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THE STABILITY OF THE FREE AMINO ACID POOL IN ISOLATED PERIPHERAL NERVES OF *CARCINUS MAENAS* (L.)

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INTRODUCTION

Little information exists on the stability of the free amino acid pool of isolated peripheral nerves from the common shore crab, *Carcinus maenas*, when incubated in saline. The results of such a study would seem to be an essential prerequisite to the interpretation of influx and efflux rates obtained for selected amino acids using tracer techniques. Lewis (1954) observed that the rate of leakage of the amino acids from freshly dissected, resting nerves of this species was very low. After about 2 h, however, he reported that the nerves began to leak amino acids more rapidly, an effect which was accompanied by an equivalent increase in potassium leakage. He suggested that this latter increase might have been due to a deterioration of the preparation, probably in the smallest fibres of the whole trunk.

The fluctuations in the free amino acid content of isolated ligatured nerves of the chinese crab, *Eriocheir sinensis*, have been studied, by Gilles & Schoffeniels (1969), in relation to variations in the salinity of the bathing medium. They concluded that the isolated nerves responded to salinity fluctuations in a similar fashion to those in the intact animal. They suggested that fluctuations in the levels of essential amino acids were regulated by changes in membrane permeability whilst the so-called 'non-essential' amino acid levels were controlled at the level of their metabolism. However, they presented no data on the ratio of influx to efflux of the amino acids they studied.

It was decided in the present study to investigate the effects of incubation in saline on the levels of free amino acids in the peripheral nerves of *Carcinus*. The effects of adding various blood concentrations of metabolites to the incubation medium such as glucose (Binns, 1969) and selected amino acids (Evans, 1972) have also been studied. These results are compared with an experiment in which fresh crab blood was used for the incubation medium. The efflux of various amino acids has been estimated by both chemical and radio-isotope techniques. An attempt has also been made to identify any effects due to isolation from the thoracic ganglion of the crab on the amino acid levels in the peripheral nerves.

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MATERIALS AND METHODS

Samples of peripheral nerve were dissected out in saline from the walking legs of *Carcinus* as described by Evans (1973*a*). The ends of the nerve bundles were then ligatured with hairs and the nerves transferred to vials containing 1 ml of the specific incubation medium. The nerves were transferred to fresh media at 15 min intervals. After the required incubation period, at room temperature, the nerves were removed from the solution, blotted carefully and the ligatured ends cut off. The remaining portion was weighed, the amino acids extracted from it and estimated on a Technicon Automatic Amino Acid Analyser as described by Evans (1973*a*). All the solutions used in the incubation were aerated prior to, but not during, the course of the incubations and they were agitated at regular intervals.

The composition of the saline used throughout this study was as follows: Na^+ 494 mM, K^+ 11·3 mM, Ca^{2+} 12·6 mM, Mg^{2+} 18·3 mM, $SO^2{}_4^-$ 18·3 mM, Cl^- 532 mM and $HCO^-{}_3$ 2·5 mM. All solutions were adjusted to pH 7·1.

The effects of various incubation media on tissue amino acid levels have been investigated. These have included *Carcinus* saline, *Carcinus* saline plus 7·2 mg D-glucose/100 ml (Binns, 1969), *Carcinus* saline plus various amino acids at blood-plasma concentrations (Evans, 1972). These results have been compared with those obtained by the incubation of nerves in samples of fresh *Carcinus* blood. In another series of experiments samples of blood, peripheral nerve and central connective were taken from specimens at random times after feeding, to see whether there was any correlation between the tissue and blood-plasma levels of amino acids. The tissue samples were prepared as described above and a description of the sampling, extraction and analysis techniques for amino acids from the blood plasma of *Carcinus* is given by Evans (1972).

The efflux of amino acids has been measured chemically using an apparatus in which ligatured nerves were suspended from a clamp such that their cut ends were just above the surface of a 1 ml aliquot of saline which was agitated continuously. The solutions were changed at regular intervals and the amounts of the various amino acids released were estimated by evaporating the sample to dryness under vacuum and then applying the sample to the top of the autoanalyser column in the normal fashion. Initial efflux rates were calculated from the plots of amino acid efflux against time. The results are expressed as μM of amino acid released per μl of cell water per minute. Efflux experiments have also been performed using ^{14}C-labelled amino acids as tracer molecules. Nerves were incubated for 20 min in saline containing either ^{14}C-L-glutamate (U) or ^{14}C-L-aspartate (U) (S.A. 270 and 231 mCi/mM respectively; Radiochemical Centre, Amersham) at a concentration of 0·1 μmoles/ml and 1 μCi/ml. After a light blotting the nerves were weighed prior to transfer through a series of vials each containing a 1 ml aliquot of saline. The radioactivity released into each vial was estimated and the final vial in each series contained 0·5 ml of hyamine hydroxide (0·5 M in methanol) (Koch-Light Ltd.) in which the nerves were solubilized for 1 h at 50 °C. Ten ml of a scintillation fluid consisting of a 0·8% solution of butyl-PBD (Koch-Light Ltd.) in a 1:2 mixture of Triton X100:toluene was added to each vial. The radioactivity was estimated on a Packard Tri-Carb Liquid Scintillation Spectrometer, correction for quenching being made by reference to the external standard.

