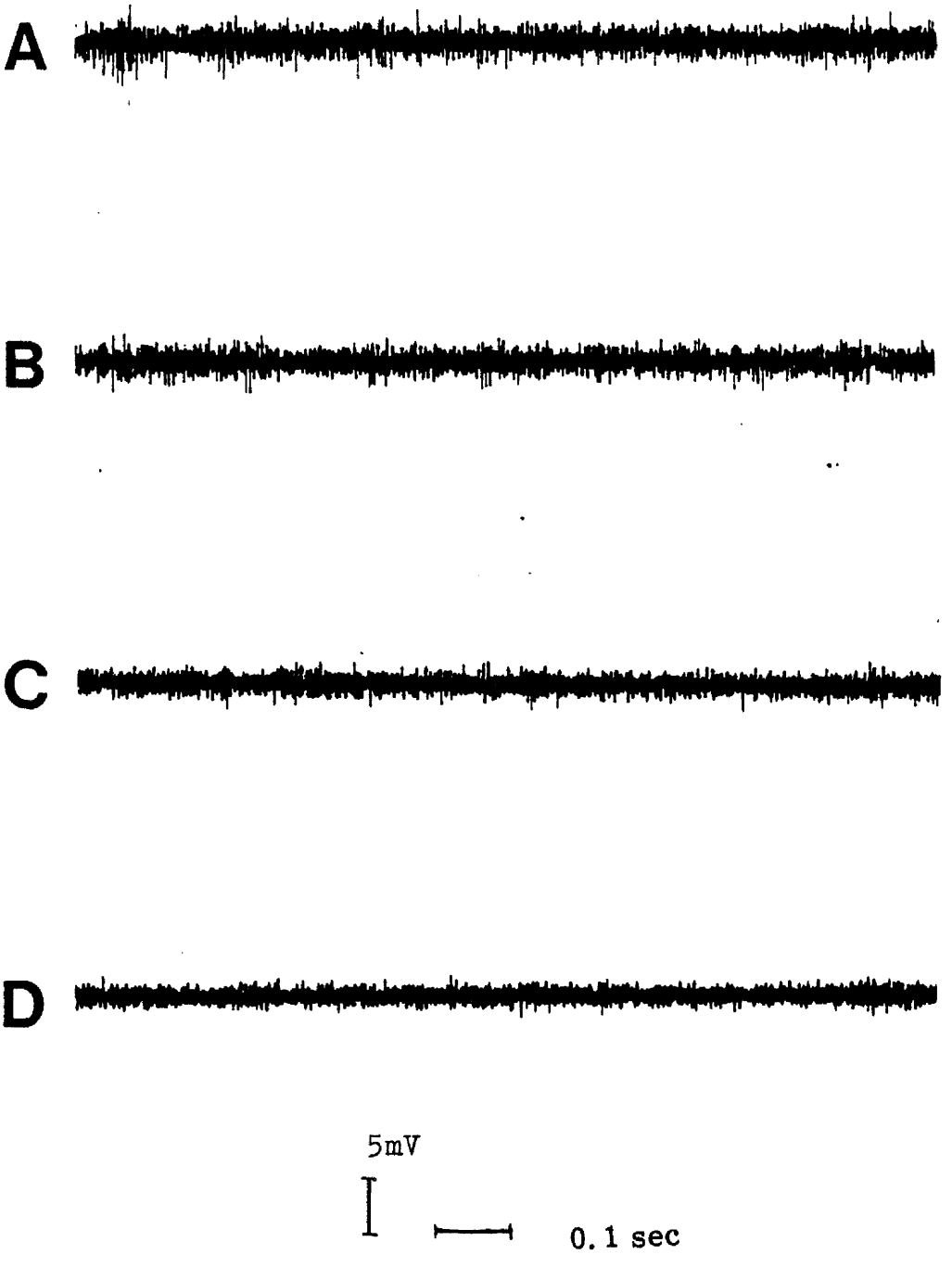


Fig. 61

Fig. 62 - Continuous record of the frequency of spiking in the isolated nerve cord of american cockroach showing the effects of A = 2 x 10^{-3}M taurine, B = 5 x 10^{-3}M taurine and C = 10^{-2}M taurine on spontaneous nervous activity.
T = time of application of test solution and
W = time when the nerve cord was washed with saline.
The details of recording procedure are given in Section 2.4.17.

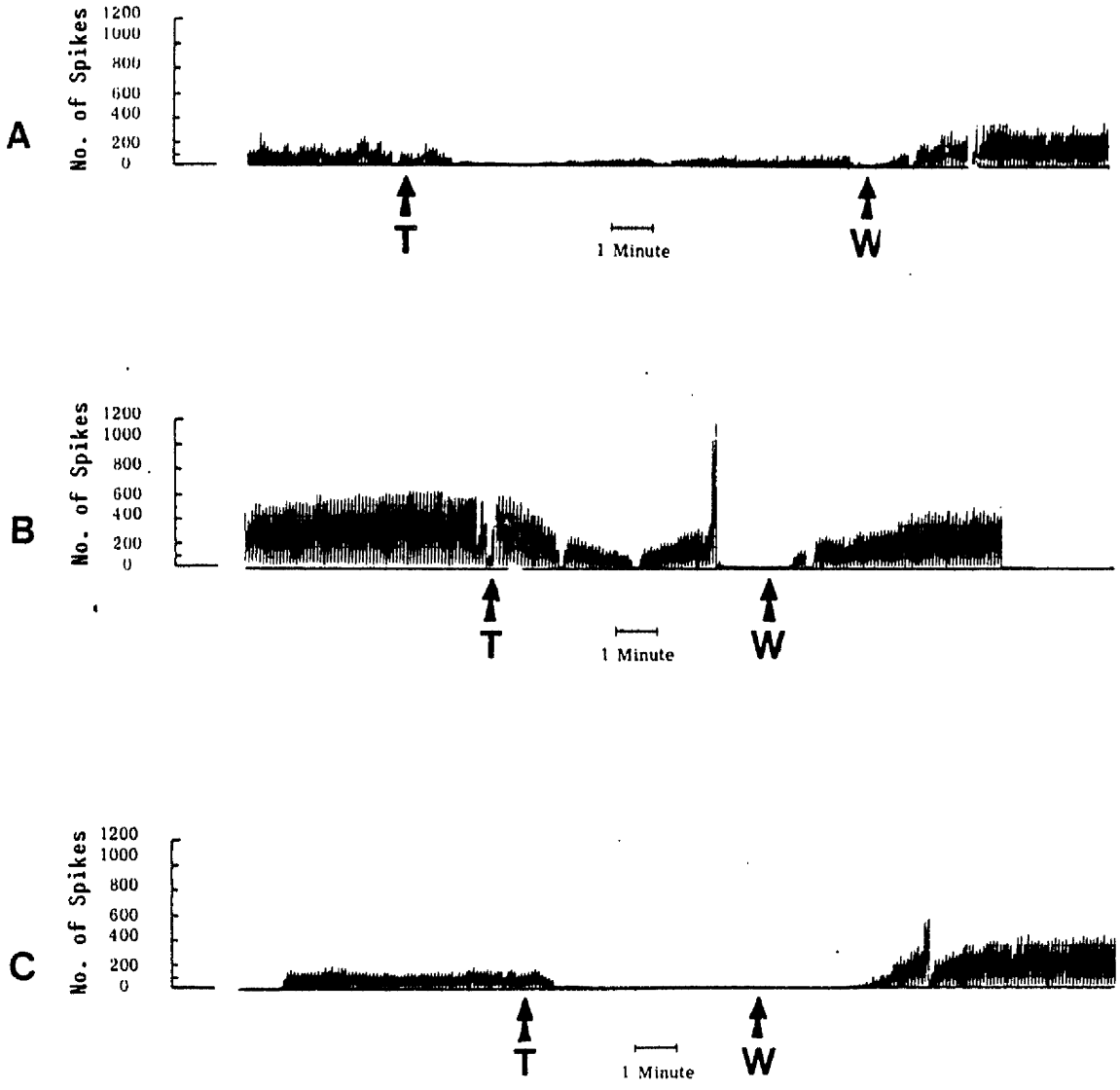

A
No. of Spikes
1200
1000
800
600
400
200
0
T
1 Minute
W
B
No. of Spikes
1200
1000
800
600
400
200
0
T
1 Minute
W
C
No. of Spikes
1200
1000
800
600
400
200
0
T
1 Minute
W

Fig. 62

were arginine, and threonine. The statistical analysis however, showed that this elevation in concentration was non-significant (i.e. $P>0.1$, <0.2). When the nerve cord was perfused separately with 10^{-6}M leucine, and 10^{-6}M iso-amylamine (The concentration used by Tashiro et al. 1975), $3x10^{-3}$M arginine and $1.5x10^{-2}$M threonine (the concentration of arginine and threonine found in the haemolymph of poisoned insects) an increase in firing rate of the nerve cord was observed (Fig. 63, 64) with all the amino acid tested.

To summarize, the isolated nerve cord from the paralysed insects show an abnormal higher rate of spontaneous activity. The nerve cord preparation responds to the externally applied effectors like nicotine in a predictable manner. Taurine, the amino acid whose concentration increases most markedly in the haemolymph of stressed insects, supresses the spontaneous nervous activity. While the other amino acids studied i.e. leucine, iso-amylamine, arginine and threonine all show an excitatory effect. The haemolymph from a poisoned insect also produces an increased rate of neuronal firing in the isolated nerve cord.

Fig. 63 - Response of the isolated nerve cord of american cockroach to perfusion with leucine and iso-amylamine. A; appearance of activity before the application of amino compounds; B; after perfusion with 10^{-6}M leucine; C; appearance of activity of another cord before applying amino compound; D; after perfusion with 10^{-6}M iso-amylamine.

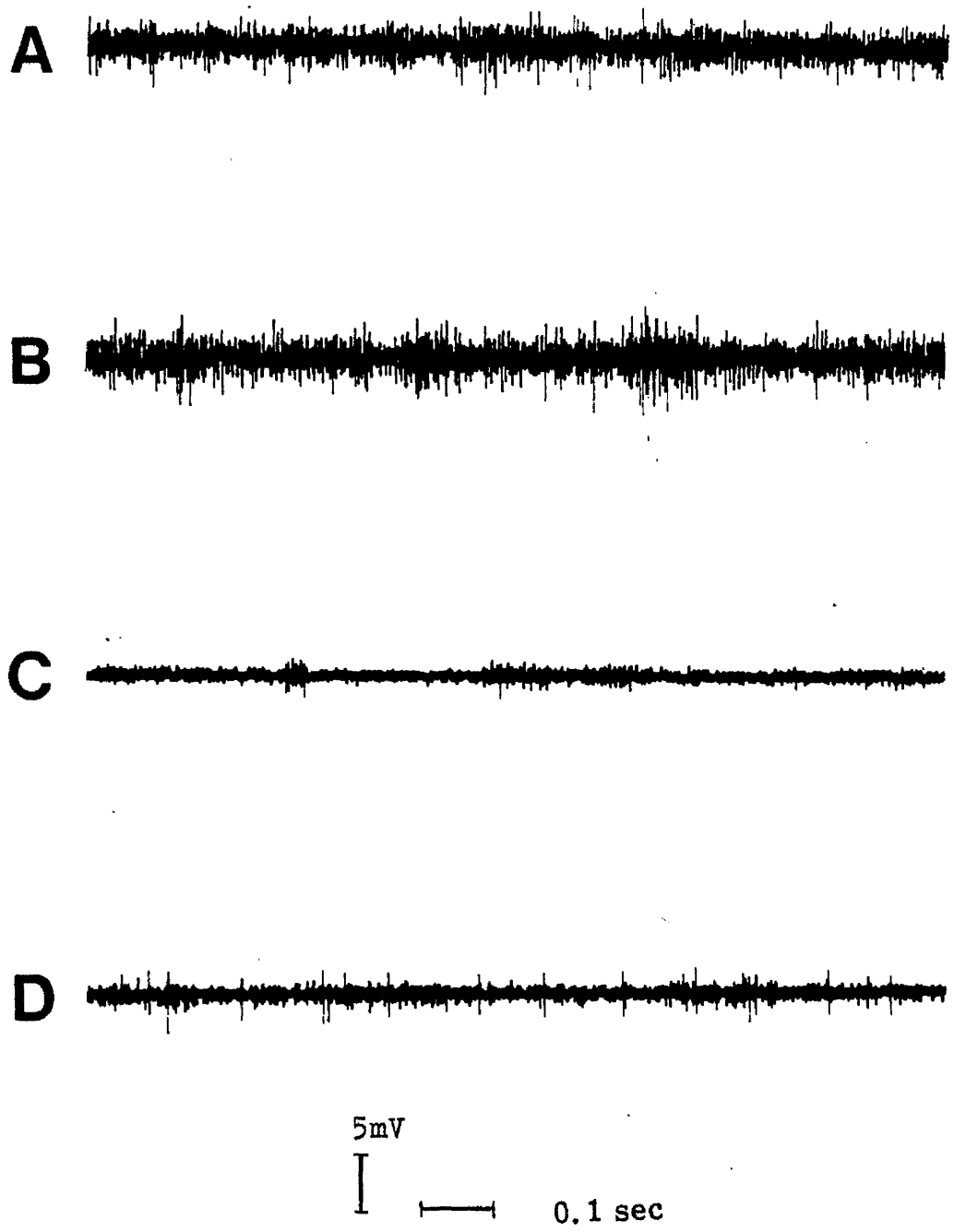


Fig. 63

Fig. 64 - Response of the isolated nerve cord of american cockroach to perfusion with threonine and arginine. A; normal appearance in insect saline; B; after perfusing with 1.5×10^{-2}M threonine; C; normal appearance of another nerve cord, and D; after perfusion with 3×10^{-3}M arginine.

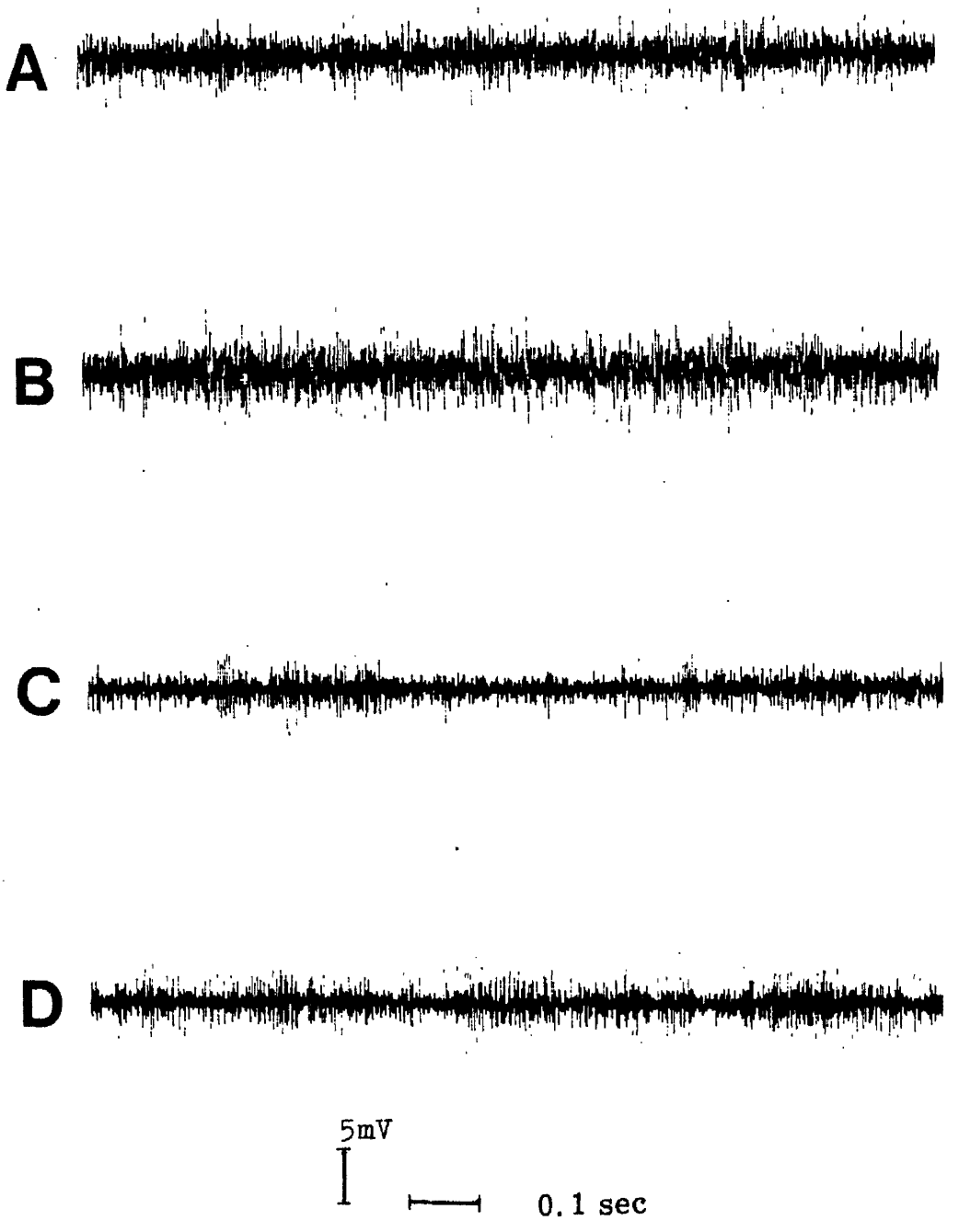


Fig. 64

PART THREE

METABOLISM OF AMINO ACIDS AND AMINES BY THE NERVOUS TISSUE OF LOCUST

3.3.1.1 Synthesis of alanine by the nervous tissue of Schistocerca americana gregaria

Alanine is one of the major constituents of the free amino acid pool of the nervous tissue. During the course of in vitro incubation and the application of insecticides to living insects it was observed that alanine always showed a substantial increase in concentration in the CNS. The concentration of alanine rose from 11.03 to 21.86 µmol/g wet weight tissue when thoracic ganglia were incubated in insect saline for 30 minutes at 37°C under aerobic conditions (Fig. 65).

This increase was more marked (rising to 37.89µmol/g wet weight tissue) when 10mM glucose was included in the medium.

The elevation of alanine always coincided with the decrease in glutamine and aspartate (from 6.74 and 9.97µmol/g to 2.73 and 5.98µmol/g wet weight of tissue respectively) (Table 26). At the same time a rise in the free ammonia from 3.28 to 6.13µmol/g wet weight was observed. During all the incubations the concentration of glutamic acid did not show any significant change.

