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# Separation of Rat Muscle Aminopeptidases

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By means of chromatography on DEAE-Sephadex, two arylamidases (hydrolysing L-arginine 2-naphthylamide) and three dipeptidyl peptidases (hydrolysing dipeptide 2-naphthylamides) were distinguished in extracts of rat muscle. However, the arylamidase from the larger peak also hydrolysed the dipeptide 2-naphthylamides. Glycyl-L-arginine amide, an alternative substrate for dipeptidyl peptidase I, was not hydrolysed by arylamidase. L-Leucine amide was hydrolysed by an enzyme, presumed to be leucine aminopeptidase, from a separate peak, but was also hydrolysed by arylamidase. Arylamidase, dipeptidyl peptidase III and most of the leucine aminopeptidase could be extracted from the muscle with a neutral salt solution, but dipeptidyl peptidase I was extracted only in the presence of Triton X-100; dipeptidyl peptidase II showed an intermediate extraction behaviour.

A number of aminopeptidases have been found in animal tissues and studied to varying degrees, but a precise classification of these enzymes is not yet possible. As well as peptides, various amino acid derivatives, including esters, amides, 4-nitroanilides and 2-naphthylamides, have been used to detect and study their activities.

Patterson *et al.* (1963) first showed that L-leucine 2-naphthylamide, which had been commonly used as a substrate for the histochemical detection of leucine aminopeptidase (EC 3.4.11.1), was instead hydrolysed by a separate enzyme and suggested the temporary use of the term 'arylamidase' to denote enzymes that were active on the arylamides of amino acids. This term has been frequently used by subsequent workers. Enzymes hydrolysing these substrates are found in brain (Marks *et al.*, 1968; Suszkiw & Brecher, 1970), pancreas (Behal *et al.*, 1965), liver (Behal *et al.*, 1965; Hopsu *et al.*, 1966), kidney (Felicetti *et al.*, 1971), pituitary (Ellis & Perry, 1966) and cultured HeLa cells (Lundgren & Roos, 1974). A dependence on thiol groups and bivalent cations and inhibition by low concentrations of puromycin are commonly observed characteristics of these enzymes.

Another group of enzymes, the dipeptidyl peptidases (McDonald *et al.*, 1971), remove dipeptides from dipeptide 2-naphthylamides or from the *N*-terminal end of peptides of a certain length. Four such enzymes have been distinguished. Dipeptidyl peptidase I, which appears to be identical with cathepsin C, has a fairly broad specificity towards the dipeptide moiety, has maximum activity at pH 5.0–6.0 and is activated by thiols and by $Cl^-$ ions. Dipeptidyl peptidase II hydrolyses the 2-naphthylamide of L-lysyl-L-alanine (which is not attacked by dipeptidyl peptidase I), but not the 2-naphthylamides of glycyl-L-phenylalanine, L-seryl-L-tyrosine or glycyl-L-arginine, which serve as substrates for dipeptidyl peptidase I. It is optimally active at pH 4.5–5.5 and no activators are known. Dipeptidyl peptidase III splits the 2-naphthylamide of only L-arginyl-L-arginine; this is not hydrolysed by dipeptidyl peptidase I or II. It has maximum activity at pH 8.0–9.0 and is activated by thiols and inhibited by EDTA. Dipeptidyl peptidase IV hydrolyses glycyl-L-proline 2-naphthylamide and has relatively slight or no activity on other dipeptide 2-naphthylamides; some hydrolysis of this substrate by dipeptidyl peptidase II, but not by dipeptidyl peptidase I or III, was reported. Dipeptidyl peptidases I and II appear to be located in lysosomes, whereas dipeptidyl peptidase III was associated with cell sap and dipeptidyl peptidase IV was found in a microsomal fraction.

We are interested in the role of aminopeptidases in muscle protein catabolism, but these enzymes have not been closely studied in muscle. Joseph & Sanders (1966) purified leucine aminopeptidase from pig muscle and showed its properties to be generally similar to those of the well-known leucine aminopeptidase from kidney. Bury & Pennington (1973) separated two arylamidases and leucine aminopeptidase from autopsied human muscle. A survey of rat tissues for dipeptidyl peptidases (McDonald *et al.*, 1971) showed that aqueous extracts of muscle possessed activity towards the substrates for all four enzymes. There is, however, evidence that the dipeptide 2-naphthylamides are not specific substrates for dipeptidyl peptidases, since arylamidase from kidney (Vanha-Perttula *et al.*, 1965) and pituitary (McDonald *et al.*, 1966) can hydrolyse these compounds (by sequential removal of the two amino

acids). This was shown to be the case in human muscle by Bury & Pennington (1975), who, however, showed by ion-exchange chromatography that dipeptidyl peptidases I and II were present in this tissue.