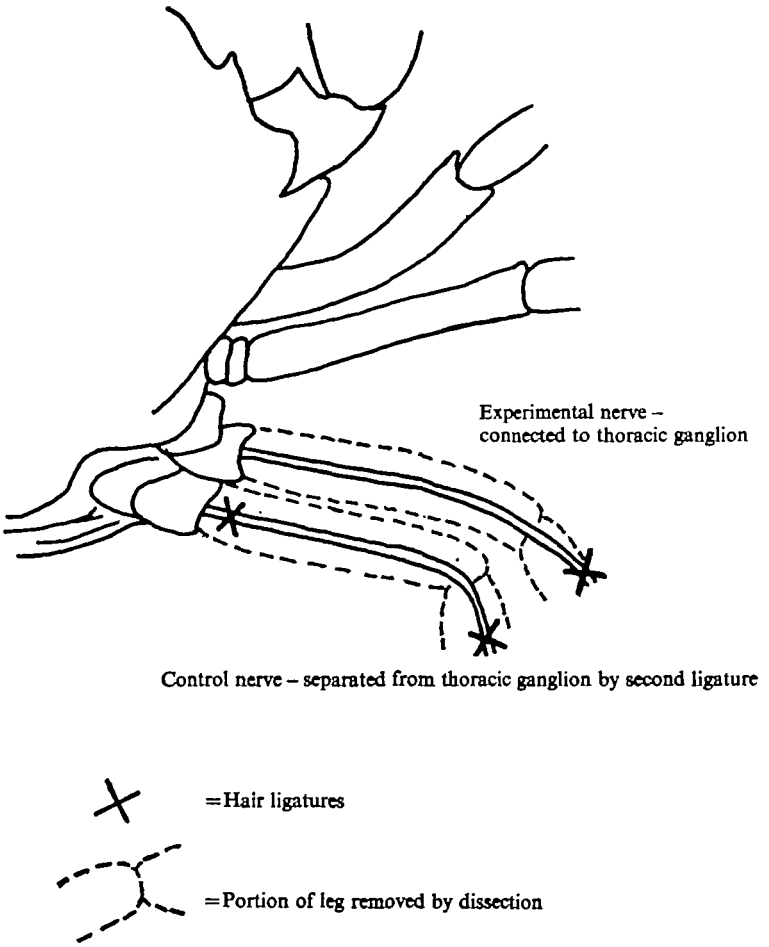

Fig. 1. Diagram to show the position of the hair ligatures on experimental and control nerve incubations, in experiments to determine the effects of maintained connexion to the thoracic ganglion on the levels of free amino acids in peripheral nerve.

The efflux curves for glutamate and aspartate were analysed into their various components and the effects upon the two effluxes of including D-glucose at 7·2 mg/100 ml in the efflux medium was examined.

The effects of isolation of the peripheral nerve from the thoracic ganglion has been investigated in a number of preliminary experiments. Some of the walking legs were dissected free from their peripheral nerves which were left still connected to the body of the crab, retaining their connexion with the thoracic ganglion. This was achieved by cutting around the coxa-basi-ischium joint of a walking leg to prevent the crab autotomizing the leg. The meropodite and carpopodite portions of the leg were removed from the nerve by careful dissection and the end of the nerve was secured with a hair ligature. The peripheral blood vessel which runs along the leg, parallel to the nerve, was cut short and also ligatured. The cut end of the leg was sealed with petroleum jelly. Some of the nerves were then ligatured a second time at the point where the nerve emerged from the cut end of the leg. Fig. 1 shows a diagram of the

dissection for experimental and control incubations. During the dissection, which was carried out in an aerated saline containing amino acids at blood-plasma concentrations (Evans, 1972), the crab was secured to a perspex plate by its chelae and its last pair of walking legs.

When the dissection was complete the crab was transferred to the incubation chamber still attached to the perspex plate. The incubation apparatus consisted of an inner chamber into which the crab was introduced and which contained an aerated saline containing amino acid at the blood-plasma concentrations, and an outer chamber containing iced water which reduced the incubation temperature to 12 °C.

The crab was kept in this apparatus, and samples of experimental and control nerves were taken at appropriate times. The effect of introducing D-glucose at 7·2 mg/100 ml was also investigated on this preparation. During the incubation the petroleum jelly seals effectively prevented loss of blood from the ends of the legs. The crabs could be maintained in this apparatus for extended periods, the final observations being made after 6 h.

RESULTS

The effects of incubation in saline on the free amino acid levels in the peripheral nerves of *Carcinus* are shown in Figs. 2 A and B. It can be seen that the levels generally remained steady for about 1 h, after which time they started to decline. The aspartate values showed a larger variability and gave a more erratic time course than those for the other amino acids. The weight changes of the peripheral nerves were also followed in saline to discover whether any of the apparent changes in levels could be accounted for in terms of swelling or shrinkage of the tissue. The results of this experiment are shown in Fig. 3, and it can be seen that there was only a slight initial shrinkage which was over in the first few minutes.