The addition to the medium of 10mM amino oxyacetic acid, an inhibitor of aminotransferases, blocked the formation of alanine and disappearance of glutamine and aspartate. The possibility of the assimilation of free ammonia to yield alanine could easily be ruled out because the incubation of the tissue in the presence of 10mM ammonium acetate did not result in any additional increase in alanine, while the concentration of aspartate still declined. Incubation with 10mM glutamine resulted in a very large increase in the concentrations of alanine, glutamate, Gaba and free ammonia and

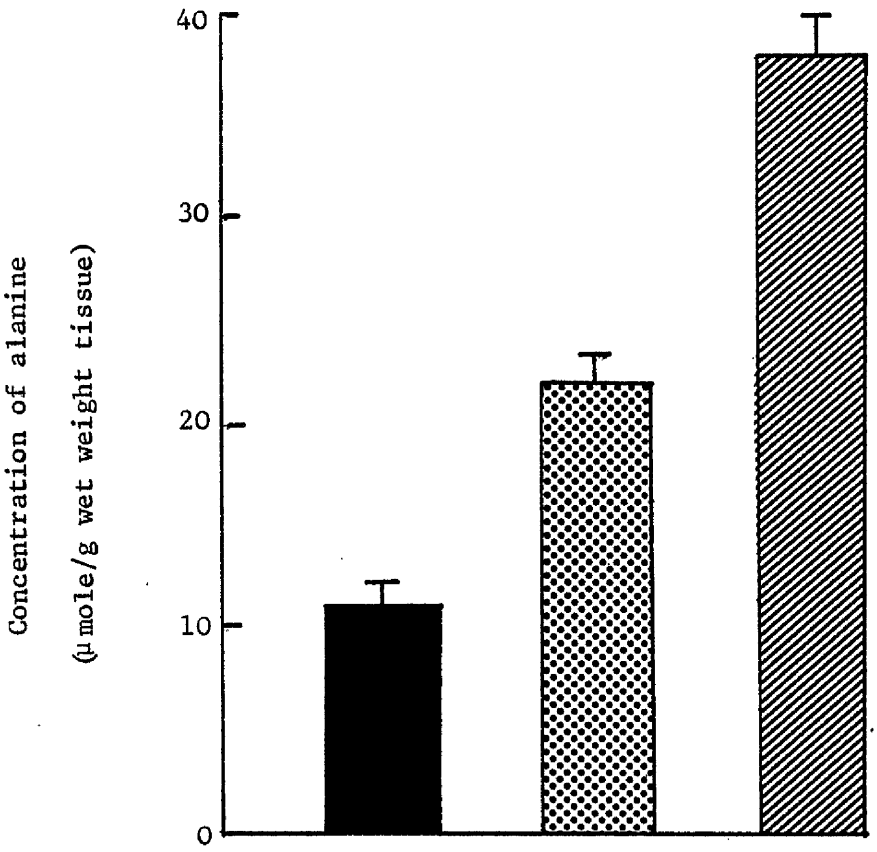


Figure 65 Concentration of free alanine in the thoracic ganglia of locust at the end of incubation for 30 minutes at 37°C.

Media were - ■ = Concentration in freshly dissected tissue; ⊠ = insect saline

▨ = saline + 10mM glucose

Vertical bars indicate standard deviation from the mean.

Table 26. Total free amino acids in thoracic ganglia of locust after 30 minutes incubation at 37°C.

	CONTROL (No incubation) Mean ± S.D.	INSECT SALINE Mean ± S.D..	Iso-osmolal Saline + 10mM Glucose Mean ± S.D.	Iso-osmolal Saline + 10mM Glucose + 10mM AOAA Mean ± S.D.	Iso-osmolal Saline + 10mM Glucose + 10mM Ammonium Acetate	Iso-osmolal Saline + 10mM Glucose + 10mM Glutamine
Alanine	11.03±0.54 (4)	21.86±1.41 (3)	37.89±2.00 (3)	13.05±0.97 (4)	22.69±3.07 (4)	41.22±1.22 (3)
Ammonia	3.28±0.85 (7)	6.13±0.91 (7)	6.68±1.54 (6)	10.51±1.68 (4)	*	23.28±4.50 (3)
Aspartic Acid	9.97±1.09 (4)	5.98±0.24 (3)	6.16±0.30 (3)	10.56±1.32 (3)	3.71±0.88 (4)	8.63±1.01 (3)
γ-Aminobutyric acid	2.89±0.14 (4)	3.36±0.06 (3)	4.52±0.72 (3)	3.18±0.22 (4)	3.48±0.52 (4)	5.03±0.46 (3)
Glutamic acid	10.88±1.40 (4)	11.98±0.19 (3)	13.12±0.37 (3)	14.30±1.98 (4)	9.16±0.64 (4)	19.97±2.99 (3)
Glutamine	6.74±1.68 (4)	2.73±0.03 (3)	3.47±1.30 (3)	8.47±0.44 (4)	5.78±1.40 (4)	**
Proline	12.38±0.65 (4)	10.36±0.20 (3)	12.11±0.59 (3)	11.00±1.31 (4)	12.13±0.87 (4)	12.22±0.51 (3)

AOAA = Amino oxyacetic acid

* = Exogenously NH_4^+ present

** = Exogenously Glutamine present

The concentrations are given (Mean ± S.D.) as μmole/g wet weight nervous tissues.

The figures in parenthesis represent the number of separate experiments.

also partially prevented the decline in the concentration of aspartic acid.

3.3.1.2 Time course for the synthesis of alanine

The literature contains many examples of alanine accumulating in anaerobic conditions in invertebrates (see Introduction section). Therefore, the synthesis of alanine in the nervous tissue in vitro was followed by sampling at 5, 10, 20 and 30 minutes both in the presence and absence of oxygen. Fig. 66 shows that alanine accumulates at a faster rate for the first 10 to 20 minutes under both conditions yielding as much as a 2 fold increase in O_2-saturated medium. This increase was more pronounced under anaerobic conditions i.e. 2.5 times in the fresh tissue. During this period there was a steady increase in free ammonia from 3.28 to 4.86µmol/g under anaerobic circumstances and an elevation from 3.28 to 10.5µmol/g wet weight tissue in the presence of oxygen. A corresponding fall in the concentration of both aspartic acid from 7.8 to 3.86µmol/g wet weight tissue and glutamine from 6.74 to 3.8µmol/g wet weight tissue was recorded. Again this decline continued for up to 20 minutes. When the medium was saturated with O_2 slight change in the concentration of aspartate occurred. The Gaba levels remained unaltered throughout the incubation.

Under anaerobic conditions no significant alteration in the concentration of proline could be detected, whereas there was a significant fall in its concentration when the tissue was oxygenated. This decline was more marked in the first 5 minutes of incubation, falling from 9.75 to 6.61µmol/g wet weight of tissue. After that it remained more or less at the same level.

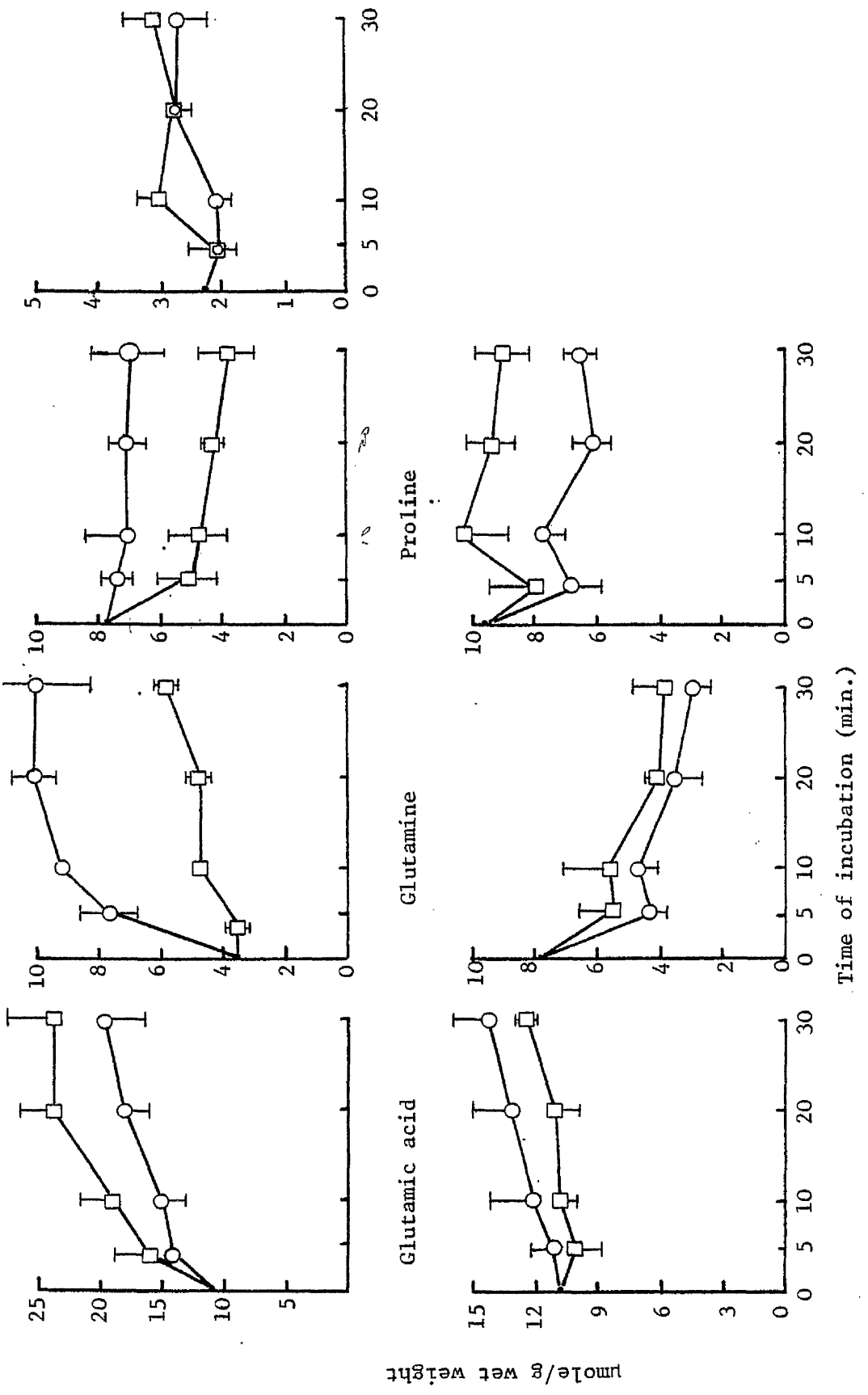


Figure 66 Concentrations (μmol/g tissue) of various free amino acids in the thoracic ganglia of locusts at various intervals of incubation at 37°C; (O), in the presence of O_2, and (□), in the presence of N_2.

These observations were consistant with the findings of Bursell (1963) from experiments carried out on the flight muscle of tsetse fly. In insect flight muscle (colorado beetle) Sacktor (1965) and mantle tissue of molluscs (Zwann, 1977), alanine is thought to be the end product of anaerobic glycolysis and the metabolism of proline, glutamate, aspartate, and alanine could be linked to a variety of schemes which replace the formation of lactate as end product with the resultant regeneration of NAD^+ (section 4.5). These schemes have in common the conversion of OAA to malate, its transport into the mitochondria; and its transformation to fumarate and succinate (see Fig. 5). In the other shuttle mechanism operative in invertebrate tissues and insect (Musca domestica) flight muscle (Estabrook and Sacktor, 1958) α - GP is formed from dihydroxyatetonephosphate (DHAP) and is transported into mitochondria and thereby converted back to DHAP, regenerating NAD^+ necessary for the maintenance of the glycolytic pathway. The locust thoracic ganglia have been shown to possess α-GPDH activity (Strang et al., 1979).

3.3.2.1 Energy metabolism in insect nervous tissue

In an attempt to determine the relative importance of the two shuttle mechanisms in the locust CNS and functional importance of alanine formation in both aerobic and anaerobic conditions, the tissue was incubated in saline saturated with O_2 or N_2. Various metabolites which might be expected to accumulate under anaerobic conditions ie. α-GP, malate, fumarate and succinate were estimated in the tissue and in the medium under both conditions.

As shown in Table 27 succinate was not detectable in freshly dissected or rapidly frozen tissue of locust nervous system. The concentration of α-GP, malate and fumarate were 0.84, 2.78 and 0.67 µmol/g wet weight respectively.

The incubation of nervous tissue for 1 hour in the presence of O_2 resulted in a substantial increase in α-GP, malate and succinate (from 0.84, 2.18 and "undetectable" to 2.86, 4.92 and 1.99µmol/g wet weight tissue respectively); whereas the fumarate disappeared altogether. The values under anaerobic conditions were 3.31µmol for α-GP; 2.28µmol for malate, 1.75µmol for succinate with undetectable fumarate. The addition of AOAA to block the transamination of aspartate to yield OAA which might be further metabolized to malate, fumarate or succinate, did not affect the metabolite concentration.

3.3.2.2 Rate of change in the concentration of metabolites of intermediary energy metabolism during incubation

The synthesis of alanine and catabolism of aspartate show a direct relationship to the period of incubation. Therefore, in order to find a link between the metabolism of these amino acids and the intermediates of the TCA cycle, the changes in the concentration of these metabolites were followed by analyzing the tissue and incubation media after 10 and 20 minutes of incubation. In the presence of O_2 a significant change was observed in all the metabolites 10 minutes after incubation which did not show further change at 20 minutes (Table 28). On the other hand the anaerobic metabolism showed a rise in α-GP from 0.84µmol/g to 1.86µmol/g wet weight tissue at 10 minutes and subsequent decline to a level of 0.97µmol/g

Table 27. Concentrations of TCA cycle metabolites, α-GP and alanine after the *in vitro* incubation of thoracic ganglia of locust for 60 minutes under aerobic and anaerobic conditions.

Incubation conditions (Insect Saline +)	αGP	Malate	Succ-inate	Fumar-ate	Alanine
Control (0 time)	0.84	2.18	N.D.	0.67	11.03
O_2	2.86	4.92	1.99	N.D.	19.44
N_2	3.31	2.28	1.75	N.D.	23.63
N_2 + 10mM AOAA	3.49	2.82	1.33	N.D.	13.45

The concentrations (in the medium and tissue) are the average of at least two separate estimates and are expressed as μmol/g wet weight of tissue.

Table 28. Concentrations of metabolites of TCA cycle, α-GP, lactate and alanine in thoracic ganglia after 10 and 20 minutes of incubation in aerobic and anaerobic conditions.

Incubation conditions	αGP	Malate	Succinate	Lactate*	Alanine
0	0.84	2.18	N.D.	0.00	11.03
O_2 - 10 min.	1.31	5.96	1.46	1.00	14.67
- 20 min.	1.48	3.78	1.47	1.50	18.00
N_2 - 10 min.	1.86	5.80	1.39	2.5	18.95
- 20 min.	0.97	4.58	3.91	3.8	23.60

The values are the average of two separate experiments and are expressed as μmole/g wet weight of tissue.

* from Strang et al., 1979.

wet weight. Malate showed higher concentrations both at 10 and 20 minutes, whereas succinate increased from being undetectable to 1.39μmol/g after 10 minutes and showed further elevation to 3.91μmol/g wet weight by 20 minutes.

In short, the concentrations of α-GP, malate and succinate show increases over those found in freshly dissected tissue, after incubations under both aerobic and anaerobic conditions. Malate and succinate are found at a higher concentration in media saturated with nitrogen, while α-GP in these circumstances after 20 minutes incubation was found at the same concentration as normally present in the nervous tissue.

3.3.3 Enzymes associated with the metabolism of amino acids

Table 29 shows the specific activities of some of the important enzymes involved in the metabolism of amino acids in the nervous tissue of the locust. Alanine and aspartate aminotransferases were the most active enzymes. The high specific activity of these aminotransferases indicates their importance to amino acid metabolism and is consistent with their importance in "malate shuttle". No appreciable activity was detected for $NADP^+$-dependent malic enzyme, alanine dehydrogenase, pyruvate carboxylase and phosphoenol pyruvate carboxykinase. The other enzymes studied, proline dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, NAD^+-dependent malic enzyme and oxalo-acetate decarboxylase were present with significant specific activities. The presence of these enzyme shows the capability of the tissue to use amino acids as a source of energy.

Table 29. Specific activities of enzymes of the thoracic ganglia of locust (Schistocerca americana gregaria)

Enzyme	EC no.	Maximum specific activity (μmol/g wet weight/min.)
Proline dehydrogenase	No number	5.26 ± 0.52
Glutamate dehydrogenase	1.4.1.2	3.71 ± 0.23
Malate dehydrogenase	1.1.1.37	8.94 ± 0.21
Malic enzyme (NAD^+)	1.1.1.39	4.01 ± 1.34
Malic enzyme ($NADP^+$)	1.1.1.40	N.D.
Alanine dehydrogenase	1.4.1.1	N.D.
Aspartate aminotransferase	2.6.1.1	65.0*
Alanine aminotransferase	2.6.1.2	41.0*
Oxaloacetate decarboxylase	4.1.1.3	0.25 ± 0.04
Pyruvate carboxylase	6.4.1.1	N.D.
Phosphoenol pyruvate-carboxykinase	4.1.1.32	N.D.

N.D. = not detectable

The specific activity quoted ± S.D. are averages of at least 3 estimates with the exception of the two results indicated thus *, which are the average of maxima obtained by the extrapolation of Linweaver-Burk plots from two separate experiments. All estimates were made at 37°C.

3.3.4 Uptake of O_2 by mitochondrial preparations and whole isolated nervous tissue of locust

Before giving the results obtained with mitochondrial preparation it would be necessary to draw the attention of obtaining pure mitochondrial preparation from different tissues as experienced by various investigators. The difficulty with preparing mitochondria from nervous tissue is that the mitochondrial fraction is contaminated with nerve-ending particles (synaptosomes, Marchbanks, 1975). The LDH is not a good marker for cytoplasm in locust CNS (Strang et al., 1979) but there is no other specific marker for cytoplasm in this tissue. It should have been detectable in occluded form (Marchbanks, 1975) in the mitochondrial/synaptosomal fraction P_2, capable of release by detergent, but no activity was detectable at all even on the addition of 0.1% Triton x-100. This is probably due to low activity and small amounts of tissue. Table 30 gives the activities of the marker enzymes associated with various fractions of mitochondrial preparation. Proof that the mitochondrial fraction is contaminated with synaptosomes is given by the high RSA (Relative specific activity = % of the total recovered enzyme activity in a fraction/% of total recovered protein in that fraction) of acetylcholine esterase, which is marker for cellular outer (plasma) membrane.

Nevertheless, under the conditions of preparing mitochondria about 80-90% cellular breakdown is achieved according to the release of LDH, and about 70% of the recovered succinate dehydrogenase (an enzyme bound to inner membrane of mitochondria,

Table 30. Summary of tissue fractionation from locust ganglia in the course of mitochondrial preparation.

Fraction	PROTEIN in Fraction	PROTEIN % of Total	ACETYLCHOLINE ESTERASE Sp.Act µmol/mg Prot./min.	ACETYLCHOLINE ESTERASE Total activity µmol/min	ACETYLCHOLINE ESTERASE % of total recovered	ACETYLCHOLINE ESTERASE R.S.A.	LACTATE DEHYDROGENASE Sp.Act µmol/mg Prot/min.	LACTATE DEHYDROGENASE Total activity µmol/min	LACTATE DEHYDROGENASE % of total recovered	LACTATE DEHYDROGENASE R.S.A.	SUCCINATE DEHYDROGENASE Sp.Act µmol/mg Prot/min	SUCCINATE DEHYDROGENASE Total activity µmol/min	SUCCINATE DEHYDROGENASE % of total recovered	SUCCINATE DEHYDROGENASE R.S.A.
P_1	0.65	30	0.33	0.215	52	1.73	0.012	0.008	17	0.56	0.024	0.0156	29	0.97
P_2	0.52	24	0.30	0.160	39	1.63	N.D.	N.D.		-	0.070	0.0364	66	2.75
S	0.96	45	0.04	0.037	9	0.20	0.041	0.040	83	1.84	0.003	0.0028	5	0.11
Total recovered	2.13			0.412				0.048				0.0548		
Total in fractionated homogenate	2.95			0.500				0.060				0.0590		
% recovery	72			82				80				93		

R.S.A. (Relative Specific Activity) is calculated as % recovered activity in fraction/% recovered protein in fraction.
A figure above one indicates enrichment.

Hatefi and Stigall, 1976), is in the mitochondrial/synaptosomal fraction. Moreover, the increase in the activity of fumarase from 0.09µmol/min/mg mitochondrial protein to 0.134 µmol/min/mg mitochondrial protein after disruption of mitochondria with sonication and from 0.19 µmol/min/mg mitochondrial protein to 0.21 µmol/min/mg mitochondrial protein by disintegrating the mitochondrial membrane with Triton X-100 shows the intactness of mitochondria in the present preparation.