The separation of some of these enzymes from rat skeletal muscle is described in the present paper.

## Experimental

### *Animals*

Wistar rats of both sexes, weighing about 200g, were used.

### *Chemicals*

L-Leucine 2-naphthylamide, glycyl-L-phenylalanine 2-naphthylamide, glycyl-L-phenylalanine amide, benzoyl-L-arginine 2-naphthylamide and L-leucine 4-nitroanilide were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, U.K. The 2-naphthylamides of L-lysyl-L-alanine, L-arginyl-L-arginine, glycyl-L-arginine, L-seryl-L-tyrosine and glycyl-L-proline were from Bachem Feinchemikalien AG, Liestal, Switzerland. Glycyl-L-arginine amide was from Fox Chemical Co., Tucson, AZ, U.S.A. L-Arginine 2-naphthylamide was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. L-Leucine amide was from BDH Chemicals, Poole, Dorset, U.K. DEAE-Sephadex (type A50) was from Pharmacia (G.B.) Ltd., London W.5, U.K.

### *Preparation of muscle extracts*

The animals were killed by a blow on the head and the gastrocnemius and anterior tibialis muscles removed, chopped finely and combined. The muscle was homogenized with 10mM-$K_2HPO_4$/$KH_2PO_4$ buffer, pH7.2, containing 0.2M-NaCl and 1mM-dithiothreitol (medium A: 9ml/g of muscle). An Ultra-Turrax homogenizer was used, at a speed of 18000–20000rev./min; to minimize any rise in temperature the tube was kept in ice and the muscle was homogenized for three periods of 20s. The homogenate was centrifuged at 45000rev./min for 30min in the no. 50 rotor of a Spinco ultracentrifuge. The pellet was resuspended with a Potter–Elvehjem homogenizer in medium A containing 0.3% Triton X-100 (4ml/g of muscle) and centrifuged (45000rev./min, 30min). The two supernatants were dialysed overnight against medium A and, unless stated otherwise, were combined for chromatography.

### *Chromatography of extracts*

This was carried out in a cold-room. The extract (15ml) was applied to a column (60cm×0.9cm) of DEAE-Sephadex (A50) previously equilibrated with medium A. Proteins were eluted, by using a peristaltic pump, first with medium A (approx. 120ml), then with 100ml of the same solution containing a linear gradient of NaCl (0.2–0.5M); fractions (3ml) were collected. A flame photometer was used to monitor the concentration of NaCl in the fractions.

### *Enzyme assays*

All assays were carried out in tubes shaken at 37°C.

The hydrolysis of arginine 2-naphthylamide and leucine 2-naphthylamide was measured in a medium (total vol., including enzyme, 0.5ml) containing substrate (0.25mM), 3.0mM-dithiothreitol and 50mM-$K_2HPO_4$/$KH_2PO_4$ buffer, pH7.5. After 30 min incubation 1ml of ethanol was added and the tubes were stood in ice for a few minutes and centrifuged (800*g*, 10min). The fluorescence of the liberated 2-naphthylamine was measured (excitation 339nm, emission 403nm).

For measurement of hydrolysis of the 2-naphthylamides of glycyl-phenylalanine, glycyl-arginine and seryl-tyrosine the medium (0.5ml) contained substrate (0.5mM), 1.8mM-dithiothreitol, 50mM-$KH_2PO_4$/$Na_2HPO_4$ buffer, pH6.0, and 5mM-NaCl. For glycyl-phenylalanine 2-napthylamide and seryl-tyrosine 2-naphthylamide the medium contained a final concentration of 4% (v/v) dimethyl sulphoxide used to prepare the stock solutions of the substrates. Incubation was for 2, 1 and 2h respectively for the three substrates and 2-naphthylamine was measured as above. Identical procedures were used with lysyl-alanine 2-naphthylamide and arginyl-arginine 2-naphthylamide, except that the buffers (50mM) were citric acid/sodium citrate, pH5.5, and Tricine [*N*-tris(hydroxymethyl)methylglycine], pH9.0, respectively, and NaCl was omitted; incubation was for 2h.