The effect of adding D-glucose to the incubation medium, in the concentration reported to be present in crab blood, 7·2 mg/100 ml (Binns, 1969), is shown in Fig. 4. This treatment caused an immediate and rapid drop in the levels of amino acids present in large quantities in the crab peripheral nerve, especially aspartate. The levels of alanine and glycine conversely showed slight initial rises and then a gradual decline. Addition to the incubation medium of the major amino acids present in crab blood at the concentrations found in blood plasma (Evans, 1972) produced little change in the form of the time course of the amino acid levels from that shown for incubation in saline alone (Figs. 2 A and B) or for incubation in crab blood itself (Fig. 5). During the first hour of the incubation all the levels of amino acids remained approximately steady. Peripheral nerves were also incubated in samples of fresh crab blood which were obtained as described in Evans (1972). The results of such experiments are shown in Fig. 5. The levels of aspartate, taurine, and glutamate tended to remain steady for the first hour and to then decline, whereas the levels of alanine, proline and glycine rose gradually over the 3 h incubation period used.

The results of the sampling experiment where animals were chosen for analysis at random times after feeding is shown in Fig. 6. It can be seen that there does not appear to be any apparent correlation between the levels of amino acid in the blood-plasma samples and the corresponding levels in the peripheral nerve and central connectives of the same crab. This suggests that metabolic processes in the nerves

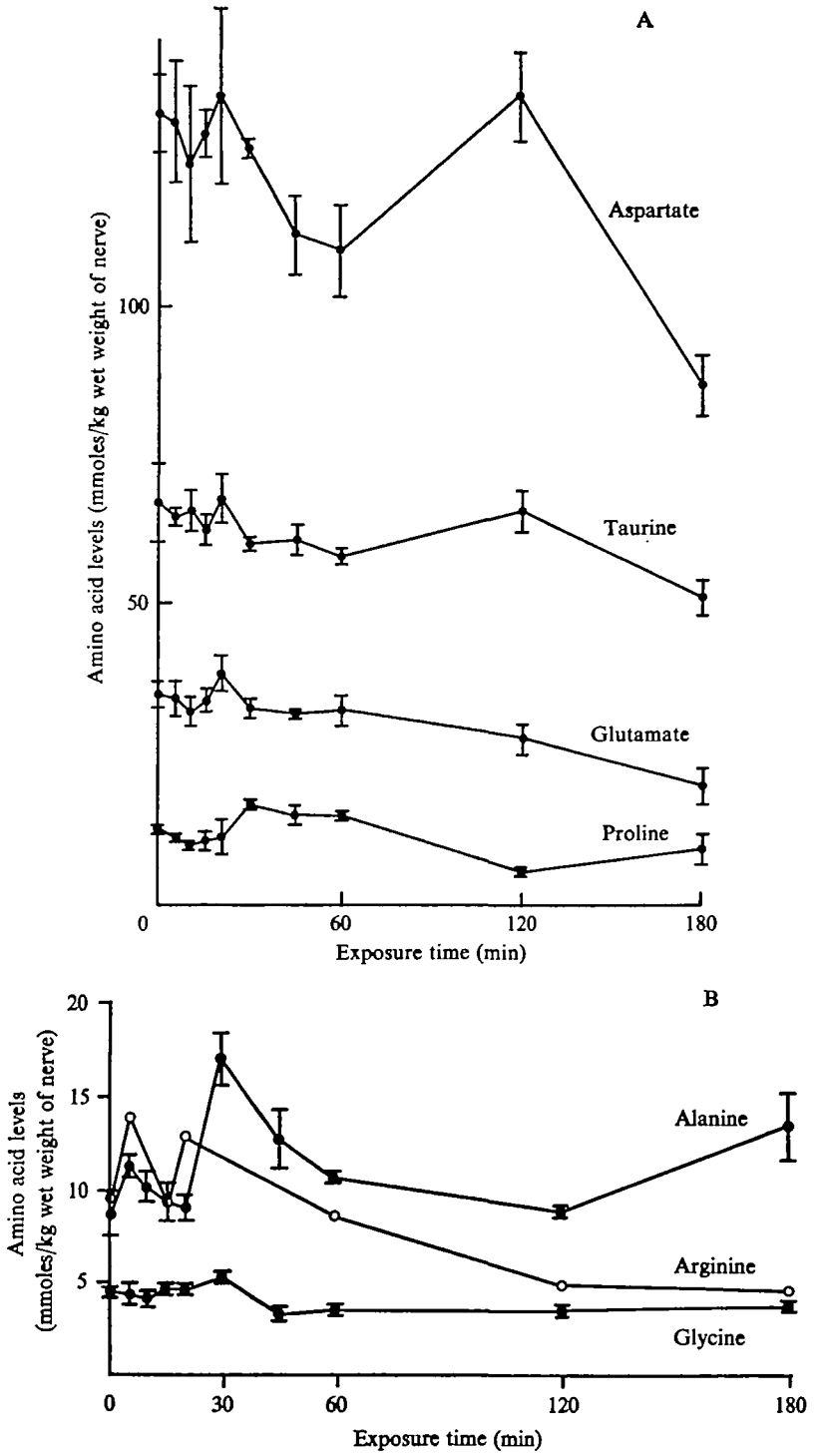

Fig. 2. The free amino acid levels of isolated, ligatured peripheral nerves incubated in saline, plotted for various incubation times. The nerves were transferred to fresh incubation saline every 15 min. The bars represent 2 × S.E. and $n = 5$.