As regards the impurity of the mitochondrial fractions, further fractionation by density gradient centrifugation (Marchbanks, 1975) was considered too lengthy and unsuitable for such small amounts of material, and the attempts to follow the method of Stahl et al. (1963) with differential centrifugation in sucrose containing 8% "Ficoll" resulted in preparations still somewhat contaminated, and with very low rates of O_2 uptake. Anyhow, it could be argued that the presence of synaptosomes in the mitochondrial fractions should not much affect the rate of O_2 with different substrate, as there seem to be no problem of differential penetration by the different compounds.

The results from a comparative study of various substrates in terms of O_2 uptake after their addition to the mitochondrial preparation of locust nervous tissue are given in Table 31. Proline was found to be the best substrate, being oxidized at a rate equivalent of 0.18µmol O_2/min/mg mitochondrial protein followed by succinate at 0.15 µmol O_2 and the rest, glutamate, α-GP and pyruvate oxidized at a comparable rate of 0.09, 0.11 and 0.10µmol O_2/min/mg mitochondrial protein respectively. It was noticed that the addition of ADP to the

Table 31. Rate of utilization of various substrates by mitochondrial preparations from the nervous tissue of locust as measured by O_2 uptake.

Substrate	µmolO_2/min/mg of mitochondrial protein	% inhibition by 2mM AOAA
Proline	0.18 ±0.02	29
Glutamate*	0.09 ±0.02	100
α-GP*	0.11 ±0.002	0
Succinate	0.15 ±0.07	0
Pyruvate	0.10**	0

All substrates were present at a concentration of 10mM in the final assay. ADP at a concentration of 1mM was also present in each assay.

* Same rate of oxidation was observed with a substrate concentration of 5mM.

** The result is an average of two separate estimates whereas the rest are mean ± S.D. of at least three separate experiments.

assay medium accelerated the rate of utilization of all the substrates and particularly glutamate showed an absolute requirement for ADP for its metabolism by mitochondria. It could be due to the reports that ADP is necessary to stimulate the GAD in such preparations (Hansford and Sacktor, 1970).

The addition of 2mM AOAA, an aminotransferase inhibitor, reduced the rate of proline oxidation by 29% while it completely stopped the O_2 uptake due to glutamic acid. No effect of AOAA on the oxidation of α-GP, succinate and pyruvate was observed.

Parallel experiments with the whole isolated thoracic ganglia (Table 32) showed that the tissue oxidizes 10mM proline at a faster rate (332μmol O_2/g wet weight/hour) than 10mM glucose (241μmol O_2/g wet weight/hour). This later figure is very similar to that obtained by Clement and Strang, (1978). The presence of both 10mM proline and glucose in the medium did not produce an additive effect. These results suggest that proline is a better substrate than glucose or pyruvate for the mitochondria and whole nervous tissue of locust; and that the role of proline is not simply an anaplerotic one to allow a faster rate of oxidation of glucose utilization.

10mM glutamate and 10mM glutamine were both tried as substrates for the intact ganglia. In neither case did the addition of substrate stimulate the uptake of O_2 by the tissue above that due to endogeneous substrate. As a result it is impossible to say how effective are these amino acids as substrates for energy metabolism in intact nervous tissue.

Table 32. - Rate of utilization of glucose and proline by the isolated intact thoracic ganglia of locust as measured by O_2 uptake.

	10mM Glucose	10mM Proline
	240	312
	235	334
	247	350
Mean ± S.D.	241 ± 6	332 ± 19

Results are presented as $\mu mol O_2/g/hour$

$P<0.002$

3.3.5 Synthesis of taurine by the nervous tissue of locust

a) from unlabelled methionine and cysteine

Taurine shows some of the characteristics of a 'neurotoxic factor' in that it accumulates in haemolymph under stressful conditions and has an effect on spontaneous nervous activity. As such factors have been reported to originate in the nervous system, the possibility of the synthesis of taurine in the nervous tissue was studied by incubating the isolated thoracic ganglia of locust in saline containing 10mM methionine and 10mM cysteine as precursors. Table 33 shows that even after 4 hours incubation with methionine there was no increase in the concentration of taurine, whereas a slight but a rise of low significance from 0.55 to 0.79 was observed in the presence of cysteine.

b) From [^{35}S]-methionine

The study was extended to the use of more easily detected radiolabelled precursor. For this purpose [^{35}S]-methionine was injected to the live locusts and the nervous tissue was dissected after 6 and 24 hours. The analysis of the tissue by ion-exchange column chromatography, monitoring radio-label in the eluted fractions, failed to show any significant recovery of the radio-activity in taurine above background, even after 24 hours.

3.3.6 Metabolism of tyrosine by the nervous tissue of locust

Tyrosine is the amino acid precursor of catecholamines both in vertebrate brain tissue (Cooper, et al., 1976) and insect nervous tissue (Mir, 1981). During preliminary studies for this thesis the metabolism of tyrosine was studied by incubating the isolated thoracic ganglia of locust with [^{3}H]-tyrosine for four

Table 33. *In vitro* synthesis of taurine from L-methionine and L-cysteine by the thoracic ganglia of *Schistocerca americana gregaria*.

	Concentration of taurine
Control	0.55 ± 0.19
10mM Methionine	0.52 ± 0.05*
10mM Cysteine	0.79 ± 0.22**

The incubations were carried out for 4 hours at 37°C in iso-osmolal saline containing the appropriate substrate. No substrate was present in controls. The results (μmol/g wet weight) are expressed as mean ± S.D. of 4 separate estimates.

*P = 0.770 and **P = 0.153.

hours. The radioactivity was monitored in the products resolved either by electrophoresis or by HPLC and by both techniques combined together. The results (Table 34) show that over 70% of the radioactivity could be found in the N-acetyl derivatives of monoamines i.e. NADA, NAOA and NATA. This is consistent with the findings of Dewhurst et al. (1972), Evans and Fox (1975), Vaughan and Neuhoff (1976) and Mir (1981) who showed that monoamines (e.g. TA) are rapidly converted to their N-acetyl derivatives by the nervous tissue of locust in vitro. At present no specific inhibitors for the N-acetyltransferase are known (Mir, personal communication), therefore, it is difficult to get any appreciable accumulation of monoamines in the nervous tissue of insects. This was a further disincentive to pursue work on the amines. Moreover, as a detailed study on the metabolism of tyrosine by the thoracic ganglia of locust was carried out by A.K. Mir in a parallel project no further experimentation was done in this field.

3.3.7 Presence and metabolism of putrescine in the nervous tissue and haemolymph of locust

3.3.7.1 Identification and estimation of putrescine

During the analysis of amino acid both in nervous tissue and haemolymph of locust an unidentified substance appeared in significant amount on the chromatographs, which had the same chromatographic behaviour as of standard putrescine (Gould and Cottrell, 1974). By using the same method of double labelling and co-chromatography, as used for taurine (Section 3.2.7, Fig. 67, Table 35) the identity of the compound as putrescine was strengthened.

Table 34. Metabolism of [^{3}H]-tyrosine by the isolated thoracic ganglia of locust (Schistocerca americana gregaria).

Metabolite	% of recovered radioactivity			
	After HPLC		After Electrophoresis	
	A	B	A	B
N-acetylamines	73	81	73	71
Monoamines	7	8	1	1
Tyrosine	20	11	26	28

Two pairs of thoracic ganglia were incubated in 10µl insect saline containing 4µCi [^{3}H]-tyrosine for 4 hours at 37°C. The incubations were terminated by adding 10µl of 0.1M $HClO_4$ and tissue was further processed and metabolites resolved as given in Material and Methods section.

N-acetylamines = NADA, NAOA, NATA

Monoamines = DA, OA, TA

A and B represent different experiments.

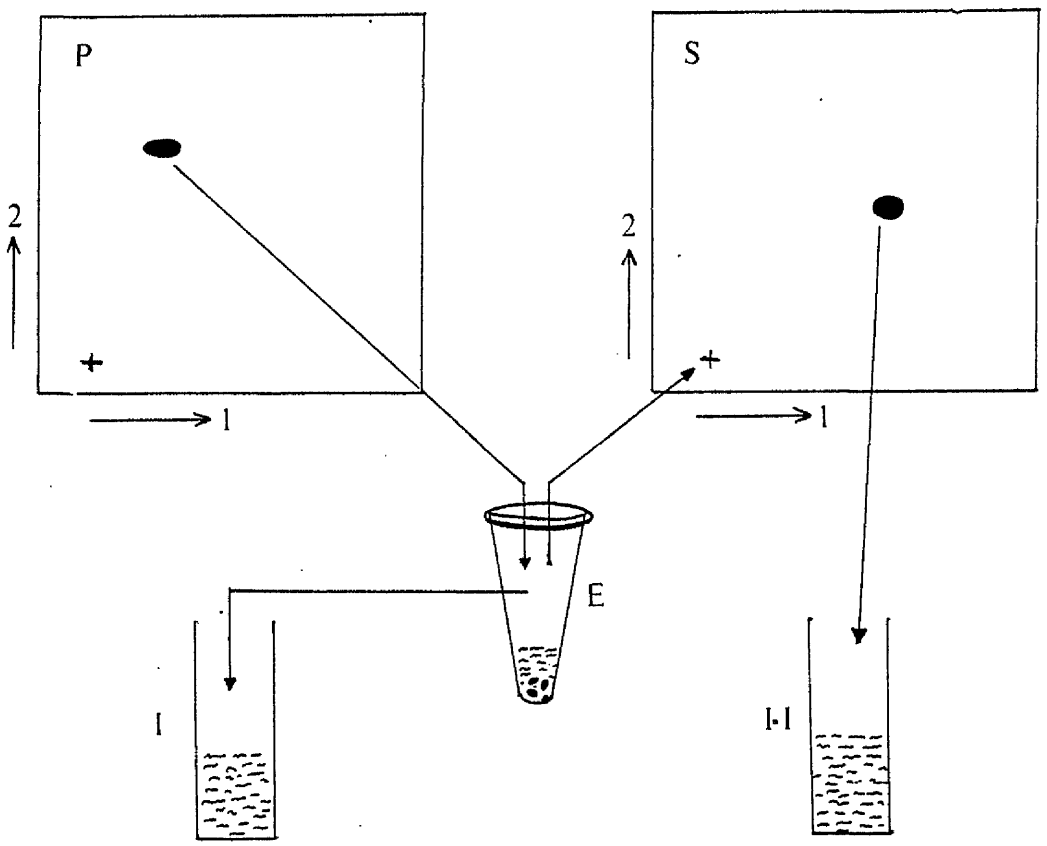


Figure 67 Diagrammatic scheme illustrating the procedure followed for identifying putrescine in the haemolymph of locust.

Standard [^{14}C]-Putrescine was reacted with unlabelled Dans-Cl, and the extract of haemolymph reacted with [^{3}H]-Dans-Cl. Both reaction mixtures were applied to same 5x5cm polyamide sheet (P) and subjected to two dimensional chromatography as mentioned in section 3.1.4 . The spot corresponding to Dans-putrescine was cut out and eluted with Ethyl acetate/toluene/acetic acid (5:9:1 v/v/v) (E). A portion of eluant was directly transferred to scintillation vial (I) and the rest applied to 5x5cm silica gel (S) and further developed in ethyl acetate/cyclohexane (3:2 v/v) in first direction and in chloroform/triethylamine (10:1 v/v) in 2nd dimension.

Cont .....

The U.V. fluorescent spot on silica gel was scraped off into mini vial (II) and radioactivity monitored by liquid scintillation counting. The results are presented in Table 35.

Table 35. Radioactivity associated with Dans-[^{14}C]-putrescine and following multiple chromatography [^{3}H]-Dans-unknown substance.

Sample		Radioactivity (dpm)		
		^{14}C	^{3}H	^{14}C/^{3}H
1	I	622	1006	1.62
	II	1240	1860	1.50
2	I	619	698	1.13
	II	1172	1135	0.97

I and II represent the sample eluted from polyamide sheet and silica gel respectively as described in Fig. 67.

Putrescine was also found to be present in the cockroach nervous system and haemolymph though the concentration seemed to be less compared to the locust. The quantitative analysis showed that putrescine was present in the thoracic ganglia of locust at a concentration of 2.45 µmol/g wet weight tissue while haemolymph had a concentration of 3.88µmol/ml (Table 36). Though these concentrations are far higher (about one order) than those found in vertebrate nervous tissue they are quite comparable to silk moth haemolymph (4.25µmol/ml; Wyatt et al., 1973).

3.3.7.2 Metabolism of putrescine in isolated nervous tissue

Putrescine has been shown to be a precursor of Gaba in the brain tissue of vertebrates (Seiler and Al-Therib, 1974). In order to assess a possible similar function of these high concentrations of putrescine in the nervous tissue of locust, the isolated thoracic ganglia were incubated with 10mM putrescine. The results (Table 37) show no increase in the concentration of Gaba at the end of one hour's incubation. Blocking the possible catabolism of Gaba to succinic semialdehyde by 10mM AOAA did not result in any accumulation of Gaba, even though an inhibition was noticed in the alanine aminotransferase and aspartate aminotransferase. In order to remove any barrier for access of putrescine to its metabolizing enzymes, the tissue was disrupted by homogenizing in insect saline containing 10mM putrescine and the homogenate incubated for 1 hour in the presence of NAD^+ (a cofactor for diamine oxidase, see Fig. 11 - Section 1.7). (The locust nervous tissue does show some diamine oxidase activity, R.H.C. Strang, personal communication). Controls were carried through the same procedure. Again no synthesis of Gaba could be found.

Table 36. Concentration of putrescine in the thoracic ganglia and haemolymph of locust (Schistocerca americana gregaria).

Locust No.	CNS (µmol/g wet Weight)	Haemolymph (µmol/ml)
1	2.76	4.2
2	1.57	4.1
3	3.24	3.92
4	2.24	3.30
Mean ± S.D.	2.45 ± 0.72	3.88 ± 0.40

The compound was estimated by means of [^{14}C]-putrescine standard, using the double isotope dansylation method.

Table 37. Effect of putrescine and AOAA on the concentration of free amino acid in the thoracic ganglia of Schistocerca americana gregaria.

Amino Acid	WHOLE THORACIC GANGLIA				HOMOGENATE	
	Control	+ 10mM Putrescine	+ 10mM AOAA	+ 10mM AOAA + 10mM Putrescine	+ 10mM NAD^+	+ 10mM NAD^+ + 10mM AOAA
Alanine	28.20±3.71	29.29±2.77	19.16±1.04	16.16±1.34	12.60±0.79	14.42±0.20
Aspartate	4.43±1.39	4.18±0.84	9.35±2.82	8.61±2.60	7.36±0.72	6.57±1.67
Gaba	4.08±0.07	4.75±0.50	3.55±0.61	3.59±0.35	3.28±0.56	3.28±0.44
Glutamate	9.88±1.32	12.97±0.12	8.48±1.86	9.71±1.50	10.25±1.50	10.07±1.76
Glutamine	1.83±0.29	3.21±0.96	7.80±0.51	5.66±0.83	5.08±1.61	2.97±1.31

The incubations were carried out in insect saline for 60 minutes at 37°C. The results (Mean ± S.D.) are expressed as µmol/g wet weight tissue.

It could be concluded that in the present in vitro study there was no evidence for putrescine serving as precursor of Gaba in the nervous tissue of locust.

3.3.8 Metabolism of [^{14}C]-putrescine in live locusts

Experiments with the live insects using radiolabelled putrescine failed to show any conversion of putrescine into Gaba either in the nervous tissue or haemolymph (Fig. 68). The presence of radioactivity in spot corresponding to standard putrescine in thoracic ganglia showed that there was no problem in the penetration of putrescine into nervous tissue. In the haemolymph there was evidence of mono-acetyl-putrescine as early as 3 hours after injection (not shown here) and persisting till 26 hours (Fig. 68).

From these results and the ones obtained with unlabelled putrescine in vitro it is impossible to ascribe to putrescine a role as a precursor of Gaba in the nervous tissue of insects.

Fig. 68 - Autoradiograph of the metabolites of the [^{14}C]-putrescine resolved electrophoretically on 20 x 5 cm silica gel sheets. The details of procedure are given in Section 2.4.9.1.b. A = deproteinized extract of nervous tissue, and B = the deproteinized haemolymph of locust. The tissue and haemolymph were obtained 6 and 26 hours after injection of [^{14}C]-putrescine into the insects. Bottom trace shows the electrophoretic separation of the standard polyamines (after Seiler and Al-Therib, 1974), the positions are indicated by arrows to assist in identification of metabolites.

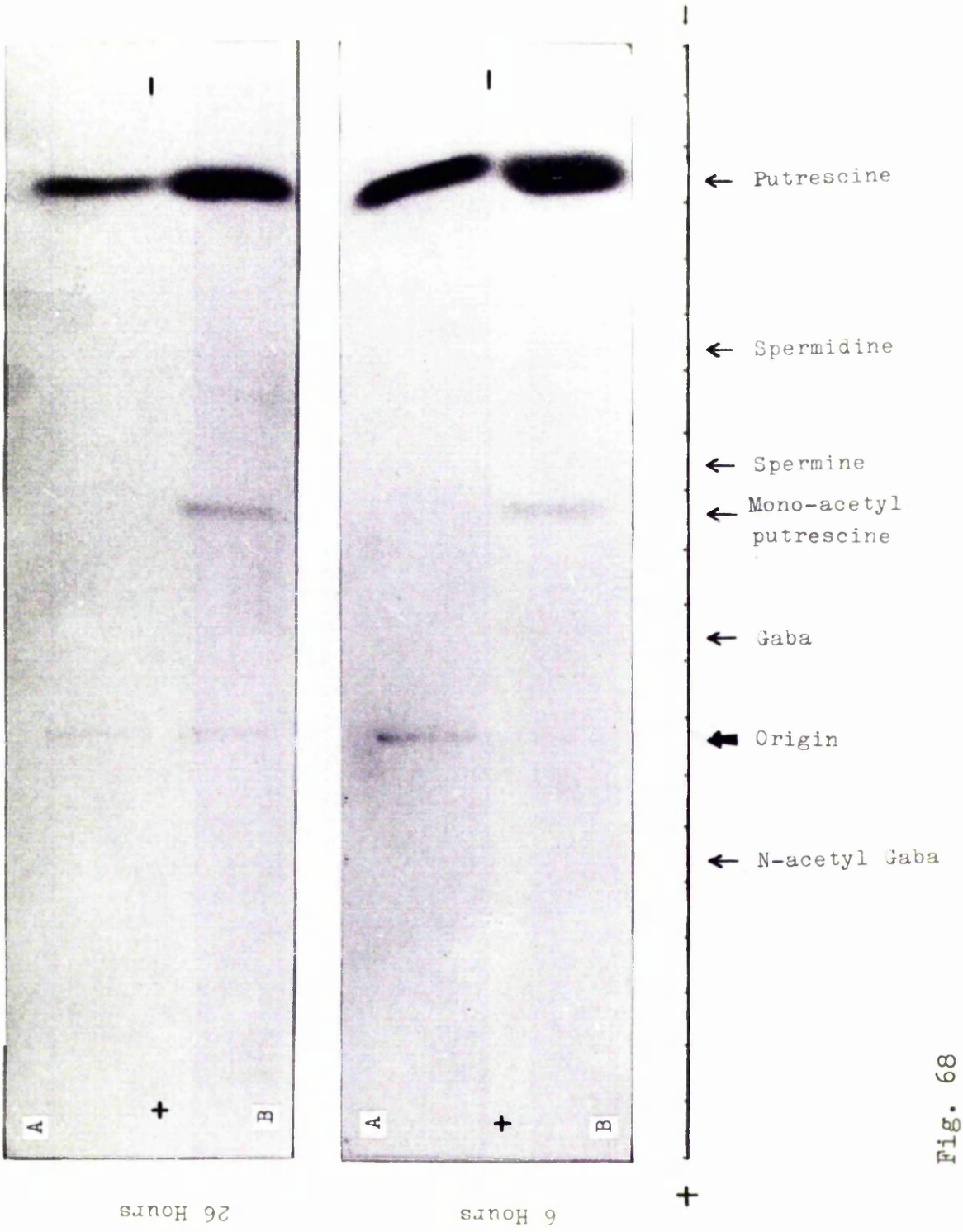

Putrescine
Spermidine
Spermine
Mono-acetyl putrescine
Gaba
Origin
N-acetyl Gaba
A
B
+
6 Hours
26 Hours

Fig. 68

SECTION FOUR

DISCUSSION

PART I

4.1 Improvement in the separation of dansyl derivatives

Dansylation, introduced by Weber in 1952, because of its sensitivity and the elegant ease with which most of the derivatives can be separated by chromatography on polyamide layers, has often been used in quantitative and qualitative studies of the free amines and the amino acids of the nervous tissue (Casola et al 1969, Neuhoff et al 1969; Neuhoff, 1970; and Neuhoff & Weise, 1970). Although much of the reported work is concerned with mammalian tissue, the method has been extensively developed for work with small quantities of invertebrate tissues also (Osborne, 1973; Dolezalova et al 1973). The modifications reported here were intended specifically to adapt the method for use with insect tissues, especially nervous tissue. By first separating the complex mixture of material which will react with Dans-Cl into a number of groups by electrophoresis (found more suitable than the chromatographic and phase-separation methods tried), the final chromatographic separation of the dansylated derivatives is made simpler and less ambiguous. The most commonly used solvents separate either one category (the amino acids) or other (the amines), but not both of these simultaneously (Dolezalova et al 1973). Therefore, the preliminary step makes it possible to concentrate upon one category of compounds. This is particularly useful when the compounds occur in widely differing concentrations. In the insect thoracic ganglia, for instance, the amines of neurochemical interest are present in about 1000 fold lower concentration than the amino acids. In the case of an unknown compound the use of a preliminary step has the added advantage of being a useful guide to its identification.