Assays with leucine amide as substrate were carried out in a total volume of 0.3ml including substrate (4mM), 5mM-$MgCl_2$ and 40mM-Tris/HCl, pH8.5. The tubes were incubated for 1h, 0.3ml of 10% (w/v) trichloroacetic acid was added and the tubes were stood in ice for a few minutes and centrifuged (800*g*, 10min). $NH_3$ was measured in the supernatant (0.1ml) by the sensitive fluorimetric method of Taylor *et al.* (1974), which involves reaction of $NH_4^+$ ions with phthalaldehyde and mercaptoethanol.

For measurement of hydrolysis of glycyl-arginine amide and glycyl-phenylalanine amide the medium (0.25ml) contained substrate (10mM), 0.1M-sodium acetate/acetic acid buffer, pH5.0, 1.8mM dithiothreitol and 5mM-NaCl. Incubation was for 2h. The substrates were found to interfere with the determination of $NH_3$ by the method of Taylor *et al.* (1974) or by the Indophenol Blue reaction (O'Donovan, 1971); therefore the $NH_3$ was first separated by microdiffusion. A portion (0.2ml) of the reaction mixture, immediately after incubation, was added to 0.4ml of 0.2M-$Na_2CO_3$/$NaHCO_3$ buffer, pH10.0, in small bottles (approx. 5cm×2.5cm diam., of the type used for liquid-scintillation counting) with central glass

rods smeared with 0.05M-$H_2SO_4$ (Seligson & Seligson, 1951). To shorten the time for diffusion (to minimize alkaline hydrolysis of the substrate), thick rods were used, roughened to assist the spread of the acid. The 0.05M-$H_2SO_4$ (40$\mu$l) was saturated with $MgSO_4$ to prevent its evaporation. The turntable carrying the bottles was rotated at 78rev./min for 15min. The $H_2SO_4$ was washed off the rods with 0.76ml of $NH_3$-free water and $NH_3$ was measured in 0.1 or 0.2ml portions by the method of Taylor *et al.* (1974).

Leucine 4-nitroanilide was used as a substrate (0.5mM) in a total volume of 0.5ml, containing 50mM-Tris/HCl, pH7.3 (at 37°C), and 1.2mM-dithiothreitol. After 30min incubation, 0.25ml of 50mM-sodium acetate/acetic acid buffer containing 12.5% (w/v) trichloroacetic acid (pH adjusted to 3.5) was added. The tubes were centrifuged and the $E_{400}$ of the supernatant, corresponding to liberated 4-nitroaniline, was measured.

As stated above, the incubation periods for the various assays varied from 30min to 2h. In order to facilitate an approximate comparison of the rates of hydrolysis of the various substrates the values in the Table and Figures represent substrate hydrolysed per h, assuming that the degree of hydrolysis is proportional to the incubation time. (In none of the assays did the proportion of the substrate hydrolysed exceed 50%.)

Protein was measured fluorimetrically with fluorescamine (Böhlen *et al.*, 1973).

## Results

The hydrolysis of several aminopeptidase substrates by rat muscle homogenate and the activity extracted by medium A and subsequently by medium A containing Triton X-100 are shown in Table 1. Most of the activity towards arginine 2-naphthylamide, arginyl-arginine 2-naphthylamide, glycyl-proline 2-naphthylamide and leucine amide was obtained in the first supernatant. This was true, to a lesser degree, for the hydrolysis of glycyl-phenylalanine 2-naphthylamide and lysyl-alanine 2-naphthylamide, but for glycyl-arginine 2-naphthylamide the greater part of the activity was released only in the presence of the detergent.

Fig. 1 shows the results of the chromatography of the enzymes hydrolysing arginine 2-naphthylamide and two of the substrates for dipeptidyl peptidase I. Hydrolyis of arginine 2-naphthylamide (denoted, for convenience, by 'arylamidase') occurs largely in a single, relatively acidic, peak; this is preceded by another, much smaller peak. The highest specific activity of the enzyme found in the fractions of the major peak was 150 times that of the original extract. Dipeptidyl peptidase I activity appeared in an early peak, roughly coinciding with the elution of most of the protein, but the substrates of this enzyme were also hydrolysed by the enzyme in the arylamidase peak. The latter accounted for most of the hydrolysis of glycyl-phenylalanine 2-naphthylamide, but only a small proportion of the activity towards glycyl-arginine 2-naphthylamide. Similar results (not shown) were obtained by using seryl-tyrosine 2-naphthylamide; in this case about four-fifths of the activity appeared in the arylamidase peak.