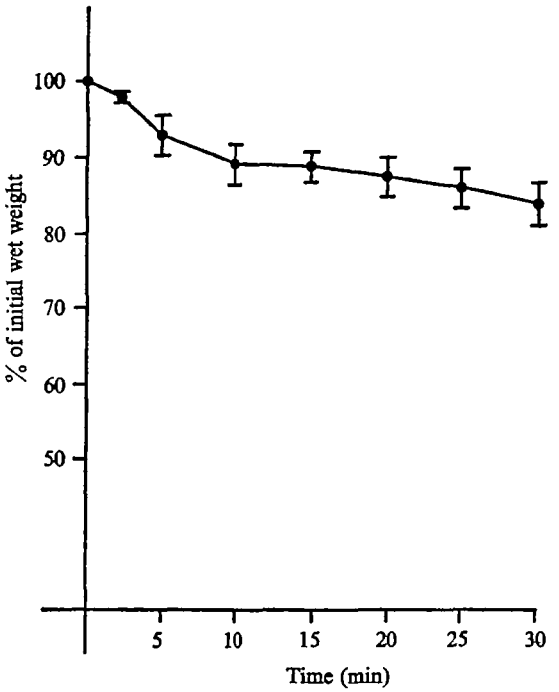

Fig. 3. The time course of weight changes in isolated, ligatured peripheral nerves incubated in saline. The results are expressed as a percentage of the wet weight of the tissue at zero time. The bars represent 2 × S.E. and $n = 10$.

Table 1. *The initial rates of efflux of amino acids from the peripheral nerves of* Carcinus

(The results were obtained by chemical measurement of the amounts of each amino acid released into saline with time, the initial rates being calculated at zero time.)

Amino acid	Efflux (μM/g tissue/min)	Rates (μM/μl cell H_2O/min)	Conc. in peripheral nerve (mM/kg wet weight)
Aspartate	0·923	1648×10^{-6}	198·62
Taurine	0·377	673×10^{-6}	75·45
Glutamate	0·209	373×10^{-6}	36·10
Proline	0·100	179×10^{-6}	24·37
Alanine	0·068	121×10^{-6}	21·64
Arginine	0·052	92×10^{-6}	13·99
Glycine	0·056	$100{\cdot}5 \times 10^{-6}$	7·31

could be more important in maintaining the high levels of free amino acids in the nervous tissue of this species than specific uptake processes for amino acids, dependent upon their concentrations in the medium around the nerve. An example of such a specific transport mechanism has been described for glutamate and other dicarboxylic amino acids in the peripheral nerve of *Carcinus* by Evans (1973*b*).

In experiments in which the nerve was suspended with its cut ends out of the incubation medium the amount of amino acid released into the medium was calculated as the number of μmoles of amino acid released per gram of tissue into 1 ml of bathing medium. When these values were plotted against exposure time a bi-phasic

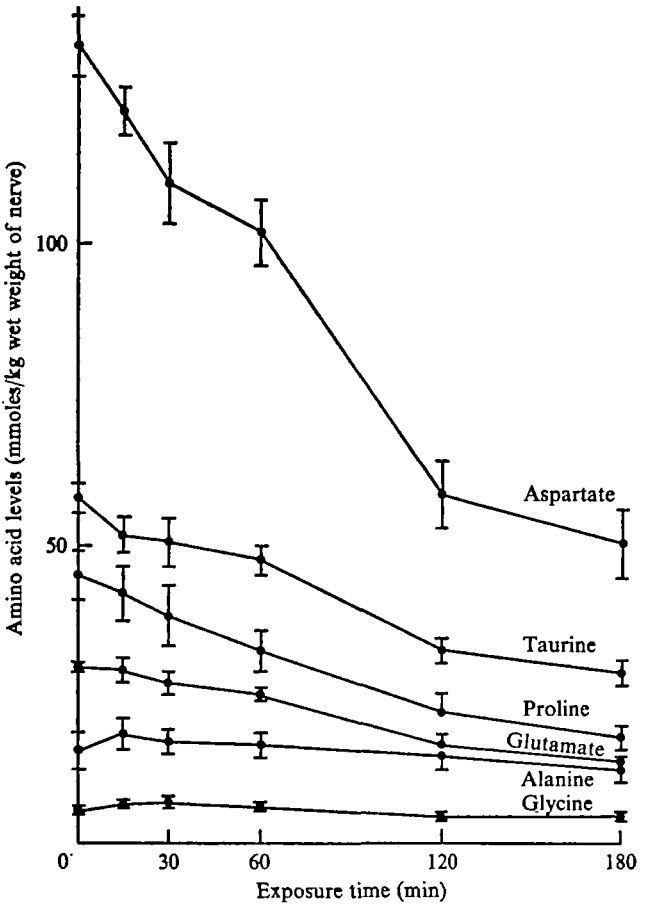

Fig. 4. The time course of the free amino acid levels of isolated, ligatured peripheral nerves incubated in saline containing D-glucose at 7·2 mg/100 ml. The nerves were transferred to fresh incubation medium every 15 min. The bars represent 2 × S.E. and $n = 5$.

efflux plot was obtained with an initial fast phase followed by a slower component. The initial efflux rates were calculated at zero time for the major amino acids present in the peripheral nerves of *Carcinus*, and these are set out in Table 1 along with the mean concentrations in the peripheral nerve of this species (Evans, 1973*a*). It can be seen that the initial efflux rates of the amino acids measured in this way were directly proportional to their concentrations in the peripheral nerve.