Using 15x15cm polyamide sheets Dolezalova et al (1973) were able to separate more than 70 different spots present in an extract of snail ganglia, of which as many as 42 were chromatographically identifiable. In the thoracic ganglia of locust the present author could detect 39 different dansyl derivative (24 identifiable) after separation on 5x5cm polyamide sheets.

Some previous authors have used Dans-Cl labelled either with [^{14}C] or [^{3}H] to obtain a quantitative estimate, without the use of an internal standard (Osborne & Neuhoff, 1974). As has been pointed out previously, the vagaries of the dansylation reaction makes this method only semi-quantitative (Bert & Snodgrass, 1975). A great variability in the extent of dansylation both between different compounds within a mixture and from one experiment to another was experienced during the present study. Consequently, the double isotope method was adopted. The addition of [^{14}C]-standards before the electrophoretic separation eliminated the problems of losses due to incomplete elution and dansylation.

Dansylation was found to be less effective as a means of studying amines than amino acids, due to the low reactivity, and the formation of multiple products. The sensitivity of the method for amines and N-acetylated derivatives was found to be only about one tenth of that for the amino acids. This, coupled to the low concentration of the monoamines in the tissue, meant that while the amino acids could be easily estimated in 0.5mg of the tissue and 50-60nl of haemolymph (consistent with the findings of Weise & Oken, 1978), larger amounts of tissue were required in the case of amines.

Despite these drawbacks, the method is sensitive, cheap and accurate way of studying simultaneously a wide range of amino compounds in small amounts of insect nervous tissue. The separation and elution of derivatives takes only 6-8 hours. If required, a dozen or more chromatograms may be run simultaneously, and since so little material is used, any doubtful analysis can easily be repeated (Brown & Perham, 1973). It is always evident from the fluorescent spot before excision and counting whether it is necessary.

4.2 Extraction of amino compounds from nervous tissue

The metabolite extraction procedures are of prime importance for any quantitative estimation. At the moment there is no single reagent which can extract for analysis equally well all the components of interest (e.g. all the free amino compounds) in the nervous tissue. So one has to compromise on the optimum yield for most of the substances or maximum yield of the single compound of interest. Generally, the reagent should be able to; a) act as a suitable medium for thorough tissue homogenization and extract maximally the metabolites of interest; b) separate completely the structural components of the tissue; c) stop, or at least minimize, any subsequent metabolic changes; d) cause no chemical modifications; and e) allow easy estimation of compounds of interest.

Keeping these ideas in mind, a variety of reagents were tried for the extraction of amino compounds from locust nervous tissue. The use of 0.1M HCl as extracting agent and protein precipitant did not yield satisfactory results because the tissue homogenized poorly in HCl, becoming a paste at the bottom of the homogenizing tube. 0.3M $HClO_4$ resulted in a better homogenization and it

precipitated the proteins at the same time. Moreover, it had the advantage of inactivating the enzymes and stopping the metabolism the moment the tissue is homogenized. As the subsequent dansylation, however, had to be carried out at alkaline pH (Seiler, 1970), it was necessary to remove the acid. Neutralization of $HClO_4$ with K_2HCO_3 in the normal way resulted in excessive formation of $KClO_4$ which always interfered with the final chromatography of dansyl derivatives on polyamide sheets. Evaporation of $HClO_4$ under vacuum was unsuitable as there was a danger of explosion as the acid became more concentrated. Moreover, the acid has oxidizing properties, and it could not be used in the analysis of easily oxidizable substances like dopa and dopamine.

Finally, the extraction was carried out in two steps by homogenizing the tissue in insect saline and later precipitating proteins with an organic solvent i.e. acetone. Acetone is an equally good protein precipitant as the more widely used picric acid (Ohara & Ariyoshi, 1979), and is also a suitable solvent for carrying out dansylation reaction (Seiler, 1970). Though all the three procedures gave fairly comparable yields of amino acids (Table 12), the third procedure was adopted during the present work because of its practical advantages.

4.3 Detection and estimation of amino compounds

Qualitative and quantitative analysis showed the presence of nearly all the important amino acids and biogenic amines i.e. DA, OA, TA. It is of interest to find tyramine in the tissue as the synthesis of DA in the thoracic ganglia of locust occurs by the hydroxylation of TA (Mir & Vaughan, 1978 and Mir, 1981) rather than decarboxylation

of L-Dopa as is the case with vertebrates (Nagatsu et al 1964). The occurence of DA and OA is in accordance with the findings of other workers (Murdock, 1971; Robertson & Steele, 1974; and Evans, 1978). The insect nervous tissue i.e. thoracic ganglia of locust and ventral nerve cord of cockroach are shown to synthesize OA from labelled tyrosine in vitro (Mir, 1981; and Robertson & Steele, 1974). Octopamine plays the role of neuromodulator or neurotransmitter both in vertebrates and invertebrates. It increases glycogen phosphorylase activity and stimulates glycogenolysis in the cockroach nerve cord (Robertson & Steele, 1972), possibly through activation of an octopamine-sensitive adenyl cyclase which has been demonstrated in this tissue (Nathensen & Greengard, 1973). Dopamine has been found to inhibit spontaneously active neurones when applied ionophoretically to single cells in the insect brain (Steiner & Pierri, 1969). The inhibitory effect of dopamine on a number of tissues in various arthropods is well documented (Leake & Walker, 1980). Dopamine has a strong inhibitory effect on the stretch receptor neurones of the crayfish (McGeer et al 1961), and when the nerve to the salivery gland of the cockroach is stimulated, inhibitory potentials can be recorded from the ascinar cells of the gland (House, 1973). Dopamine has been shown to be present in these nerve terminals (Fry et al 1974) and is possibly released in response to stimulation of these nerves.

The presence of NAOA and NATA supports the suggestion that the biogenic amines are inactivated in insect nervous system by N-acetylation (Sekeris & Karlson, 1966; Anderson, 1971; Mir & Vaughan, 1978, 1981; and Mir, 1981). The results of Table 33 are indicative of the extent and rapidity with which the amines are

converted to their N-acetylated products in locust VNC ganglia. This inactivation process of amines by acetylation is quite different from that found in vertebrate nervous tissue in which the amines are further metabolized by the action of enzymes monoamine oxidase or catechol-o-methyltransferase (Blaschko, 1973). The radiolabel chase experiments showed that using [^{3}H]-tyrosine as precursor more than 70% of the radioactivity was recovered in N-acetylamines. The N-acetylamines are the end product of the metabolism of tyrosine in insect CNS (Vaughan & Neuhoff, 1976; Mir & Vaughan, 1978 and Mir, 1981).

Chromatograms of the extract of the locust thoracic ganglia show the presence of all the amino acids common to mammalian brain. Some amino acids e.g. proline, valine, alanine, glycine, glutamic acid, aspartic acid, phenylalanine, tyrosine, taurine and glutamine are found at a considerably higher concentration than others i.e. cysteine, lysine, tryptophan, methionine, cystine, arginine and histidine. It is difficult to single out any general role to the considerable pool of free amino acids. In the insect CNS the concentration of amino acids is generally 2-3 times of that found in the mammalian brain (Table 1 Section 1.4). Obviously one major function would be the provision of precursor for protein synthesis, although rapid uptake of glucose and trehalose and incorporation of ^{14}C from the sugars into amino acids (Treherne, 1960) suggests a general metabolic role for at least some of these amino acids. In mammalian nervous tissue ^{14}C from glucose is largely incorporated into the free amino acids (Gaitonde et al 1964). Shortly after the subcutaneous injection of glucose into cats and rats 48-75% of the radioactivity in the brain is found associated with the amino acids

(Gaitonde et al 1964; 1965). The observed distribution of the label as % of the total [^{14}C]-glucose metabolized 30 minutes after injection was alanine 2%, Gaba 4%, aspartic acid 9%, glutamine 9% and glutamic acid 37%. Similarly during in vitro studies it was observed in rat brain slices that after 22 minutes of incubation with uniformly labelled [^{14}C]-glucose the conversion of the ^{14}C of sugar to amino acids was alanine 1.5%, Gaba 3%, aspartate 2.4% and glutamate 9% (Beloff-Chain, 1955). Similarly in the insect and other invertebrate nervous tissue the major labelled metabolites from [^{14}C]-glucose are alanine, aspartate, glutamate and glutamine (Bradford et al 1969). The % radioactivity detected in these metabolites in locust CNS was alanine 33%, glutamate 6%, aspartate 1% and glutamine 2%. These observations demonstrate how dynamic is the metabolism of amino acids in both mammalian and insect nervous tissue. This is consistent with the high specific rate of activity of the amino transferases in the tissue which allows rapid equilibration of the ^{14}C derived from the main pathways of energy metabolism with the amino acid pool.

Outstanding in the amino acid pool of the locust CNS is proline, whose high concentration both in the cerebral and thoracic ganglia is comparable with that in other insect nervous tissue (Ray, 1964; Werman, 1972; Osborne & Neuhoff, 1974). The significance of this high concentration of proline is not clear but in the flight muscle of some insects e.g. tsetse fly and colorado beetle, this amino acid plays a vital role in energy metabolism (Bursell, 1963; 1981 and Weeda et al 1980). There is evidence for a "proline cycle" (Bursell, 1977 as discussed later) in these insects which emphasizes its importance in this respect. The high proline level (about 25%

of the total amino nitrogen) in the nervous system of locust is suggestive of such a role in this tissue as well (section 4.5a). Certainly there is evidence to suggest that proline is a major energy source for locust flight muscle (Mayer & Candy, 1969; Worm & Beenakers, 1980 and Strang, 1981).

Metabolites entering the CNS must come from the haemolymph which is the circulatory fluid in insects. As there is no vascular system in insects at all, as compared to vertebrates, it bathes the outside of tissues and organs. A considerable difference exists between haemolymph and nervous tissue in the concentration of glycine, phenylalanine, glutamic acid, aspartic acid and alanine. The significance of high titre of the free amino acids in insect haemolymph is poorly understood. The concentration of most of the amino acids usually varies with the nutritional state (Firling, 1977). The possible neurotransmitter amino acids (eg. Gaba, glutamate and aspartate) are virtually absent from the haemolymph. The tissue to haemolymph ratio for glutamate and aspartate in insects is even greater than found for tissue to blood in the vertebrates. At the insect neuromuscular junctions glutamate has an excitatory effect and similarly aspartate has also been tentatively categorized in the class of amino acids causing excitation (Section 1.5.2). Apart from the neurotransmitter role glutamate and aspartate lie so close to the tricarboxylic acid (TCA) cycle in metabolic terms that simple transamination would yield the α-keto acid; α-ketoglutarate and oxaloacetate respectively. These keto acids are intermediates of TCA cycle and would readily participate in the process of energy production necessary for the maintenance of nervous activity.

This fact is exemplified by the recovery of radioactivity in metabolites such as CO_2; lactate, α-ketoglutarate, malate and amino acids (alanine, aspartate, glutamine and Gaba) when the CNS of locust is incubated in vitro in the presence of [^{14}C]-glutamate (Bradford et al 1969).

Glycine, an inhibitory neurotransmitter in the mammalian CNS (Davidoff et al 1967; Graham et al 1976) is found at rather higher concentration in the haemolymph than the nervous tissue of locust. In the thoracic and cerebral ganglia it is present at a concentration of 2.5 and 4μmol/g tissue respectively. This amino acid might have a similar transmitter function in locust CNS but the above mentioned fact of lower values in tissue than haemolymph would cast doubt on such role. Further, glycine in cockroach muscle and haemolymph is higher than in the nervous tissue (Osborne & Neuhoff, 1974). These authors are of the opinion that glycine may not be an insect neurotransmitter, but may instead be involved in intracellular osmotic regulation, as is the case with echinoderms (Osborne, 1972) and other marine invertebrates (Lewis, 1952). At this stage the significance of the amino acids in the oxidative metabolism of insect tissues cannot be fully discussed but is likely to be of importance. In another tissue, locust fat body, in vitro incubation with [^{14}C]-glycine and [^{14}C]-leucine showed that 50% of the ^{14}C from glycine and about 75% of ^{14}C from leucine appeared in CO_2 during a 4 hour incubation period (Clements, 1959). This indicates the ability of insects to metabolize these amino acids for the generation of energy as happens in vertebrate liver. Similarly in rat brain slices the metabolism of [^{14}C]-leucine into CO_2 was 32 times more rapid than incorporation into proteins (Chaplin et al 1976), suggesting that

these amino acids have a major function in energy provision in that nervous tissue. The energy production role of amino acids with reference to locust nervous system gets support from the general fall in the concentration of the various amino acids (especially proline, Section 3.1.14) studied after incubation of thoracic ganglia in the medium devoid of glucose.

Turning from general energy metabolism to that of some specific relevance to nervous tissue, tyrosine, a precursor of the neurotransmitter monoamines, dopamine and octopamine (Mir, 1981) can itself be generated from phenylalanine (Moloo et al 1974). Only sketchy information is available about the significance and metabolism of those amino acids found as a minor constituent of the locust CNS. Tryptophan (precursor of serotonin) alongwith lysine, leucine and phenylalanine has been observed to be oxidized by the mitochondria of gill and mantle tissue of see mussel, Mytilus edulis (B.N. Zaba, personal communication). The proposed points of entry of the amino acids into the intermediary metabolism through TCA cycle as found in vertebrate tissues are shown in Fig. 69. In the absence of direct evidence to the contrary it is assumed that most of the amino acids would follow the same metabolic route in the insect CNS. The first step in their oxidation seems to be the transamination and this tissue possesses very high amino transferase activity.

In short, the amino acids generally present in higher concentration in the insect nervous tissue (Proline, alanine, aspartate, glutamate and glycine) correlate closely with those that can be synthesized by most animals (White et al 1973).

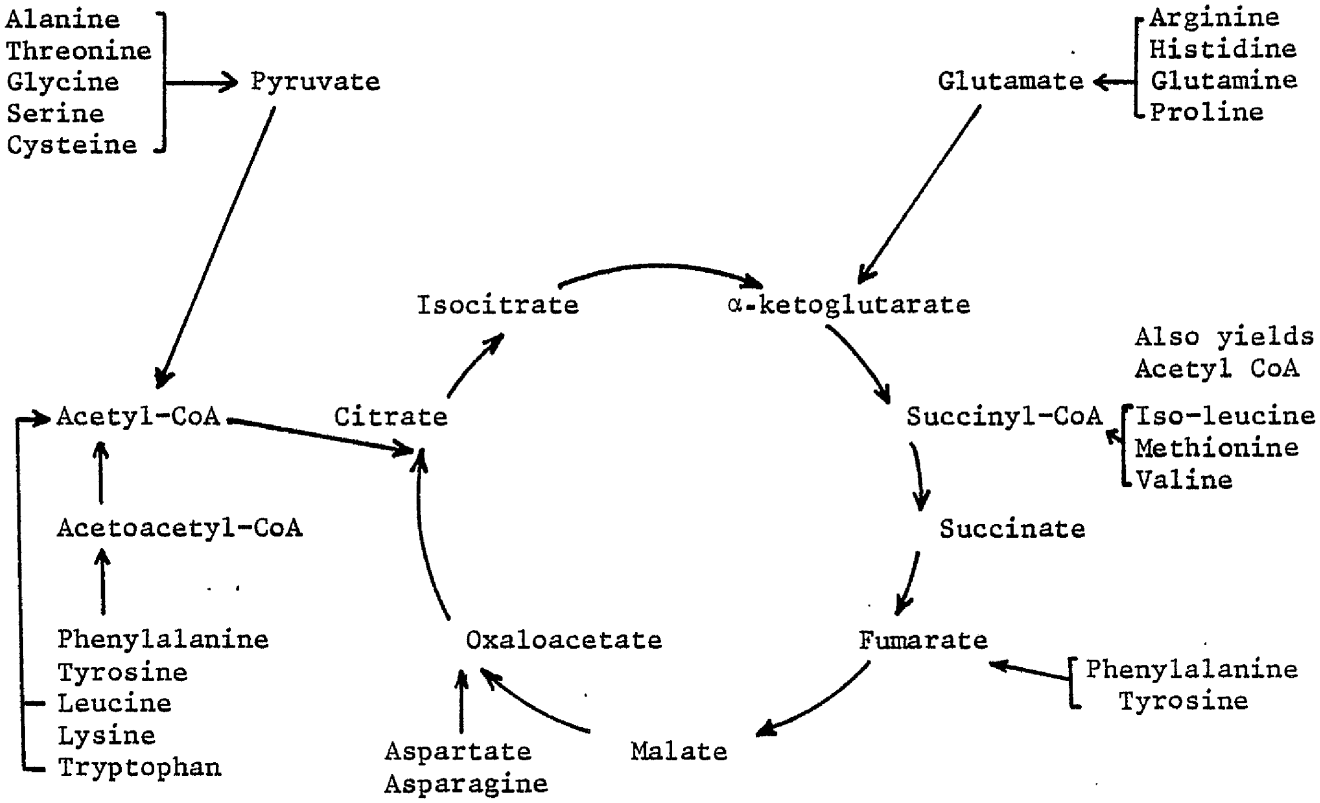


Figure 69 Pathways by which the carbon skeletons of amino acids enter into the tricarboxylic acid cycle in vertebrate tissue.

Adopted from Lehninger, 1978.

This suggests that these amino acids were selected in invertebrate neurones for intracellular functions requiring high concentrations because these concentrations could be maintained by intracellular synthesis. However, this does not rule out the possibility that the intracellular pools of these amino acids may in part be maintained by uptake from body fluids. This is also evident from the uptake in glycine, phenylalanine, tyrosine, and proline against concentration gradient after the incubation of the isolated thoracic ganglia of locust in saline containing amino acids. Therefore, it can be stated that the amino acid pool of haemolymph would be as important as that of the nervous tissue itself. Some of the points are further discussed in the next sections.