The efflux of specific amino acids has been studied in more detail using radioactively labelled amino acid tracer molecules. Fig. 7 shows a typical efflux curve for ^{14}C-L-glutamate from the peripheral nerve bundle of *Carcinus*. It can be seen that extrapolating the slow phase to zero time and correcting for the amount of radioactivity known to be present in the extracellular space (assumed to be 30% of the total water space, Evans, 1973*a*), still leaves about 50% of the total intracellular activity unaccounted for. This suggests that we are not dealing with the efflux of label from a single intracellular compartment, but that the efflux is complex. If the slow phase, which accounts for 51% of the total intracellular label, is deducted from

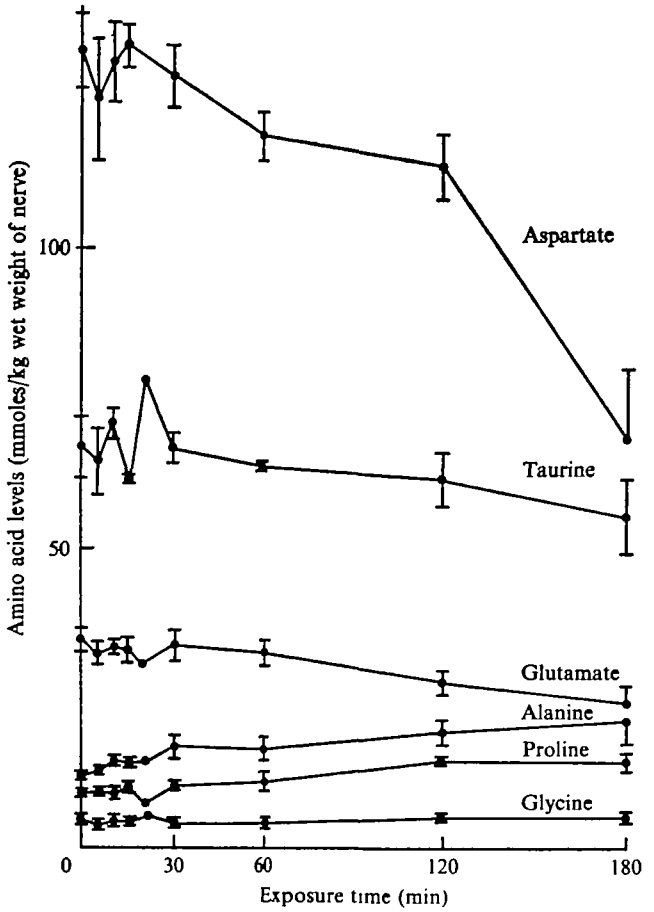

Fig. 5. The time course of free amino acid levels of isolated, ligatured peripheral nerves incubated in fresh crab blood. The nerves were transferred to fresh samples of blood every 15 min. The bars represent 2 × S.E. and $n = 5$.

the total activity a second fast phase can be demonstrated which accounts for a further 39% of the total intracellular activity at zero time. If we assume that 5% of the total intracellular activity in the nerves is sequestered into a fraction insoluble in 60% aqueous ethanol (Evans, unpublished) then 95% of the total intracellular activity taken up by the nerve can be accounted for.

Table 2 shows the $T_{0.5}$ in minutes for the fast and slow components of the glutamate

Table 2. *The half-times of radioactive efflux of amino acids from the peripheral nerves of* Carcinus

(The results show the half-times, in minutes, of the components of the efflux of radioactivity from the peripheral nerves of *Carcinus* loaded with ^{14}C-labelled aspartate or glutamate, and incubated in saline in the presence or absence of 7·2 mg D-glucose/100 ml.)

	Slow	Fast
Glutamate into saline	77·7	3·1
Glutamate into saline + glucose	67·0	3·1
Aspartate into saline	75·0	2·8
Aspartate into saline + glucose	118·3	2·7

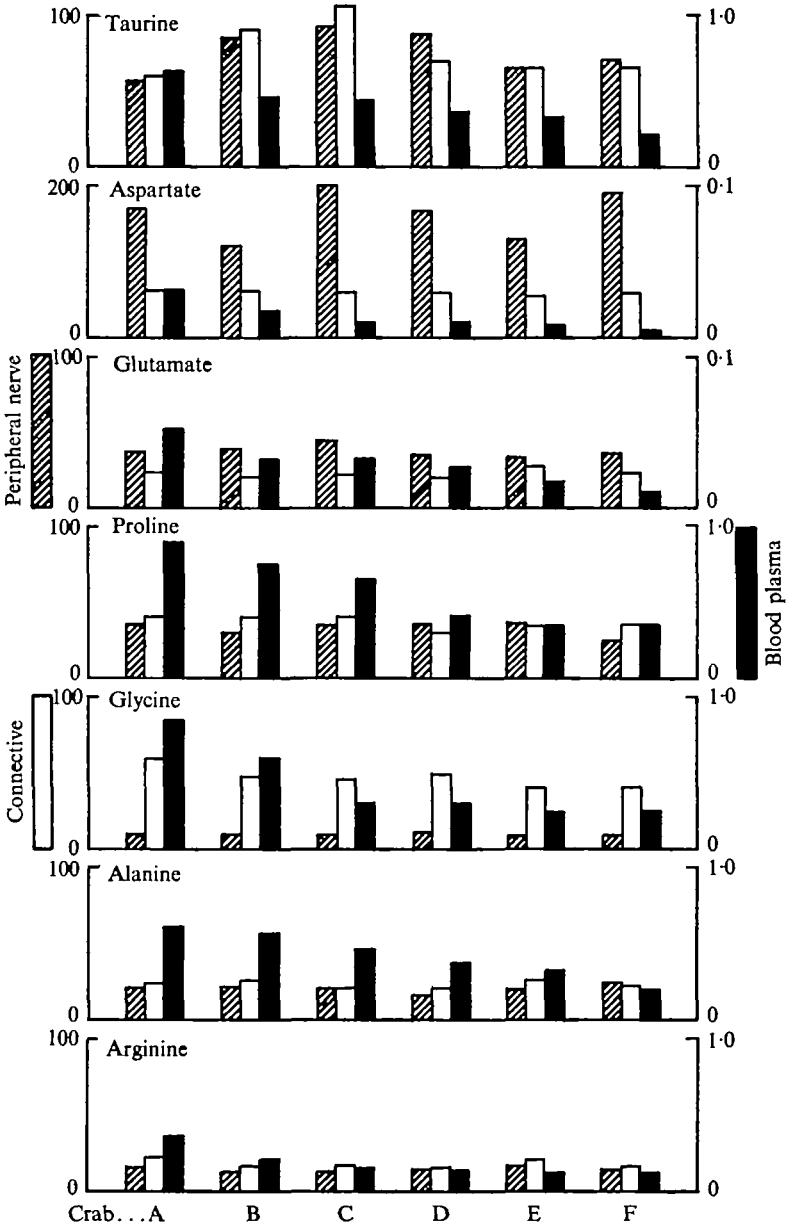

Fig. 6. The free amino acid levels of peripheral nerve, connective and blood plasma of individual crabs sacrificed at random times after feeding. The results are expressed as m-moles/kg wet weight of nerve for the peripheral nerve and the connective and as m-moles/l of blood for the plasma fraction.

and aspartate effluxes into normal saline and into saline plus 7·2 mg D-glucose/100 ml. It can be seen that the presence of glucose did not significantly alter the rates of efflux of glutamate from either compartment but that it tended to slow down the efflux of aspartate from the slow compartment. A possible explanation of this phenomenon is suggested in the discussion.

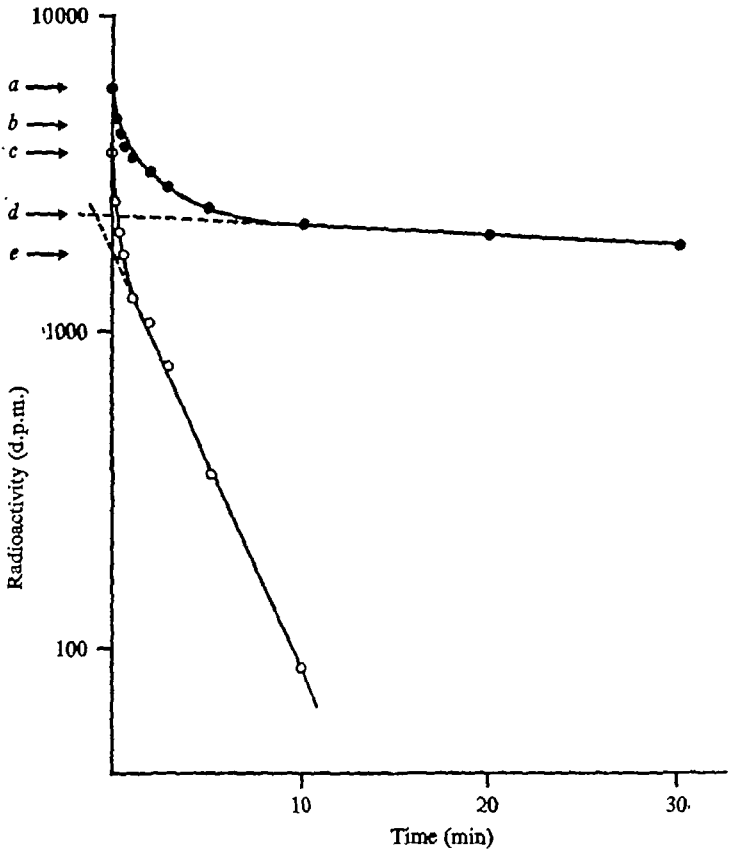

Fig. 7. A typical time course of efflux of radioactivity from an isolated, ligatured peripheral nerve incubated for 20 min in saline containing ^{14}C-labelled glutamate (1 μCi/ml and 0·1 μmole/ml). After blotting and weighing the nerve was transferred through a series of vials containing cold saline and the amount of activity released into each vial was estimated. (*a*) Total activity taken up by nerve. (*b*) Total activity minus extracellular space fraction. (*c*) Total activity minus slow phase. (*d*) Slow phase extrapolated to zero time. (*e*) Fast phase extrapolated to zero time.