4.4 Optimization of the conditions for *in vitro* incubation studies with thoracic ganglia of locust

4.4.1 Effect of incubations on the concentration of amino acids in the nervous tissue

The prime objective of the present investigations was to follow the release of amino compounds from the nervous tissue under a variety of (chemical or electrical) stimuli. Therefore, it was necessary to have preliminary knowledge of the behaviour of these compounds under different condition *in vitro*. The concentration of most of the amino acids studied decreased when the thoracic ganglia of locust were incubated *in vitro* for 1 hour in a medium devoid of sugars. This fall in the total concentration of amino acids during incubation is consistent with the amino acids being used as fuel (briefly discussed in previous section). Eliminating oxidative metabolism by blocking the electron transport chain with KCN, diminishes the oxidation of amino acids like proline

(consistent with the idea that proline is a substrate under aerobic conditions - see section 4.5a). The KCN - insensitive metabolism of aspartate, glutamate and alanine suggests the presence of pathways operative under anaerobic conditions, which might perhaps allow the tissue to maintain its activity. Adding glucose to the medium and making the saline iso-osmotic with the haemolymph result in lower utilization of the amino acids. This is further evidence of a role in the oxidative metabolism but only in the absence of of a carbohydrate alternative. Alanine increases substantially in vitro presumably due to the synthesis by the aminotransferase system (Section 4.5a), with the rising concentration of pyruvate (Clement & Strang, 1978). A much greater proportion of ^{14}C from glucose is incorporated into alanine than into any other amino acid by locust and other invertebrate nervous tissue (Bradford et al 1969). This offers an interesting contrast with mammalian CNS where lactate is a major end product. The above mentioned authors also observed that alanine was in fact, the major metabolite formed from glucose in Helix and Schistocerca nervous system, and was second only to lactate in Eledone. Lactate and alanine were also produced from glutamate in substantial quantities. The concentration of alanine increases even more when glucose is added to the medium. This enormous accumulation of alanine is suggestive of this amino acid being a major end product of glycolysis (Section 4.5). Another possible contribution to increasing concentration of alanine is the oxidation of proline. This fact was first realized in tsetse fly flight muscle (Bursell, 1963, 1965) and has also been observed in other invertebrates (Weeda et al 1980).

4.4.2 Effect of incubation on the efflux of amino acids from the nervous tissue

The free amino acids of CNS exchange to varying degree with the fluids surrounding the tissue. Proline, alanine, iso-leucine, phenylalanine are the amino acids which most rapidly diffuse out of the tissue into media devoid of amino acids, but aspartate, glutamate and Gaba are mostly retained under these circumstances. The concentration of this later group of amino acids is considerably higher in the tissue than haemolymph. Their retention during the in vitro incubation would favour the argument that these amino acids have neurotransmitter or other role of unique importance in CNS. This is also consistent with the finding of Evans (1975) that in cockroach CNS there is a specific energy requiring uptake mechanism that leads to a high concentration of glutamate within the nerve cord. The difficulty in accepting an amino acid like glutamate as transmitter is its central role in general cell metabolism. In these preliminary experiments the difficulty lies in discriminating between these two functions. Kravitz et al (1970) are of the view that there is no known reason why a compound cannot serve an important metabolic role as well as functioning as a transmitter. All that is required is that cell regulates the level of the compound so that it can adequately serve both purposes. This point is exemplified in the vertebrate CNS by the phenomenon of metabolic compartmentation (Balaz & Cremer, 1973; Davidson, 1976).

The efflux of the amino acids is affected by KCN, which will be expected to reduce the ATP required for their retention or uptake. A greater release is observed of glutamate, aspartate, Gaba and proline when tissue is incubated in the presence of KCN. Further evidence of the involvement of energy comes from the greater retention of the amino acids in the presence of 10mM glucose in the medium. The osmolarity of the incubation medium had little effect on the outward diffusion of nearly all the amino acids studied except aspartate which was even more retained under iso-osmotic conditions. The increased efflux of the amino acids from the tissue after pre-incubation in saline containing added amino acids could be attributed to the unusually high accumulation of various amino acids in the tissue or possibly in some extracellular compartments. These amino acids were then subsequently released very rapidly to a medium free of amino acids so that much of the efflux took place within first 5 minutes of incubation. After that the rate of release was slower possibly being a reflection of the synthesis of these amino acids in the tissue. A study with [^{14}C]-glucose in Glossina morsitum (not specifically in CNS) has shown that the insect can synthesize alanine, aspartate, cysteine, glutamate, glycine, proline and serine from glucose. Arginine, histidine, hydroxyproline, isol-leucine, leucine, lysine, methionine, phenylalanine, taurine, threonine, valine showed no radioactivity and are probably nutritionally essential. Although tyrosine and hydroxyproline are not synthesized from glucose, they are at least partially dispensable nutrients for this insect because they can be synthesized from phenylalanine (Moloo et al 1974). In the locust nervous tissue alanine, aspartate, glutamate and glutamine can be synthesized from glucose

(Bradford et al 1969).

The exception to the steady efflux of most of the amino acids was the almost complete retention of aspartate, and, following an initial surge of Gaba efflux, a decline in its concentration in the perfusion medium. This subsequent disappearance of Gaba from medium may be due to reuptake into the tissue. Cutler & Young (1979) reported the same effect after in vitro incubation of cerebral cortex slices of rat. Gaba and glutamate declined in concentration in the medium between 5-40 minutes of incubation after initial rapid efflux. These authors presumed that these two amino acids were reaccumulated by the tissue after an initial high rate of release. The release of Gaba from the brain to surrounding fluids has been demonstrated both in vivo and in vitro in vertebrates. It was released on local superfusion of the monkey brain (De Feudis et al 1970); to fluids in contact with the neocortex of cats (Mitchell & Srinivasen, 1969) and also to those of fourth ventricle (Obata Takeda, 1969). In this last situation the Gaba release was increased three fold by electrical stimulation of cerebellum. Glutamate is also released in response to excitation of brain tissue (Katz et al 1969) and synaptosomes (Bradford, 1970). Similarly glycine is one of the amino acids liberated in vivo on superfusion of parts of the monkey brain (De Feudis et al 1970).

The efflux of amino acids at higher temperature would seem to indicate something more complex than simple diffusion, but as many are involved in both general metabolism and neurotransmission the effect is unsurprising. In the locust CNS the efflux of amino acids has a positive relation with the temperature i.e. increasing with the rise in temperature from 21°C to 32°C, but further

increase in temperature to 37°C has non-significant effect on the comparative release at 32°C. This increased efflux of amino acids with rising temperature may be due to the permeability changes of the neuronal membrane or possibly because of the higher excitability of tissue at higher temperature compared to low temperature (Kerkut & Taylor, 1956; and Bernard et al 1965). The body temperature in insects is not regulated as it is in warm blooded animals. In nature the insect tissues have to cope with varying temperatures (Heinrich, 1980). During the flight the insects quite often sustain body temperature between 30-40°C (Church, 1960). Moreover, the ambient temperature for Schistocerca gregaria is 25-35°C (Weis-Fogh, 1956). Therefore, the in vitro studies carried out at 37°C may not be that unnatural for the locusts.

Briefly these in vitro studies, though preliminary in nature, nevertheless act as a pointer towards the nature of the functions and metabolism of various amino acids. Answers to some of the questions arising from these investigations will be found in the next few pages of this text. It was concluded that the best situation for the nervous tissue to be studied in vitro would be to dissect out the ganglia over short periods not exceeding 30 minutes, and keeping the tissue at 0°C for as short as possible time prior to incubation. The tissue to be incubated in iso-osmotic saline containing glucose as energy source. The pre-incubation of the tissue in the presence of amino acids, however, results in altering the amino acid profile to some extent compared to that in freshly dissected tissue. This must raise some question about how closely the conditions in vitro reflect those in vivo, nevertheless,

pre-incubation had the practical advantage of eliminating the variations in the concentrations of amino acids found among tissues obtained from different insects. This made the results obtained after various treatments more readily comparable.

PART II

4.5 Metabolism of amino acids in locust CNS

The study of the role of amino acids in general metabolism (particularly the energy metabolism) of the locust nervous system was a secondary objective of this work, arising naturally from the changes in concentration of different amino acids when the tissue was incubated under a variety of conditions *in vitro*. Several features emerged which helped to place the locust nervous system in a general context of insect metabolism. Broadly speaking the amino acid metabolism which will be discussed here can be divided into two categories i.e. aerobic and anaerobic energy metabolism.

4.5.1 Aerobic amino acid metabolism of locust CNS

Perhaps the subject of most immediate biological interest is the role of amino acids in the normal (aerobic) energy metabolism of the nervous system. Outstanding in this respect, at least in potential, is proline.

The previously reported studies on the energy metabolism in the locust nervous system, had indicated that in contrast to mammals, the tissue was rather poorly equipped, in terms of the characteristics of rate-limiting enzymes, to utilize circulating carbohydrates, mainly glucose and trehalose (Strang & Clement, 1980; Strang, 1981). Obvious alternatives as potential major substrates are the amino acids, the high concentrations of which in the haemolymph of

different insects has been remarked on for many years as one of the major differences in physiological biochemistry between insects and mammals (Gilmour, 1961).

The pioneering studies of Bursell (1963, 1965, 1967) with the tsetse fly flight muscle has opened the way to viewing proline as a major oxidizable substrate. Many subsequent studies have confirmed that this amino acid is an important substrate for flight muscle in a variety of insects (Sacktor & Childress, 1967; Hansford & Johnson, 1975; Pearson et al 1979 and Weeda et al 1980a). These studies have also revealed, however, that in this as in many other aspects of their metabolism, great variety exists among different species.

In general the concentration of proline in the haemolymph of resting insects is high. In the case of the locust it is 9.5μmol/ml and in cockroach it has been found at 10.5 μmol/ml (Osborne & Neuhoff, 1974). In the thoracic ganglia of locust it is present at similar concentrations i.e. 7.5-12μmol/g tissue. The concentration in the cerebral ganglia is 9.5μmol/g tissue. Proline is likely to be present in the diet of phytophagous insects such as the locust, although it is a rather low proportion of the ingested amino-acids (representing about 4-10% of the amino acid content of various proteins of plant origin, Colin & Edsall, 1943; and Block, 1947). The proline of the haemolymph of locusts constitutes a higher proportion, variously estimated at 10-35% of the free amino acid pool. In any case proline is not considered to be an essential amino acid in insects, indicating an adequate biosynthetic capacity in the intact insects. The major site of this biosynthesis is likely to be the fat body, as has already been confirmed in the case of colorado beetle (Weeda et al 1980b) and tsetse fly (Bursell, 1977).

The high rate of O_2 uptake reported here for intact thoracic ganglia in the presence of 10mM proline suggests that the imino acid is an excellent substrate for oxidation by the nervous system of the locust. The rate of uptake, 332 µmol O_2/g/hr (5.5 µmol/g/min) was significantly (38%) higher than the maximum rate obtained with glucose as substrate.

Using intact pieces of tissue, it is impossible to say whether the supplied exogeneous substrate is the only one used by the tissue, which will contain unknown endogenous reserves, but the excellence of proline as energy substrate is confirmed by the use of a mitochondrial preparation. Related to protein content of the preparations the rate of O_2 uptake found for the mitochondrial preparations from nervous tissue compares remarkably well with similar preparations from other insect tissues, including flight muscles known to use proline as main substrate e.g. tsetse fly (Bursell, 1965, 1967, 1981) and colorado beetle (Weeda et al 1980a).

As mentioned previously, among insects diversity of metabolism rather than uniformity is the general rule. In the case of proline metabolism several schematic variations have been discerned. The main distinction is between those insects tissues which utilize proline as the main substrate, such as the tsetse fly flight muscle, or where it serves only an anaplerotic role in support of carbohydrate metabolism such as is found in the case of blowfly flight muscle. Within those insects for which proline is the major substrate, a range of variations exist in the route by which the proline is oxidised. This latter point will be explored in greater detail later.

In the case of the locust nervous system, the information available at the moment is insufficient to determine the relative importance of, say, glucose and amino acids in the living insects, but it might be expected that if proline functioned simply to allow the maximum utilization of carbohydrates, then there would be an additive effect of glucose and proline on O_2 uptake by intact ganglia. This was not found to be the case, nor did Bradford et al (1969) find any additive effect when they combined 10mM glucose with 10mM glutamate using locust ganglia. (In fact, as will be discussed later, glutamate may not have been a very good choice of amino acid as energy substrate). The efficacy of proline by itself as substrate was confirmed by the studies with the mitochondrial preparations. In addition, two rather preliminary experiments, not reported here, indicated that the addition of pyruvate to a mitochondrial preparation respiring on proline did not have a stimulatory effect. It seems reasonably clear that while the locust nervous tissue can utilize glucose and proline separately, their relative utilization under different conditions, and the manner of the control mechanisms, require to be further investigated.

Some general indications as to the relative importance of carbohydrate and proline may be obtained from the maximum specific activities of the enzyme Proline dehydrogenase (PDH) and α-glycerophosphate dehydrogenase (GPDH). It is this latter enzyme present in both cytoplasm (where it requires NAD^+ as cofactor), and mitochondria (where it carries a flavin moiety), which is considered to be of great importance, in many insect tissues especially flight muscle, in reoxidizing cytoplasmic NADH and thus ensuring a high rate of glycolysis.

This shuttle mechanism was first proposed for the blow-fly flight muscle (Sacktor & Dick, 1962). This tissue has a very high rate of glycolysis, using glucose as the only substrate for energy metabolism. In the locust flight muscle, a tissue highly dependent, at least at the onset of flight, on glucose metabolism, the activity of cytoplasmic GPDH (100-200µmol/g/min at 37°C and that of mitochondrial enzyme 25-80µmol/g protein/min) are much higher than that of PDH, the maximum activity of which is less than 4% of the activity of the mitochondrial enzyme. In the locust nervous system the GPDH in the cytoplasm is 10µmol/g/min, and that of mitochondria 1.1µmol/g/min. Thus the PDH is closer to the activity of the cytoplasmic GPDH. Perhaps the best that can be said at the moment about the energy metabolism of the locust nervous system, is that the pattern of enzyme activities does not indicate any extreme specialization for one substrate.
As the maximum specific activity of the mitochondrial GPDH is slightly higher than that of PDH, in the locust CNS, it may be wondered that proline seems to support a more vigorous rate of O_2 consumption by mitochondrial preparations than does α-glycerophosphate (α-GP). The explanation does not lie in the K_m of the enzymes, which favours GPDH (1.3mM compared to 4mM for proline dehydrogenase). Both enzymes are likely to be saturated at 10mM substrate. The explanation may lie in the fact that the oxidation of α-GP will release only a pair of electrons, which will reduce only ½mol O_2, while proline oxidation will require up to 5.5mol O_2 for the oxidation of a mol of proline. Also that the two enzymes may be under some control i.e. GPDH inhibited when PDH is active and vice versa.

The other point of variation concerns the pathway by which the proline is oxidized. A number of pathways for energy metabolism during the flight of insects exist (Bursell, 1981). These are shown in Fig. 70 for comparison and reference. The energy metabolism in insects can be divided into three main classes. a) In blowfly the total oxidation of glucose is the main pathway and proline metabolism has a minor role; b) in some beetles the supplementation of energy during flight with the complete oxidation of proline is a more developed phenomenon. This would involve the generation of pyruvate from malate by the NAD-linked "malic enzyme" [malate dehydrogenase (decarboxylating)]. By this route the oxidation of one mol of proline yields 29 ATP, and finally c) in tsetse fly and colorado beetle proline forms the main fuel for flight and the major end product is alanine. The overall reaction for partial oxidation of proline in these insects is:

$$\text{Proline} + 3H_2O + Pi + GDP + 2.5\ O_2 \longrightarrow$$

$$\text{Alanine} + 2CO_2 + GTP + 5H_2O$$

Energy yield =

$$5 \times 2H^+ + 2\varepsilon \begin{cases} 2 \text{ via Fp} \longrightarrow & 2 \times 2\text{ATP} = 4\text{ATP} \\ 3 \text{ via NAD} \longrightarrow & 3 \times 3\text{ATP} = 9\text{ATP} \end{cases}$$

$$+ 1 \text{ substrate level (GTP)} = 1\text{ATP}$$

$$\text{TOTAL} \quad \overline{14\text{ATP}}$$

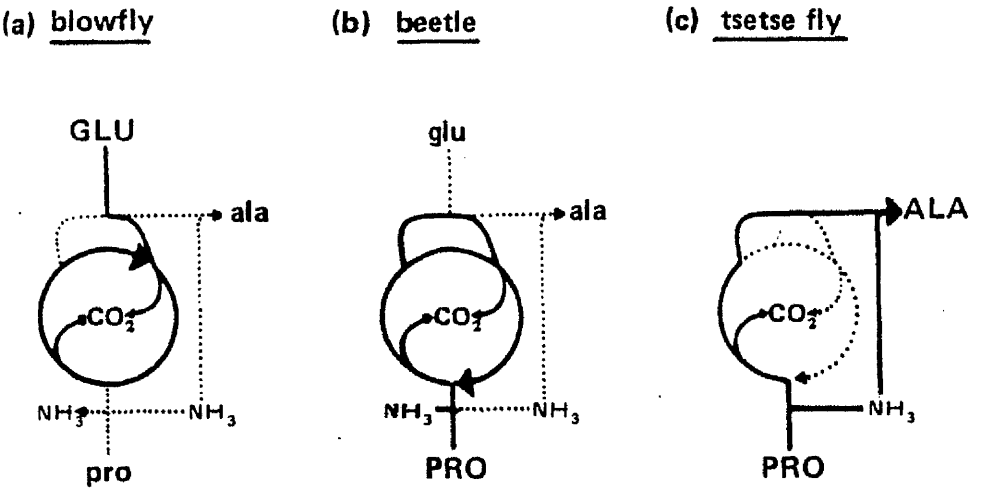


Fig70 Schematized comparison of flight metabolism in: the blowfly (*Phormia*), the Japanese beetle (*Popillia*), and the tsetse fly (*Glossina*). Main pathways are shown in heavy, and subsidiary pathways in dotted, lines. glu: glucose; ala:alanine; pro: proline.

Basically these three pathways differ in the varying degree of regulation of the common enzymes of the glycolysis and the key enzyme of proline metabolism i.e. proline dehydrogenase; "malic enzyme" and alanine 2-oxoglutarate transaminase (Bursell, 1981).

A study of the kinetics of various enzymes involved may give some clue to the relative importance of pathways in a particular tissue. The first and obvious requirement for the oxidation of proline in any tissue is the presence of the mitochondrial flavoprotein enzyme proline dehydrogenase. This is present in the locust thoracic ganglia with maximum specific activity of 5μmol/g/min at 37°C. Depending on the route of oxidation this activity would allow a theoretical O_2 consumption of 12.5 or 25 μmol O_2/g/min which is much higher than the maximum rate of O_2 uptake actually found with intact ganglia at 10mM proline, (5.5 μmol/g/min at 37°C). The metabolism of proline in whole tissue seems to be under control and also possibly due to the partial aerobic conditions in vitro ganglia is not utilizing proline to its full capacity.

It is with the entry of glutamate, formed by the oxidation of proline to the TCA cycle that the main possibility of variation lies. The alternatives are catalyzed by the two enzymes glutamate dehydrogenase (GDH) and alanine aminotransferase (AlAT) and it is their characteristics that will determine the pathway followed. In common with many other insect tissues the activity of aminotransferase is very high (40 μmol/g/min). In contrast the activity of the GDH is low (4μmol/g/min). For convenience, this activity was measured in the reductive direction, and it has generally been found much lower in the oxidative direction (about 1% of reductive direction, Hansford & Johnson, 1975). On the basis of relative activities of the

enzymes it is the pathway involving transamination that is more likely to be operative. This suggestion receives further support from the fact that glutamate oxidation by the mitochondrial preparation is completely inhibited by the use of the aminotransferase inhibitor, AOAA. This would argue that transamination is the only adequate means of glutamate entering the TCA cycle. The effect of the inhibitor on the oxidation of proline is to inhibit it by only 30%. It may be argued that on the basis of the possible pathways involved that inhibition of the TCA cycle should prevent a much larger part of the total oxygen uptake (60-80% depending on the pathway followed). It is likely, however, that the mitochondria in the preparation are only partially effective, and that most of O_2 uptake derives from the first part of the pathway, outside the TCA cycle, and principally the action of proline dehydrogenase itself. The common finding with mitochondrial preparations is that they can only partially use any given substrate, due to low concentration of products in relation to the km values of the enzymes of the TCA cycle and dehydrogenases associated with it. This is especially true as the O_2 uptake studies are made immediately after the addition of the substrate, before concentrations of any products could have built up.