The typical results of an experiment where the effect of connexion to the thoracic ganglion has been investigated are shown in Fig. 8. It can be seen that at two hours and at all other sampling times the levels of the individual amino acids other than glycine and alanine were higher in the nerves which were connected to the thoracic ganglion than in their companion nerves which had a second ligature introduced just after the emergence of the nerve from the cut end of the leg. The addition of 7·2 mg D-glucose/100 ml to the incubation medium again caused an immediate and rapid drop in the amino-acid levels in both control and experimental nerves.

DISCUSSION

It can be seen from the results reported above that the levels of free amino acids in *Carcinus* peripheral nerve tended to remain fairly steady for the first hour of incubation in saline and then declined. It is possible that this drop in levels could be accounted for by the increased efflux observed by Lewis (1954) after about 2 h incubation in

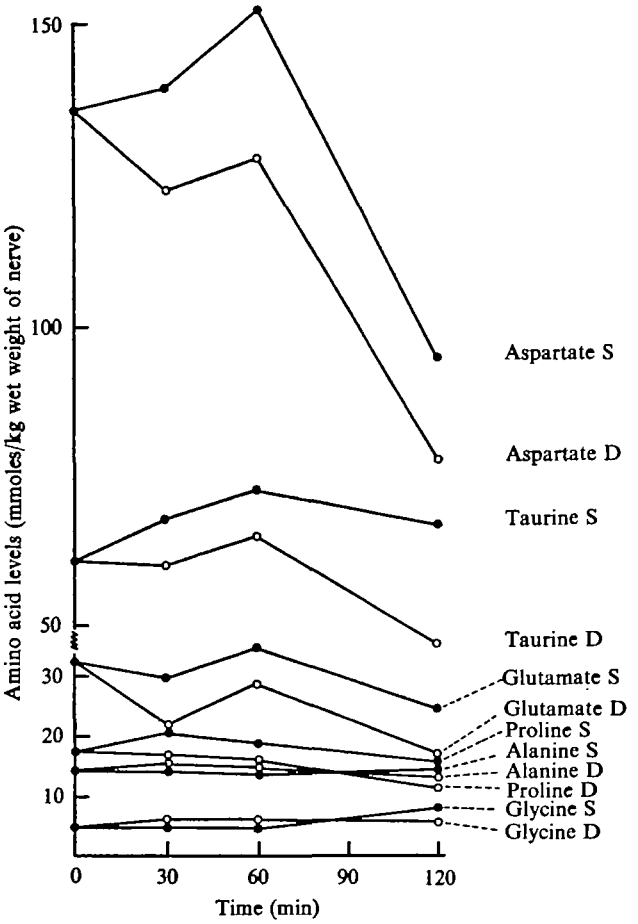

Fig. 8. A typical example of the effect of maintained connexion to the thoracic ganglion on the time course of the free amino acid levels in peripheral nerves incubated in saline containing amino acids at blood-plasma concentrations. Single-ligatured nerves (S) retain their connexion to the thoracic ganglion whereas double-ligatured nerves (D) serve as controls isolated from the ganglion.

his system. He has correlated this increased efflux with an accompanying increase in the efflux of potassium ions and suggests that they both could be due to some deterioration of the preparation, probably in the smallest fibres of the whole trunk.

The very rapid drop in amino acid levels caused by the addition of D-glucose (7·2 mg/100 ml) to the bathing medium was unexpected. As can be seen from Fig. 5, this effect was not observed when the nerves were incubated in samples of fresh *Carcinus* blood, which were thought to contain glucose concentrations of that order (Binns, 1969). It is possible that the glucose might be effectively sequestered into a blood compartment where it is not capable of mediating this effect on the nerves. The obvious candidate for such a compartment would be the haemocyte fraction. This has recently been shown to contain large stores of both glycogen and non-glycogen polysaccharides (Johnston, Spencer Davies & Elder, 1971; Johnston & Spencer Davies, 1972) as well as a large pool of free amino acids (Evans, 1972). It might well be that a

large proportion of the blood glucose measured by Binns (1969) is sequestered into the haemocyte fraction thus effectively protecting the nerves from exposure to a glucose concentration of the magnitude in question.

However, the mechanism of this glucose effect is not altogether clear. Chaplin, Huggins & Munday (1970) have shown that starvation of *Carcinus* can affect the size of the free amino acid pool in muscle and that this could more than offset any changes due to the effects of environmental salinity changes. These findings suggest that the free amino acid pools of *Carcinus* tissues are significant in supporting their energy requirements. It is known that glucose has a sparing effect on the metabolism of amino acids and thus in the presence of a large supply of glucose the enzymes synthesizing particular amino acids could be inhibited. In view of this possibility it is of interest to note that Bradford *et al.* (1969) found that the addition of glucose to the incubation medium reduced the amount of aspartate formed from ^{14}C-labelled glutamate in the locust ganglion in a similar manner to the effect observed in the mammalian cortex. Thus in the crab the addition of glucose could be also inhibiting the formation of aspartate by enzymic pathways. This suggests that the very high levels of aspartate found in the peripheral nerves of *Carcinus* could be maintained largely by a high rate of metabolic production from precursors. This is also suggested by the apparent lack of any correlation between variations in the amino acid concentrations in blood plasma and in the nervous tissues. The evidence from efflux experiments can also be interpreted in terms of this hypothesis, since the rate of efflux of labelled aspartate was reduced in the presence of glucose, the $T_{0.5}$ increasing from 75 to 115 min for the slow phase. It is also possible that the presence of glucose could reduce the ability of the neurones to take up amino acid molecules released into the extracellular spaces, since D-glucose has been shown to reduce the uptake of ^{14}C-labelled glutamate into the peripheral nerves of *Carcinus* (Evans, 1973*b*). Recently D-glucose has also been shown to reduce the uptake of ^{14}C-labelled aspartate by this preparation (Evans, unpublished).

The addition to the incubation medium of the amino acids present in crab blood plasma again failed to counteract the drop off in levels observed after 1 h, as did the incubation of the nerves in actual samples of crab blood. The latter medium was thought to be the most physiologically suited environment obtainable for the incubation of peripheral nerves. It is of interest to note that in tissue-culture experiments the neuronal ganglia of the lobster do not survive well in lobster blood and that the best incubation medium found for them appears to be a complex mammalian medium based on calf serum (Hildebrand *et al.* 1971).

The fact that the isolated nerves, even in crab blood, failed to maintain their amino acid levels after 1 h suggested that there could be some effect mediated by their normal attachment to the thoracic ganglion. It can be seen from Fig. 8 that in all cases the nerve which retained its connexion to the thoracic ganglion had an amino acid content that was larger at 1 and 2 h than the control nerves which were isolated from the ganglion by a second ligature. It is also to be noted that in the nerves connected to the ganglion the levels tended to rise in the period of the first hour. There are a number of possible interpretations of these results. It could be that the reduced levels in the double-ligatured nerves represent some kind of injury phenomenon to the nerve, but clearly this would represent a gross unspecific effect unlike the selective

effect demonstrated. Alternatively it could be that the second ligature reduces either the amount of amino acid passing down the peripheral nerve from the thoracic ganglion or the amount of an unknown substance, of which the level in the peripheral nerve controls the permeability of the membranes to amino acids or their rates of uptake from the extracellular spaces. This latter explanation envisages a similar scheme to that used to explain certain hormone actions, with the membrane permeability or uptake rates being controlled by the levels of an intermediary compound such as cyclic 3′,5′-AMP. This latter explanation has been suggested as a possible way in which the ionic composition of the incubation medium could act on the amino acid metabolism and permeability of the cell membrane to amino acids, or alternatively of the way in which some ionic species could act to directly control the activity of key enzymes involved in the metabolism of amino acids together with the permeability of the cell membrane (Gilles & Schoffeniels, 1969). Further experimentation is needed on this system particularly in relation to the rates of efflux and the rates of amino-acid transport along the peripheral nerves under the various experimental conditions. Thus until more evidence becomes available the involvement of 'messenger substances' in controlling amino acid levels must remain a matter of speculation.

The efflux of amino acids into saline in the present study was found to be greater (approx. $\times 30$) than that measured by Lewis (1954). It is possible that differences in sampling technique could account for the observed differences in amino acid effluxes, but it is difficult to make a true comparison by reason of the lack of technical details in Lewis's brief communication. The present efflux experiments showed that the initial rates of efflux of the amino acids were proportional to their concentrations in the nerves.

The radioactive efflux experiments showed that the efflux from the peripheral nerve was complex, consisting of a slow component ($T_{0.5}$ = 77·7 min) and a fast component ($T_{0.5}$ = 3·1 min) for glutamate. The slow and fast components of the glutamate efflux accounted for about 50% and 40%, respectively, of the total intracellular activity in the peripheral nerve at zero time. An autoradiographical localization of the uptake of glutamate into the peripheral nerves of *Carcinus* will be reported in a further communication (Evans, 1973*c*). The results of this study suggest that glutamate is taken up into both glial and neuronal systems. It is tempting to speculate that the fast component could represent the efflux from the thin branches of the glial system interdigitating between and investing the neurones, whereas the slower component could represent the efflux from the neuronal compartment itself. However, before this suggestion can be substantiated further, more detailed kinetic analyses will have to be performed on this system.

SUMMARY

1. Isolated peripheral nerves of *Carcinus maenas* (L.) were capable of maintaining their free amino acid levels steady for the first hour of incubation in saline.

2. The addition of a presumed blood concentration of D-glucose (7·2 mg/100 ml) caused a rapid drop in the amino acid levels, whereas incubation in actual fresh samples of blood did not. It is possible therefore that much of the glucose in crab blood might be effectively sequestered into the haemocyte fraction.

3. It is suggested that metabolism might play an important part in the maintenance of the very steep free amino acid concentration gradients across the neuronal membranes of this species.

4. The efflux of amino acids from this tissue is complex. It consists of a slow component ($T_{0.5}$ = 77·7 min) and a fast component ($T_{0.5}$ = 3·1 min) which account for about 50% and 40% respectively of the total intracellular radioactivity accumulated after a 20 min incubation.

5. In experiments where the nerves retained connexion with the thoracic ganglion, the levels of amino acids were higher than in controls which were isolated from the ganglion by a ligature. The possible significance of these findings is discussed.

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