A corollary of the importance of transamination for the entry of glutamate into the TCA cycle is the fact that a molecule of alanine must be formed for each of glutamate (derived from proline) consumed. The work of Bursell (1981) and others (Zwann, 1977) has indicated that the source of pyruvate required in transamination can be the TCA cycle. After some doubt as to the enzyme linking the TCA cycle to pyruvate, it is now considered that the NAD-linked "malic" enzyme is the most important. The activity found for this enzyme in the

locust nervous system was 4 µmol/g/min at 37°C and is thus comparable to dung beetle flight muscle at 2.5 µmol/g/min at 30°C (Pearson et al 1979). The ratio of the activities of the two enzymes which must compete for malate, malic enzyme (4 µmol/g/min) and malate dehydrogenase (9 µmol/g/min), is often an indication of proline utilization. The ratio (0.45) found for the locust CNS is quite similar to that (0.31) found for the flight muscle of the Dung beetle (a known proline utilizer) and quite different from the flight muscle of locust (0.027) or cockroach (0.021) (Pearson et al 1979).

On the basis of the results quoted in this work, there is no doubt that proline is an effective substrate for energy metabolism in the locust nervous system, but it is not possible to say yet that it is the most important one. One advantage of this amino acid in this context is that it would provide a reasonably constant reserve of metabolites in the course of long flights in a way that carbohydrates could not. Although flight muscle possesses PDH, its maximum specific activity is slightly less than that of the nervous tissue and it has a Km of 29 mM (compared to that of CNS of 4mM; R.H.C. Strang personal communication), which would mean that it has only very low activity. Although the concentration of proline in the blood of a flying locust does initially fall, it then rises again to resting levels after about 2-3 hours (Mayer & Candy, 1969). This rise suggests that resynthesis of the proline is taking place. At any rate, it is present in high concentration (8mM, Mayer & Candy, 1969) and could thus support the CNS, at a time when the concentrations of carbohydrates have fallen far below their resting levels.

As to the route by which proline is oxidized, the crucial test which remains to be done is to determine the stochiometry of alanine production. If transamination is the major route, then the alanine produced should equal the proline consumed. In view of the imperfections of the mitochondrial preparations, this may be an unrealistic hope, although claimed by Weeda et al (1980a) relating alanine production to 0_2 consumption. Certainly alanine does accumulate in ganglia incubated in the presence of 0_2. However, alanine accumulation far exceeds the proline decline, and there must be some doubt that tissue, deprived of its tracheal system, is truely aerobic. As will be discussed later, alanine accumulation is an inevitable accompaniment of anaerobic conditions in many invertebrate tissues.

The major synthetic pathway for alanine in locust *CNS* is through the transamination of pyruvate with glutamic acid (shown in Fig. 71). In no case reported here was the concentration of glutamic acid found to change during the <u>in vitro</u> incubations. The same is true for other invertebrate tissues (Zwann, 1977). Glutamate is generated by the transamination of α-ketoglutarate and aspartate and also by deamination of glutamine and oxidation of proline. There are at least 16 reactions whereby glutamate can be generated through transamination from a wide range of amino compounds (Metabolic Pathways, 1978, Boehringer Inc. London). Glutamate homeostasis, due presumably to the equilibria maintained by active transaminase enzymes in locust nervous tissue, is remarkably effective. Experiments with the isolated thoracic ganglia over half an hour have indicated that there is no significant change in total free amino nitrogen. The small decline in amino groups from 84 to 77 μmol/g tissue can be accounted

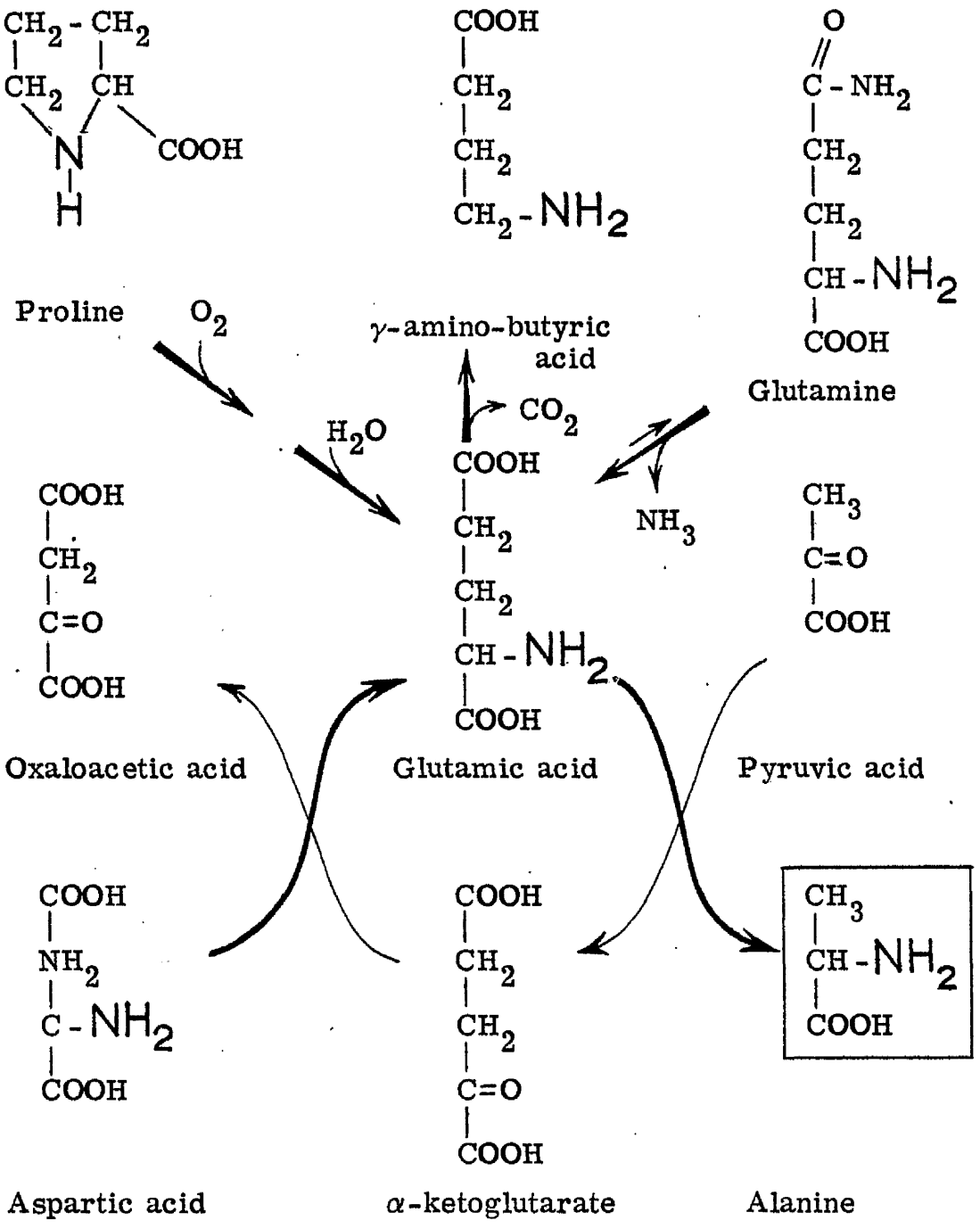


Fig. 71 Biosynthesis of alanine in the nervous system of S. gregaria
Thick arrows indicate the transfer of amino nitrogen.

for by the small increase in free ammonia, which rises from 3.5 to 6 μmol/g tissue. On the other hand there is large transfer of $-NH_2$ group from many amino acids to pyruvate with ultimate accumulation of alanine. The alanine thus formed has been found to diffuse easily out of the tissue. It is possible to speculate that the reason for this massive synthesis of alanine and its outward diffusion could be its role as a 'sink' of amino nitrogen thus preventing the accumulation of potentially toxic free ammonium ion. In the mammals the free NH_4^+ is removed from the tissue by the formation of glutamine (Krebs, 1953; Blumsen, 1957; and Meister et al 1962). The removal of ammonia by this route is an energy-consuming process; glutamine synthetase requires ATP, and glutamate dehydrogenase utilizes NADH, thus limiting the amount of NADH available for ATP formation in oxidative phosphorylation. Alanine formation by transamination in contrast does not depend on expense of energy and can proceed both under aerobic and anaerobic circumstances. On the other hand, work of Treherne (1960) who failed to find incorporation of label from glucose into alanine in the ganglia of living cockroaches would be an evidence against such a theory. In fact, the alanine accumulation may be an artefact of in vitro study. It is possible that during such incubations partial anaerobic conditions may prevail and anaerobiosis and alanine accumulation have long been associated in living invertebrates (Stokes & Awapara, 1968, Meyer 1977, 1978 and Zwann 1977).

The most likely source of the carbon skeleton of alanine in vitro is glucose entering metabolism via the pathway of glycolysis. In the Schistocerca CNS the proportion of ^{14}C derived from glucose

appearing in the alanine is as high as 33% of the total radioactivity; next only to CO_2 which accounted for 36% (Bradford et al 1969). The accumulation of alanine is stimulated both by anaerobic conditions and the presence of glucose in the medium. Over 30 minutes of incubation the concentration of alanine increases from 11 µmol to 22 µmol/g tissue under anaerobic conditions and increases even more to 38 µmol/g tissue if glucose is present as well. Rates of glycolysis under anaerobic conditions would be expected to increase leading to increased concentration of pyruvate. Such an increase has been reported for this tissue even under the best conditions of aerobiosis in vitro (Clement & Strang, 1978). All this suggests that the driving force for alanine formation under the conditions reported in this thesis is the accumulation of pyruvate. If this is so, then it would serve to highlight the importance of the aminotransferases AlAT and AspAT, which had the highest specific activities of any of the enzyme investigated, respectively having maximal activities of 41 and 65 µmol/min/g tissue. This would imply that reactions catalyzed by them are likely to be always at equilibrium. This high activity of AlAT is often characteristic of a tissue for which proline oxidation is important as is likely to be the case here. Although only two AT have been estimated in the locust nervous system, the net change in the distribution of amino groups in amino acids in the course of incubation indicates that a wide variety of AT are likely to be at work, and the rise in the concentration of alanine equals the sum of the fall in other amino compounds. The presence of many AT in locust CNS would be consistent with the findings of Kilby & Neville (1956) with a variety of other locust tissues.

Pertaining to this discussion it might be remarked that there is evidence that at least some insects possess a "Proline/alanine cycle", in which some tissues, usually flight muscle, partially oxidize proline with the formation of alanine, which is then used by the fat body for resynthesis of proline (Bursell, 1977, 1981 and Weeda et al 1980b). Maybe this cycle is present to some extent in the locust. There is an interesting analogy between this insect physiological cycle, and the "glucose/alanine cycle" which is found in the mammals under conditions of starvation, in which the skeletal muscle oxidizes amino acids with the formation of alanine, which then acts as a precursor of glucose in liver. Before, however, generalizing too much about this possibility in the locust, it must be noted that Weeda et al (1980b) found that the fat body of the migratory locust had only very low activities of one of the important enzymes of proline resynthesis, namely NADP-linked malic enzyme.

There are other possible amino acid substrates for aerobic metabolism by the CNS. Glutamate is formed in the normal course of proline oxidation and so must be a prime candidate. Bradford et al (1969) found that it supported higher 0_2 uptake than the endogeneous fuels with locust ganglia. However it is present in low concentrations in the haemolymph (0.5µmol/ml) and in the various studies in which it has been tried as substrate for mitochondrial preparations it has consistently proved to be ineffective (i.e. it supports an 0_2 consumption of 0.09 µmol/g/min as compared to 0.18 µmol/g/min with proline). One reason for its inadequacy may be that the mitochondria are not permeable to this amino acid, as was shown for the mitochondria of blowfly (Sacktor, 1970). In the absence of ADP, glutamate had no effect on the respiration of mitochondrial preparation.

It had an absolute requirement for ADP, and so do the other substrates which show high rate of respiration in the presence of ADP. This suggests that the regulation is at the level of respiratory chain similar to tsetse fly flight muscle (Bursell, 1981). In the blowfly requirement for high concentration of ADP is attributed to effect at the level of the dehydrogenase (Hansford & Sacktor, 1970).

Another possible substrate is glutamine. It is present in high concentrations in the nervous tissue and haemolymph. However, in a study with intact ganglia, 10mM glutamine did not increase respiration above endogeneous level. Even if glutamine is not considered a potential energy substrate it does contribute its amino group to the formation of alanine. The present findings agree with those of Kilby & Neville (1957) who found homogenates of locust tissues could form alanine from glutamine. During the incubations of locust thoracic ganglia in vitro glutamine concentrations fell and a massive rise in the concentration of alanine was observed in the presence of glutamine suggesting that glutamine is deaminated to glutamate an intermediate in alanine synthesis.

In the synthesis of alanine, the intermediate glutamate is generated from a number of amino compounds. The major ones being:

1. aspartate
2. glutamine and
3. proline.

There is a possibility in insect tissues of two mechanisms of amino metabolism, one aerobic and another anaerobic, both leading to an accumulation of alanine. This complicates the interpretation of the gross amino acid metabolism of the ganglia in vitro, when both aerobic and anaerobic conditions could be present at the same time.

4.5.2 Anaerobic amino acid metabolism of locust CNS

Mention of the fact that alanine accumulation invariably accompanies anaerobic conditions in insects conveniently introduces the subject of the metabolism of amino acids in anaerobic conditions.

Before discussing the evidence for the presence and function of such pathways in insects in general and in the locust nervous system in particular, it may be pertinent to discuss whether or not insect tissues are ever normally deprived of air.

The picture which has so far emerged from work on insect metabolism is of extreme aerobiosis. This may in part be due to the heavy emphasis on the metabolism of flight muscle which has long been of interest because of its astonishingly high rate of energy consumption in action. Whatever the substrate consumed, this energy metabolism is almost exclusively aerobic.

Nevertheless, if tissues other than the flight muscle are considered, there is evidence of at least some anaerobic capacity. The leg muscle of locusts contain LDH (Pearson, et al., 1979), which would be consistent with their occasional bursts of violent activity, and would indicate some parallel with mammalian skeletal muscle.

During diapause and overwintering many insects survive under highly anaerobic conditions. In fact, survival over long periods of totally anaerobic conditions, produced artificially, is a feature of insects (Conradi-Larsen and Somme, 1973). Although as with other features of insect metabolism it is unwise to generalize too broadly from the instances of a few insects, it does not fly too much in the face of their biology to expect that even the most apparently aerobic insects should have some anaerobic capacity. Returning to the

formation of alanine by transamination between pyruvate and glutamate, it is the availability of either pyruvate or glutamate which will determine the rate of alanine formation (Snell, 1979). In fact alanine is a much more sensitive indicator of anaerobiosis in the insect CNS than is the accumulation of lactate. Alanine accumulation over 30 minutes period is 4-5 times that of lactate (Clement and Strang, 1978). This is explicable on the basis of the relative activities of the respective synthetic enzymes. The AlAT has a specific activity of 41μmol/g/min which is about 4 times the specific activity (10μmol/g/min) of lactate dehydrogenase (Strang, 1981).

The formation of alanine under anaerobic conditions is not restricted to insects, but is a phenomenon found in many invertebrates (Stokes and Awapara, 1968; Zwann, 1977; Meyer, 197.7 and English et al., 1982). During the synthesis of alanine, glutamate pyruvate transaminase competes with the lactate dehydrogenase (LDH) for pyruvate. While NADH is oxidized to NAD in the reaction mediated by LDH, no such involvement of NADH occurs during the formation of alanine. In the locust nervous tissue no evidence was found for the assimilation of free NH_4^+ by the direct reductive amination of pyruvate to yield alanine. Attempts to find alanine dehydrogenase in this tissue were also negative. To maintain the flux of glycolysis during anoxia, Sacktor (1965) proposed a model in which three consecutive reactions could replace the formation of lactate as the final step of glycolysis and would generate NAD as well.

These reactions are;

pyruvate + glutamate ⟶ alanine + α-ketoglutarate

α-ketoglutarate + aspartate ⟶ glutamate + oxaloacetate

oxaloacetate + NADH ⟶ malate + NAD

---

pyruvate + aspartate + NADH ⟶ alanine + malate + NAD

It is possible that part of the accumulation of alanine in the locust thoracic ganglia is a result of this sort of mechanism. The evidence for it comes from the concommitant decline in the concentration of aspartate with increase in alanine during the incubation of this tissue in vitro. This decline in aspartate is more pronounced under anaerobic than aerobic conditions. As already mentioned the enzyme responsible for the reaction between α-ketoglutarate and aspartate (aspartate aminotransferase) is the most active (65μmol/min/g tissue) aminotransferase of the locust nervous tissue. The decline in aspartate observed even in the presence of glucose suggests that its metabolism is not restricted to general amino acid metabolism (in the absence of carbohydrates) but is associated with anaerobic fermentation. One of the other possible sources of alanine can be the decarboxylation of aspartate to yield β-alanine. Though in the present study it is difficult to distinguish between α-alanine from β-alanine as they co-chromatograph on polyamide sheet, this direct formation of alanine can be ruled out because the inhibition of aminotransferase by AOAA completely blocks the formation of alanine, suggesting that alanine is only formed via the aminotransferase system.

If the reason for the accumulation of alanine is the concentration of pyruvate from increased glycolysis it is unlikely to represent a specific anaerobic mechanism, in that unlike lactate formation it is not directly involved in the reoxidation of NADH. If glycolysis continues for even a short time under anaerobic conditions it must say something about the normal mechanism for the reoxidation of NADH in the cytoplasm and the importance, in the context, of the α-GP shuttle (Fig. 5, Section 1.5.1). It has been pointed out (Sacktor, 1970) that a tissue using this shuttle under anaerobic conditions must produce 1 mole of α-GP for each of pyruvate thus giving no net yield of ATP from glycolysis from glucose (although this is not true if glycogen is the source). Over 60 minutes of anaerobic incubation the accumulation of alanine (12μmol/g) far exceeds that of α-GP (2.5μmol/g). This indicates that other mechanisms for the reoxidation of cytoplasmic NADH must exist in the thoracic ganglia. One obvious mechanism is lactate formation, but its accumulation is low compared to alanine and moreover, will not contribute to alanine formation.

Other proposals for the role of alanine in the anaerobic metabolism of invertebrates include schemes in which alanine formation serves for the regeneration of NAD, replacing lactate fermentation, required to sustain glycolysis (Hochachka and Mustafa, 1972; Collicutt and Hochachka, 1977 and Zwann, 1977). As the mitochondrial membrane is impermeable to NAD or NADH two shuttle mechanisms have been proposed for the transport of cytoplasmic reducing power in the form of NADH into the mitochondria (Rognstad and Clark, 1974). One is the well known glycerophosphate shuttle, the operation of which was once widely assumed to play a similar role in all cells. However, the

significance of this cycle in various mammalian cells has been challenged (Williamson et al., 1971), and as mentioned above it can not account for all the alanine formed. Safer et al. (1971) have provided evidence for the operation of "malate-aspartate cycle" in mammals.

The possibility of such a pathway occurring in the locust nervous tissue was assessed by studying the metabolite concentration and the enzymes involved in the intermediate reactions. According to overall equation, as mentioned earlier, for the reductive transamination -

Pyruvate + aspartate + NADH $\longrightarrow$ alanine + malate + NAD

malate should accumulate besides alanine. However, in Schistocerca thoracic ganglia malate does not show a stochiometric increase (4µmol/g for malate as compared to 12µmol/g tissue for alanine). Moreover, this increase is not confined to strictly anaerobic conditions. This apparent anomaly can be explained by the earlier mentioned fact that even under best conditions in vitro semi-anaerobic conditions must prevail in the ganglia.

The fact that concentration of malate alone does not account for the increase in alanine is not at all surprising since malate penetrates the mitochondria where it can be transformed into succinate via fumarate (Fig. 5, Section 1.5.1), thus giving rise to observed accumulation of succinate. Under anaerobic conditions over 20 minutes the concentration of succinate rises to 4µmol/g tissue. The NADH necessary for the reduction of fumarate to succinate (according to the scheme of Collicutt and Hochachka, 1977) must not necessarily originate from reactions located in the cytoplasmic matrix, but can be supplied entirely from a partially operative Krebs cycle (Schroff and

Schottler, 1977). Therefore, glucose breakdown by glycolysis could proceeed to pyruvate and its redox balance could be maintained by the reductive transamination reactions characterized by the highly active aspartate aminotransferase.

The decline in concentration of aspartate (of 4μmol/g tissue) does not, however, equal the combined increase in the concentration of malate and succinate (6μmol/g tissue), and falls far short of balancing the increased amino nitrogen of alanine. There must be some other source of carbon skeleton of intermediates of the TCA cycle and of NH_3 for alanine synthesis. Part of the carbon (0.7μmol) comes from fumarate (converted to succinate) and the source of the rest (about 1μmol) is not known. On the other hand glutamine does supply a part of NH_3 group after deamination to glutamate, which after transamination with pyruvate yields alanine. As regard the metabolism of carbon the transamination between α-ketoglutarate and aspartate generates OAA which when converted to malate can either give rise to succinate or by the action of malic enzyme would yield pyruvate. But under anaerobic conditions the synthesis of pyruvate from malate seems unlikely because this reaction utilizes NAD, though the presence of "malic enzyme" at a specific activity (4μmol/g/min) in locust nervous tissue favours such a conversion in this tissue. The initial formation (10 minutes after incubation) of malate and succinate both under aerobic and anaerobic conditions may be a reflection of partial anaerobiosis as I have been emphasizing quite often. Later as the O_2 (present at saturating concentrations in the medium) may have diffused to the inner part of the tissue the metabolism may also be mostly aerobic. As shown in Table 28 the increase in succinate from 1.4 μmol/g to nearly 4 μmol/g after 10 to 20 minutes of incubation under

anaerobic conditions suggests that malate is converted only to succinate, whereas over the same period of incubation in the presence of O_2 the concentration of succinate does not show any change. The decline (of 2μmol/g tissue) in the concentration of malate under aerobic conditions at the same time may be due to the decarboxylation of malate to pyruvate by the above mentioned enzyme.

Anyhow, the reduction of OAA derived from aspartate can not entirely replenish all the NAD required for glycolysis. In the past decade information has been accumulating on the role of phosphoenol pyruvate-carboxykinase (PEP-CK) in the glycolysis in invertebrates. In bivalve tissues PEP is carboxylated to OAA which is reduced to malate (Felbeck and Grieshaber, 1980). Malate originating from either glucose or aspartate then passes into the mitochondria where as mentioned earlier either it is converted to succinate or pyruvate (via 'malic' enzyme reaction, Schroff and Schottler, 1977). The lack of PEP-CK activity in the locust nervous tissue rules out the existence of a bypass for the accumulation of malate or succinate with resultant regeneration of NAD. Moreover, if such a mechanism operates it would certainly bypass the transamination system and would not contribute to the formation of alanine.

It can be concluded from these observations that requirement for cytoplasmic NAD to maintain glycolytic activity under the anaerobic conditions in locust CNS is met by the combined operation of all the three mechanisms discussed with the end products, succinate, α-glycero-phosphate and lactate. With the information (about lactate and α-GP only) available to him in 1965, Gilmour proposed that in insect tissues more than one mechanisms were operative simultaneously. Now with more information about the third aspartate-malate-succinate pathway this

view is strengthened. Therefore, it is not surprising if insects can sustain anaerobic conditions to varying extent. It can be one of the examples of nature where the living organism has the option of a number of alternatives for maintaining metabolic activity in unusual environmental conditions. The question whether all the cells of the nervous system possess these three mechanisms or different cell types have different mechanisms of regeneration of NAD under anoxic conditions remains to be answered. On the whole the tissue seems to be well suited to adapt its energy metabolism in various aerobic and anaerobic circumstances, at least the nerve cells have a limited capacity to survive anaerobic conditions which contrasts the highly aerobic muscle tissue. Clement (1979) showed that the spontaneous nervous activity of the isolated thoracic ganglia of locust ceases after 5 minutes under anaerobic conditions.

PART III

4.6 Insecticides and stress syndrome in insects

The remarks by Beament (1958) in his elegant study with the cockroaches, that "it is possible that hyperstimulation of the nervous system, regardless of whether it is produced by chemical stimulation, mechanical, or electrical stimulation of animals or self-promoted activity through imprisonment, could all give rise to auto-catalytic metabolites which cause paralysis and death", prompted a number of scientist all over the world to investigate, what seemed a promising and interesting phenomenon that might lead to a simple common answer to the question of the mode of action of a variety of toxins. But even after nearly 25 years no single conclusive answer has been found. There are as many explanations for autotoxicity in insects as there are investigations. The more detailed the exploration carried out, the

more complications arise. Nevertheless, each study has presented some evidence which in its own way is a worthwhile contribution to knowledge of the biology of insects.

Very few people engaged in pest control seem likely to apply DDT in the future and the way it brings about the death of insects is perhaps of little practical concern to today's insecticide user. However, due to the similarity in symptons produced by poisoning with DDT and those produced by bodily stress, and also it being more used in "mode of action" studies than any other insecticide, it was employed in the present investigations as the yardstick against which other insecticides were compared.

The insecticides were injected directly into the thoracic cavity of insects to overcome the difficulty of differential effects by topical application. For example LD_{50} for topically applied DDT for american cockroach is 10µg/g, but more than 9000µg/g is needed for the grasshopper, Metanoplus femurrubrum and 100µg/g for locust, Schistocerca americana gregaria; whereas 2µg/g of DDT injected into this grasshopper is lethal (Cornwell, 1976). Table 38 gives the LD_{50} of insecticides including the ones used in this study. The amount of insecticide injected into each locust was twice the LD_{50} in order to have the maximal immediate toxic effect. The poisoning of insects with insecticides results in increased respiration which is associated with the symptoms of poor coordination. The injection of DDT or methylchlor into German cockroach at 100µg/insect causes the consumption of oxygen to increase 3-4 times above normal in half an hour (Merill et al., 1946). This is followed by prostration and

Table 38 Toxicities of different insecticides against locusts

Insecticide	Toxicity to locusts LD_{50} in µg/g	
	Schistocerca	Locusta
γ-BHC	9	2-7
dieldrin	5	2
DDT	100	100
malathion	31	24-48
parathion	2	1

The LD_{50} is the dose which when applied to a group of insects kills 50% of them.

Toxicity values are from Chapman, (1976).

ultimately the death of insects (Cook and Holt, 1974). Prostration of DDT-poisoned insects can be reproduced by inducing insects to struggle (Beament, 1958), or more quickly by direct stimulation, using mechanical, or electrical means. Heslop and Ray (1959) compared the effects of bodily stress and DDT poisoning on the oxygen consumption and neuromuscular reactions of american cockroaches. Of the two strains of P. americana used in these tests, the one which was prostrated more readily by 20µg of topically applied DDT was also the one more readily paralyzed by bodily stress. Blood taken from american cockroaches, stressed by having their mouthparts sealed or by being dehydrated by silica aerogel, administered into the abdomens of honeybees produced in them a neuromuscular reaction. In some experiments fragments of leg muscle taken from normal and headless cockroaches placed in contact with silica aerogel, were substituted for blood. These observations suggested that nervous system played a part in producing substances associated with stress (Pence et al., 1975).

4.7 Effect of insecticides on insect central nervous system

The initial response of locusts and cockroaches to poisoning by insecticides clearly indicates some impairment of the nervous system. Body tremors, twitching of the legs, great excitability and eventual paralysis all outwardly suggest an interference with nerve function. The recording of spontaneous nervous activity from VNC excised from poisoned cockroaches shows an inconsistant burst of abnormal hyper-activity. The trains of nerve impulses in the VNC of DDT-poisoned cockroaches always coincide with body tremors and frequently continue

after prostration (Heslop and Ray, 1959). In their study pulses were recorded in 30 of 45 cockroaches prostrated by DDT and in 8 of 13 paralyzed by bodily stress. These studies suggest that because the cockroaches subjected to stress produce reactions similar to those produced by DDT-poisoning, insecticides of this type which first cause convulsions and then kill very slowly, may first produce symptoms of stress, obscuring the specific response to the poison.

The insecticides (e.g. DDT) cause spontaneous discharges in the nerve fibres, of motor nerves (those carrying impulses to muscles), thus producing the characteristic muscle twitches of poisoned insects (Yeager and Munson, 1945). Since the response can be seen in amputated, DDT treated legs of cockroaches, the decapitation or separation of body segments causes no reduction in DDT tremors, it would seem that no specific target (nerve centre) is involved (Roeder and Weiant, 1946). Tobias and Kollross (1946) conclude that those parts of the insect's body necessary for the development of symptoms of DDT poisoning are contained within the lateral half of a body segment, which contains the lateral half of a ganglion, leg nerves and peripheral structures. The hyper-activity observed in the nerve cord excised from the poisoned cockroach is consistent with such a view.

4.8 Accumulation of neuroactive substance(s) in the haemolymph of stressed insects

The symptoms of poisoning in insect have been accompanied by the excessive accumulation of neuroactive substances in their blood (Sternburg et al., 1959; Colhoun, 1960; Tashiro et al., 1972). The unusually high concentration of an amino compound (confirmed as taurine -

its possible function is discussed later) in the haemolymph of locusts and cockroaches after bodily stress of enforced walking or insecticide treatment is consistent with these findings although not with the identity of previously suggested compounds. No accumulation of iso-amylamine or monoamines (e.g. octopamine, dopamine, serotonin) was detected, excluding the possible involvement of these compounds under these stressful conditions unless they were below the sensitivity of our method. Sternburg and Kearns (1952) found that DDT poisoned cockroaches contain in their blood a material which is not DDT but which on injection into normal cockroaches can be lethal. They found that toxic samples of blood from poisoned cockroaches have about equal effects when injected into either DDT-resistent or susceptible strains of houseflies. Haemolymph from DDT poisoned cockroaches injected into the leg of another cockroach quickly causes high frequency discharges in the curaral nerve; this active material accumulates in the haemolymph with the advancement of poisoning symptoms. Partially purified toxin, diluted 50 fold, greatly increases spontaneous activity in a cockroach isolated nerve cord. Higher concentrations block spontaneous activity (Sternburg et al., 1957). Hawkins and Sternburg (1964) later identified this substance as an esterified aromatic amine.

The DDT-induced toxin can also be produced in cockroaches by repeated mild electric shocks. Sternburg and colleagues suggest that because no such biologically active substance has been detected in the blood of untreated insects, the toxin might result from excessive stimulation of peripheral nerves; it may normally be involved in transmission of nerve impulses and the accumulation which occurs during insecticide poisoning may disrupt the entire nervous system (Sternburg

et al., 1957). Using chromatographic techniques, a substance produced in isolated nerve cords treated with organophosphorus compounds was found to be identical with that resulting in the intact insect following DDT-prostration.

On the assumption that insects suffering bodily stress might produce the same, or a similar toxin, capable of giving DDT-like symptoms, a detailed examination of the haemolymph of Periplaneta americana was made by Patel and Cutkomp (1968). The insects examined were either normal, stressed by forced immobilization, or poisoned with chlorinated hydrocarbon insecticides, or with organophosphates. Physical stress and chlorinated hydrocarbons both caused the production of a compound with particular fluorescence characteristics, absent in normal cockroaches and not induced by organophosphorus-poisoning. This indicated that the american cockroach did not respond in a similar biochemical fashion to the two situations. In contrast Holzhacker and Giannotti (1967) are unconvinced that the symptoms of DDT-poisoning-tremors and movements of legs are caused by a neurotoxin carried in the haemolymph. By severing the connectives of the VNC of injected cockroaches they showed that symptoms of poisoning occur away from the site of injection, only when the central nerve cord is intact. They believe that the suboesophageal ganglion is a significant site of action of DDT in insects.

Working on the same hypothesis Tashiro et al. (1972, 1975) showed that iso-amylamine (a decarboxylated product of leucine) appears in the perfusion saline when the nerve cord of cockroaches are incubated in the presence of insecticides. They also observed that this excitatory neuroactive amine accumulates as well in the haemolymph of the larvae

of silkworm, Bombix mori after treatment with either DDT or TEPP. They believe it to be the neuroactive substance appearing in blood of insects under the abnormal conditions of chemical stress.

The present author found only a few amino compounds to show a significant change in their concentration in the haemolymph of locusts treated with insecticide γ-BHC (an organochlorine insecticide most widely used these days). Among these the most pronounced accumation ($2\frac{1}{2}$ times the normal concentration) occurs in taurine (those functional and metabolic roles of which are discussed in section 4.11); the rise in alanine concomittant with the decrease in proline (section 4.5); increase in threonine and arginine. Arginine accumulated probably as a result of hydrolysis of arginine phosphate, the phosphagen of invertebrate muscles (Florkin and Jeuniaux, 1974; Van der Horst et al., 1980). Similarly while investigating the earlier reports of Sternburg et al. (1959), Colhoun (1960) showed that the blood of roaches treated with DDT and TEPP contained a number of substances in abnormal amounts. Among these was a catecholamine (not adrenaline or nor-adrenaline but it was termed 'insect adrenaline'), which was biologically active when tested against the isolated heart and nerve cord preparation of normal roaches. The main source of this 'catecholamine' in the roaches appeared to be the corpus cardiacum gland, although Gersch et al. (1957) reported its occurrence in the nerve cord. The presence of this active substance in the blood of DDT and TEPP treated roaches and the high concentration of a number of amino acids in the haemolymph of locusts after treatment with γ-BHC, coupled to excitation of the spontaneous nervous activity of isolated abdominal nerve cord of cockroach perfused with leucine, isoamylamine, threonine, arginine, glutamic acid, and aspartic acid (present study and Tashiro et al., 1972, 1975), leads to the suggestion that more than

one factor may be responsible for biological activity when blood is tested against various preparations. There seems little merit in discussing the importance of one substance relative to that of another, for in the blood of a single treated insect all abnormal factors contribute to some degree of biochemical or physiological disturbance (Colhoun, 1960).

4.9 Dehydration and symptoms of stress in paralyzed insects

Dehydration is one of the causes advanced for the final death of stressed insects, and it is one which might also be expected to cause increased concentration of compounds in the haemolymph. In the work reported here the volume of haemolymph of the paralyzed locusts compared to the untreated insects did not decrease significantly enough to cause any excessive concentration of amino acids or any other neuroactive factor. Even where the studies have been carried out over longer periods the authors (quoted below) agree that dehydration is not the prime cause of paralysis and death in insects. Burt and Goodchild (1971) carried out experiments to determine whether insects killed by pyrethrin insecticide die because of loss of water, through increased respiration or increased diuresis. The latter has been noted in some insects and may be a symptom of damage to the nervous system by the insecticides (Cornwell, 1976). Cockroaches treated with just-lethal doses of pyrethrin I were weighed every half hour for 7 hours. One hour after treatment, the insects started to lose weight faster than untreated cockroaches. By this time the poisoned insects were already prostrate, suggesting that dehydration begins only when fatality is already assured. To further establish this, these authors carried out transfusions with the hope of alleviating poisoning symptoms. However,

20-50µl of saline or haemolymph from untreated cockroaches injected four times within seven hours failed to influence symptoms and none of the injected insects recovered.

Edney (1968) calculated that when Periplaneta lost 12% of their wet weight, the haemolymph osmolarity would increase from 410-607 m-osmole (if it is assumed that there were no ion-regulating mechanisms in the haemolymph), but Wall (1970) failed to record such an increase and suggested that excess solutes are removed from the haemolymph during dessication (Djajakusumah and Miles, 1966). The observations of Cook and Holt (1974) on the measurements of haemolymph osmolarity in normal cockroaches and paralyzed insects agree with those of Wall (1970). They suggested that the notion "that the process of dehydration alone cause neuromuscular dysfunction in stress-paralyzed insects can be dismissed". Their main findings were that; 1) starved and dehydrated cockroaches did not develop the symptoms of paralysis in 7 days. Further, if insects were allowed food and water after this time they quickly returned to a normal physiological state. However, cockroaches subjected to physical stress seldom reverted to a normal condition; 2) haemolymph ion-regulating mechanisms seem to function in a normal manner in both paralyzed and normal insects. Thus excessive accumulation of ions along the outer surface of muscle fibres and nerves was not likely until the blood volume reaches a critical level or the normal tissue ion storage sites exceeded their limits; 3) as the dehydration progressed even the obvious reduction in the blood volume with its implied restriction on circulation was somewhat alleviated by the gradual appearance of a membraneous air filled sac in the abdominal cavity. Therefore, all these observations would indicate that the unusual accumulation of amino acids or neuroactive

substances in the blood of insects under the influence of chemical or physical stress is a consequence of metabolic change in the steady state concentration or excessive release of these substances rather than simply due to dehydration.

4.10 Changes in the concentration of amino compounds in the haemolymph and CNS of insects after chemical stress or electrical stimulation

Only very small differences in concentration occur in most of the amino acids in the haemolymph of insecticide treated insects. Proline is depleted in locusts after injection of γ-BHC (present study) and in the paralyzed cockroaches it has been reported to fall to one quarter of its normal content (Corrigan and Kearns, 1963). The changes in proline content of the haemolymph would appear, therefore, to be correlated with symptoms of insecticide poisoning, perhaps due to its oxidation, in the highly increased O_2 uptake reported under these conditions. This suggestion gains support from the fact that many insect tissues can utilize proline as an energy substrate (section 4.5.1) although the extent of proline utilization by locust and cockroach is unknown and there is little evidence for its use in flight muscle in these insects. The marked drop in proline concentration following insecticide poisoning appears to be associated with prostration, the insects having previously experienced great muscular, nervous and respiratory activity, probably stimulating the oxidation of proline.

No evidence emerged from the work reported here that the nervous system was the source of the main amino compound (taurine) accumulating in the haemolymph. The incubation of nervous tissue of locust in saline containing insecticide did not result in marked difference in the concentration of most of the amino acids studied. Qualitatively no

unusual compound appeared in the tissue or medium under these conditions in vitro. The increase in alanine with concommitant decrease in aspartic acid and glutamine, in the isolated nerve cord of locust in these in vitro studies, is in fact a reflection of partial anaerobic circumstances experienced by the tissue (see section 4.5.2). Ray (1964) observed that proline in the nervous tissue of cockroaches declines with the application of insecticides both in vivo and in vitro. DDT, dieldrin, DFP and O-IMPF cause a surge of respiration and enhanced muscular and nervous activity. Thus in the case of poisoning with these insecticides it seems likely that the depletion of proline simply reflects the stimulated oxidation of this amino acid. Some of the imino nitrogen probably appears as glutamine as originally suggested by Winteringham (1958). In these studies Ray (1964) reported an increase in alanine but did not discuss its significance. The increase in alanine also found in the present study under similar conditions can now be explained in the light of recent findings for the appearance of imino nitrogen from proline into alanine (section 4.5.1).

Electrical stimulation of the isolated nervous tissue of locust causes an increase (twice the concentration found in freshly dissected untreated tissue) in glutamic acid and aspartic acid. The exact cause for this elevation in concentration of these compounds is not known and needs further investigation. The only possible answer may be the net new synthesis of these amino acids in the tissue to replace their increased release into the medium observed after the electrical stimulation. Electrical stimulation and malathion have been observed to cause the rapid efflux of both glutamic acid and aspartic acid to

the incubation medium, whereas the release after the application of other insecticides does not significantly differ from control. The release of glutamate and aspartate from perfused cat brain slices in response to electrical stimulation have also been reported (Phillis, 1978). This release may be the consequence of excitation of nervous tissue under these circumstances, though virtually no information is available from locust nervous tissue, but it may be due to disruption of the ionic channels in the nerve membrane as is observed in mammalian synaptosomes (Edwardson et al., 1972; Bradford, 1970 and de-Bellroche and Bradford, 1972).

Several insecticides including γ-BHC cause a release of neurohormones in insects (Normanand Samaranayaka, 1977). Insecticide poisoning in Rhodnius, for example, results in discharges of diuretic hormone (Maddrell and Cosida, 1971) and of the plasticizing factor (Maddrell and Reynolds, 1972). Granett and Leeling (1972) have shown that in DDT-treated cockroaches a hyperglycaemic agent appears in insects' haemolymph causing the trehalose content to increase. Poisoning of the adult desert locust, Schistocerca americana gregaria, leads to the release of hyperglycaemic and adipokinetic hormones from the carpus cardiacum (Samaranayaka, 1974). Though the study of hormones was not included in the present investigation (it in itself being a full Ph.D research programme), but together these observations would support the view that symptoms of insect poisoning accompany changes in more than one factor. It would be quite interesting to analyze, if possible, these factors separately in assessing their exact role in the overall function of the insect in stressful conditions.

4.11 Possible functional aspects of accumulation of taurine in the haemolymph of insects after chemical and physiological stress

During the present investigations a significant observation was the substantial accumulation of taurine in the blood of locusts and cockroaches poisoned with insecticides and also during bodily stress induced by enforced activity. The concentration of taurine rises from 2μmol/ml to 5μmol/ml in the haemolymph of locust (Schistocerca americana gregaria) treated with insecticide γ-BHC. Similarly, sustained flight in the locust (Locusta migratoria) results in a most marked (0.53 to 2.25μmol/ml) increase in the concentration of taurine in the haemolymph (van der Horst et al., 1980). Though the exact reason for the appearance of these large amounts of taurine under the stressful conditions are still obscure, the possible function of taurine in normal physiological circumstances of invertebrates has ranged from; a) an osmoregulator (Wigglesworth, 1949); b) an energy reserve by yielding phosphagens (guanidino derivative of taurine) Jacobsen and Smith (1968), Allen and Garrett (1971) and; c) a neuro-transmitter (Kaczmarek and Davison, 1971; Oja and Kontro, 1978).

Koechlin (1955) and Lewis (1952) consider that the high concentration of taurine is necessary to help balance the high cation concentration. In the present circumstances it is possible that any change in osmolarity of haemolymph due to dessication may partly be offset by the increase in the concentration of taurine. The phenomenon of osmoregulation is well established in marine invertebrates where a close relationship between taurine content and the salinity of environment exists (Simpson et al., 1959; Awapara, 1962). The concentration of free amino acid in muscle and byssus of Mytilus transfered to 50% sea water decreases by about 33%. and the concentration of taurine in lamellibranch

muscle falls from 91 to 66 m moles/Kg total water (Potts, 1958). Lange (1963) obtained evidence that taurine concentration in Mytilus increased relatively more than other amino acid concentrations with increasing salinity, and that it thereby exerted a sparing effect on the use of the essential amino acids in osmoregulation.

On the other hand suggestions of the role for taurine as an energy reserve, analogous to arginine phosphate, have also been forwarded (Allen and Garrett, 1971). Some species of phyla Annelida and Sipuncula contain the guanido derivatives of taurine and hypotaurine, taurocyamine and hypotaurocyamine, and possess the enzymic means of forming the corresponding phosphagens (Ennor and Morrison, 1958; Thoai and Roche, 1960 and Thoai and Robin, 1965). There is little evidence at present that either taurine or hypotaurine after conversion to guanido derivatives and phosphorylation plays a significant role in the storage of energy in other than a few species of worms (Jacobsen and Smith, 1968). Though there is no evidence for such derivatives in this study, this is one of the possibilities which needs investigation. If they were found to play such a role, the increase in taurine and arginine in locust haemolymph could be the consequence of the hydrolysis of their phosphate derivatives due to hyperstimulation of insect under the stress.

Turning to a possible neurotransmitter role, little information is available concerning the function of taurine in the nervous system of arthropods (Klemm, 1976), although it has been found in remarkable quantities in locust and cockroach CNS (Frontali, 1964; Ray, 1965; Osborne, 1971). Osborne (1971) reported a concentration of 22 μmol/g tissue in locust nervous tissue which seems very high compared to those

reported here. In the present study the concentration of 0.5μmol and 0.9μmol/g tissue was found in the cerebral and thoracic ganglia respectively of the same insect. The possible reason for the reported higher concentration of taurine (Osborne, 1971) may be the use of single label dansylation used in its analysis. The demerits of the use of a single label have already been pointed out (section 4.1). Alternately the concentration could vary throughout the life span of locust (Bodnaryk, 1981). Similarly taurine is present in invertebrate nervous tissue. Large amounts of this amino acid have been detected in the leg nerve of crabs and lobsters (Koechlin, 1955) and giant nerve fibres of Squid (Lewis, 1952). A substantial amount of taurine is found in the CNS of spiders (Meyer et al., 1980).

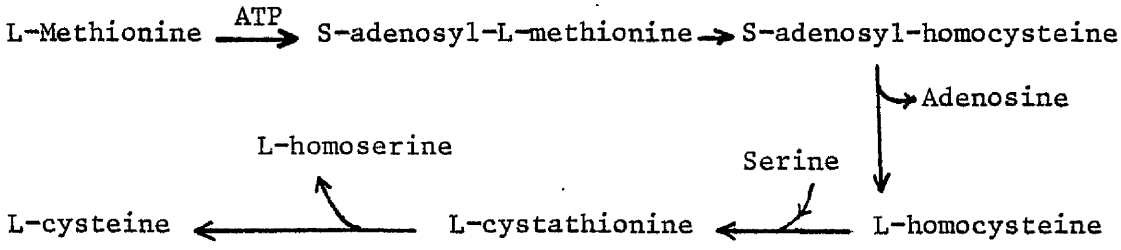
Taurine is observed to exert a reversible inhibitory effect on the spontaneous nervous activity of the isolated nerve cord of cockroach. The spontaneous spikes were depressed more with increasing concentration of taurine applied i.e. from 2×10^{-3}M to 10^{-2}M. In similar in vitro studies Pelhate et al. (1978) used 10 times higher concentration of taurine. These studies show that taurine acts on synaptic transmission in cockroach CNS, so that no effect is observed on axonal membranes (Hue et al., 1978, 1979). Their data shows that taurine has effects similar to those obtained with Gaba. Recently Goyfton et al. (1980) observed a depressive action of taurine similar to glycine on the spontaneous electrical activity of scorpion nervous system. Gruener and Bryant (1975) working with lobster giant axon show that taurine increases membrane permeability to potassium and chloride but not to sodium ions. The onset of response is rapid, the membrane permeability increases transiently by a factor of 5 during the superfusion by taurine. The membrane permeability, resting potential and action potential all

return to control levels under continued perfusion with the amino acid indicating possible receptor decensitization. Finally a reversal of potential can be discovered during taurine action. Although these characteristics are usually required of a putative transmitter, taurine in insects has not as yet satisfied all the criteria set forth by Werman (1966). The main points are that transmitter candidate; 1) should be present along with the enzymes necessary for its biosymthesis in the neuronal tissue preferably in synaptosomes; 2) should be released in response to appropriate chemical or electrical stimulii; 3) once released should have pharmacological effect (either excitatory or inhibitory) on post synaptic membranes; and finally 4) a mechanism for stopping this action be present (either by reuptake into synaptosomes or by enzymic inactivation).

From studies with vertebrate nervous tissue, Phillis (1978) concludes that taurine satisfies most of the criteria for acceptance as a synaptic transmitter. It is present in nerve terminals in the CNS, together with the enzymes for its formation. It can be released from intact or isolated preparations by appropriate neural stimulii or by elevated potassium, and once released it is removed by a high-affinity uptake system. Taurine has a pharmacological action that would be anticipated for an inhibitory transmitter in the nervous system, and its effects are antagonized by convulsants such as strychnine, picrotoxin and bicuculline. The electrophysiological findings indicate that taurine depresses the nerve spiking by increasing neuronal membrane permeability to chloride and potassium ions, and rapidity of its actions, together with their marked similarity to those of Gaba and glycine, suggest that taurine should be considered as a putative neurotransmitter rather than as a modulator.

Despite the widespread occurrence of taurine in invertebrates (Jacobsen and Smith, 1968) little is known about the biosynthesis of taurine in species in most invertebrate phyla. Its presence in the nervous tissue of insects has already been discussed. Taurine is not a component of proteins. It has been shown to be a metabolic product in invertebrates rather than a dietary component accumulated by ingestion (Awapara, 1962). Hardly any information is available about the metabolism of taurine in insects. Most of our knowledge is gathered from the studies with the vertebrates. In mammalian tissues taurine may be formed by a number of pathways shown in Fig. 72.

The experiments on the mechanism of formation of taurocholic acid in the dog and several subsequent nutritional and radioisotopic studies showed that in mammalian tissues methionine and cysteine were both rapidly converted to taurine (Virtue and Dostervirtue, 1937; Traver and Schmidt, 1942; Foster et al. 1919). The pathway of conversion of methionine to cysteine has been demonstrated to occur in the mammalian brain; [^{35}S]-methionine administered intraperitoneally to rats and added to the incubation medium of in vitro preparation of brain, is rapidly converted to form [^{35}S]-labelled-S-adenosylmethionine, S-adenosyl-homocysteine and cysteine probably through the following reaction sequences (McIlwain and Bachelard, 1971).



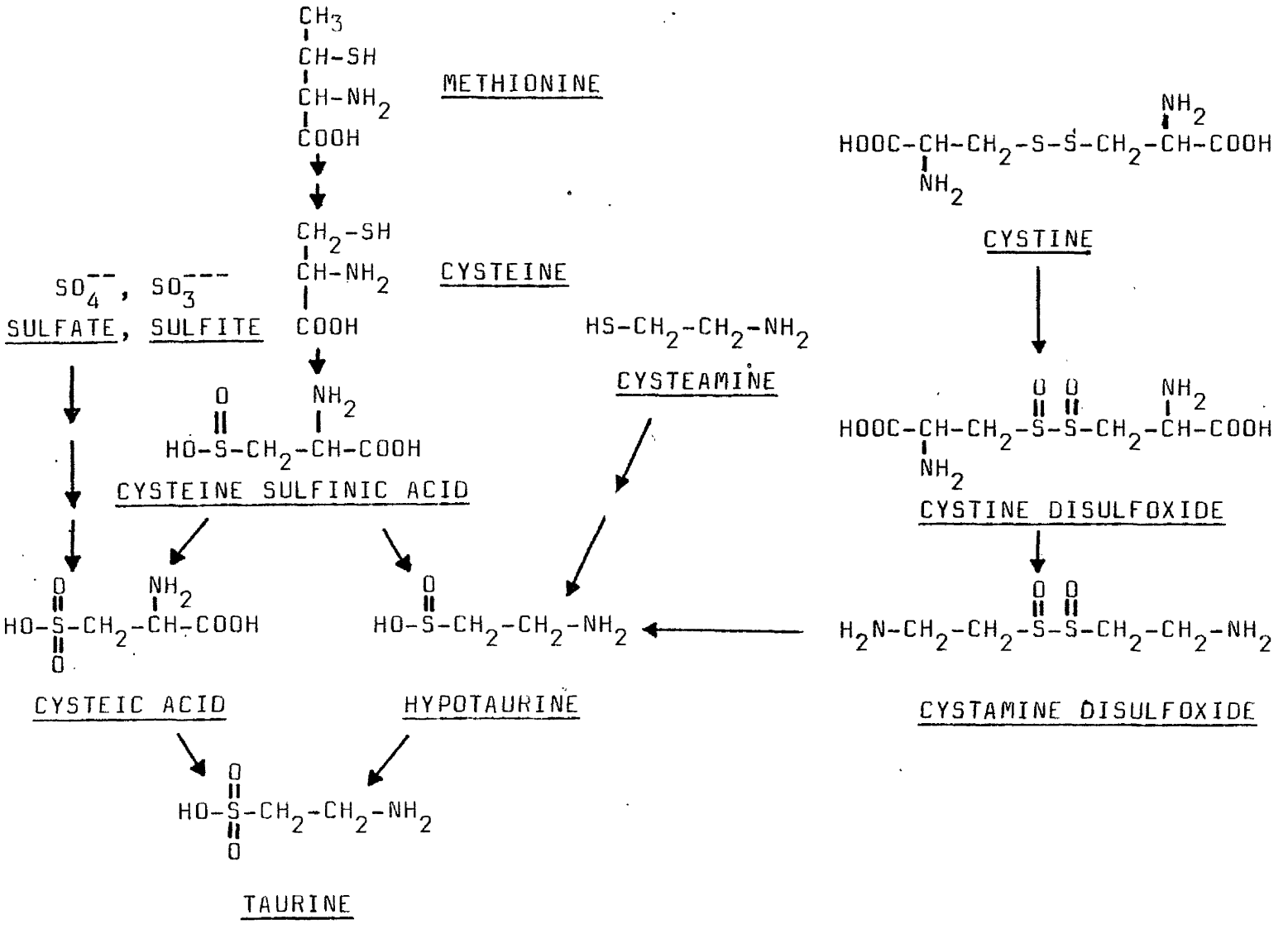


Fig. 72 Proposed biosynthetic pathways for Taurine.

(After Jacobsen and Smith, 1968)

Further metabolism may proceed via cysteic acid or hypotaurine (Fig. 72). The experiments with [^{35}S]-cysteine in Noctuid moth, Mamestra configurata, show that the major pathway for the synthesis of taurine is:

cysteine→cysteamine→hypotaurine→taurine (Bodnaryk, 1981).

The in vivo and in vitro studies carried out on the thoracic ganglia of locust in the present investigations failed to find substantial increase in taurine after the administration of cysteine or methionine. So the question of the source of taurine in the haemolymph and nervous tissue can not be answered at present and a more detailed study of various tissues of the locust and other insects is required for elucidation of the synthesis of taurine. As no evidence could be found for the rapid synthesis of taurine from cysteine or methionine to account for the excessive accumulation in stressed insects, one aspect of future study may be the investigation of its release from some preformed store, as for instance a phosphagen in the muscle tissue.

PART IV

4.12 Putrescine in the locust

The diamine, putrescine, is one of the major components of the free amino pool of the haemolymph and nervous tissue of locust and cockroach. In the locust nervous tissue and haemolymph this amine occurs at concentrations of 2.5μmol/g and 4μmol/ml respectively. Putrescine has been found to be present in nearly all kinds of living organism (Tabor and Tabor, 1972). As this diamine has strong positive charge it may have an important function in maintaining the ion-balance of the tissues. Gould and Cottrell (1974) observed that putrescine

is found at a higher concentration (independent of the tissue chosen for experiment), in the fresh water species of molluscs than marine varieties. They are of the opinion that putrescine may have some role in water retention, ion-balance or nitrogen metabolism in the terrestrial and fresh water molluscs.

In a number of studies putrescine (and other polyamines, spermidine and spermine) have been found usually associated with fast growing cells and places where rapid synthesis is taking place (Russel, 1973; Raina et al., 1980; Seiler et al., 1980). In these cells, the positive charges of these amines are thought to replace the catalytic function of cations like Mg^{++} (Tabor and Tabor, 1972; Seiler, 1981). Cellular Mg^{++} and other metal cations are derived from the circulation. Their levels are dependent on their diffusion or transport through membranes. Ion-ion interaction of metallic cations can be altered by displacement with other cations or by neutralization of the negative charge of the binding sites by metabolic reactions. In contrast, the organic polycations can be formed, conjugated and degraded within the cell, and their formation and degradation more exactly regulated. This permits subtle adjustment of intracellular concentrations according to physiological needs. Binding can in addition be terminated by reactions, which neutralize the positive charges of the polycations such as acetylation or other conjugation reactions.

The conversion of putrescine to monacetylputrescine in the haemolymph of the locust may be a consequence of such a neutralization. The insect tissues (including nervous tissue) have been reported to possess N-acetyltransferase activity (Serkeris and Karlson, 1962;

Evans and Fox, 1975). In these circumstances one would expect to find acetylated derivatives of putrescine present in the nervous tissue extract of locust as well. But no evidence for such derivatives was found although putrescine appeared to diffuse easily into the nervous tissue. The failure to recover any mono-acetylputrescine in the locust nervous tissue may be that the radioactivity in this derivative was below the limit of detection or because of the location of N-acetyltransferase. In the nervous tissue of locust the N-acetyltransferase is thought to be associated with the peripheral cells of the ganglia (P.F.T. Vaughan, personal communication). This would mean any acetylated diamine in this tissue would easily diffuse out and be removed by the circulating haemolymph.

In the mammalian brain the putrescine has been shown to be a potential precursor of Gaba (section 1.7), but no such role is found in the locust. The mammalian brain contains very low activity of diamine oxidase (DAO), Blaschko et al. (1961) and the metabolism of putrescine has been shown to occur via the formation of mono-acetyl putrescine (Seiler et al., 1979). The important step in the formation of Gaba from putrescine would be oxidative deamination by the action of the monoamine oxidase (MAO). As the insect nervous tissue has been shown to have no significant MAO activity (Blaschko et al., 1961; Colhoun, 1967; Boadle and Blaschko, 1968 and Evans and Fox, 1975) this could be the reason why Gaba is not formed in the insect tissue either in vivo or in vitro.

The locust thoracic ganglia does contain some DAO activity (R.H.C. Strang, personal communication), the enzyme carrying out the oxidation of putrescine in bacteria. But even homogenates of

locust CNS containing added NAD^+ and AOAA (to prevent further metabolism by transamination of any Gaba formed) failed to show an accumulation of Gaba (for pathway see Fig. 11, section 1.7). All these results indicate that putrescine does not contribute to the formation of Gaba in the insect CNS. This means, that Gaba formation takes place in insect nervous system as it does in the mammalian brain, by the decarboxylation of glutamic acid. The locust (Schistocerca) nervous tissue contains glutamic acid decarboxylase (GAD) activity as 0.4μmol/g tissue/hour or 0.006μmol/g/min at 25°C (calculated from Bradford et al. (1969)). This low activity is consistent with the very slow increase in Gaba concentration (from 3μmol to 5μmol/g tissue) when glutamate levels rise (from 11μmol to 20μmol/g/tissue) due to deamination of glutamine (Table 26).

Returning to the main theme of this section, the function of putrescine in the insects, it is known that insect respiration at the tissue level generally involves direct gaseous exchange between the tracheal system and various tissues through diffusion. Occurrence of a O_2/CO_2 carrier (e.g. haemoglobin) is restricted to only a few insects including the freshwater larvae of Gastrophilus intestinalis and three species of Hemiptera (Mill, 1974). The existence of a non-proteinaceous carrier has never been reported. Though they have presented no direct evidence, Cheung et al. (1982) have suggested that due to the high affinity of CO_2 for putrescine (and other polyamines) it is possible that these compounds are acting as CO_2 scavengers in the haemolymph, to maintain a balanced oxidation-reduction potential within the haemocoel. The presence of putrescine in the haemolymph and nervous tissue of locust and cockroach may well have such a function. This is one of the areas of research on the function of putrescine in insects that can be further explored.

SECTION FIVE

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