A further small activity on glycyl-arginine 2-naphthylamide, after the first arylamidase peak, is also seen; this did not invariably appear, however.

The hydrolysis of glycyl-phenylalanine 2-naphthylamide by the major arylamidase component was inhibited by 89 and 98% respectively by 0.1mM- and 1mM-puromycin. The hydrolysis of this substrate by dipeptidyl peptidase I was stimulated (15%) by 1mM-puromycin.

Table 1. *Hydrolysis of aminopeptidase substrates by homogenates and fractions of skeletal muscle*

The muscle was homogenized and extracted as described in the Experimental section. The first and second (containing Triton X-100) supernatants were assayed separately. Where indicated with an asterisk the first pellet was washed once with medium A and the value for the activity in the first supernatant includes that in the washing.

| Substrate | Activity ($\mu$mol/h per g fresh wt. of muscle) | | | |
|---|---|---|---|---|
| | Homogenate | First supernatant | Second supernatant | Final pellet |
| Arginine 2-naphthylamide | 49.08 | 52.50* | 0.92 | 1.00 |
| Leucine 2-naphthylamide | 40.49 | 33.53* | 0.70 | 2.22 |
| Glycyl-arginine 2-naphthylamide | 11.81 | 9.30* | 15.22 | 5.02 |
| Glycyl-phenylalanine 2-naphthylamide | 19.86 | 14.73 | 3.29 | 0.75 |
| Lysyl-alanine 2-naphthylamide | 6.15 | 4.94* | 1.42 | 0.25 |
| Arginyl-arginine 2-naphthylamide | 4.57 | 3.84* | 0.02 | 0.02 |
| Glycyl-proline 2-naphthylamide | 1.54 | 1.04* | 0.15 | 0.40 |
| Leucine amide | | 47.72 | 5.55 | |
| Glycyl-phenylalanine amide | 0 | 0 | 1.45 | 0.15 |

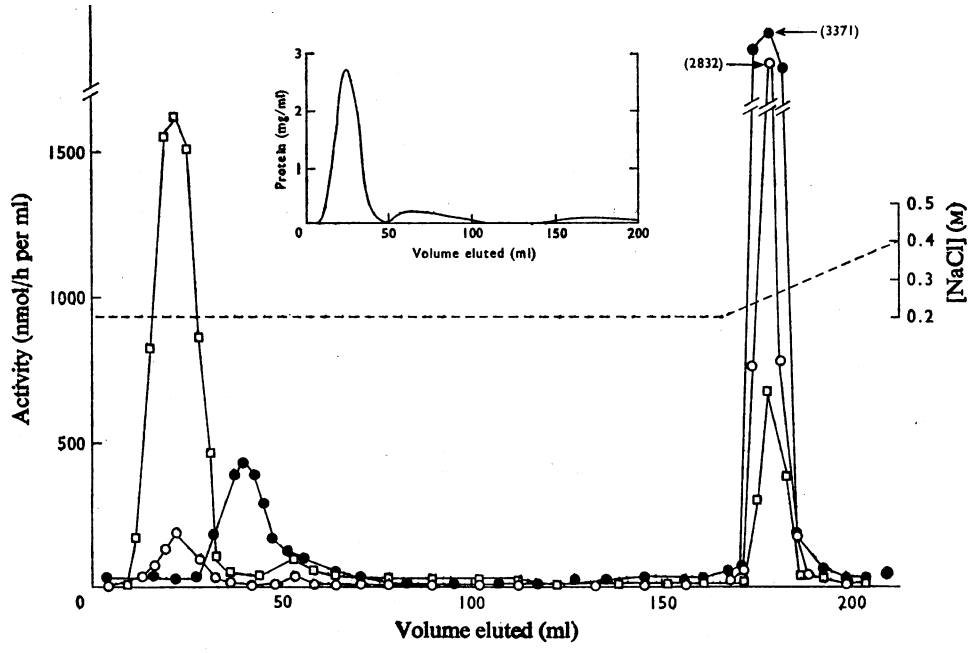


Fig. 1. *Hydrolysis of substrates for arylamidase and dipeptidyl peptidase I by fractions from DEAE-Sephadex chromatography of rat muscle extract*

For experimental details see the text. ●, Arginine 2-naphthylamide; ○, glycyl-phenylalanine 2-naphthylamide; □, glycyl-arginine 2-naphthylamide; ----, NaCl concentration. The two largest peaks have been contracted and the numbers in parentheses are the activities in the most active fractions. The inset shows the elution of total protein.

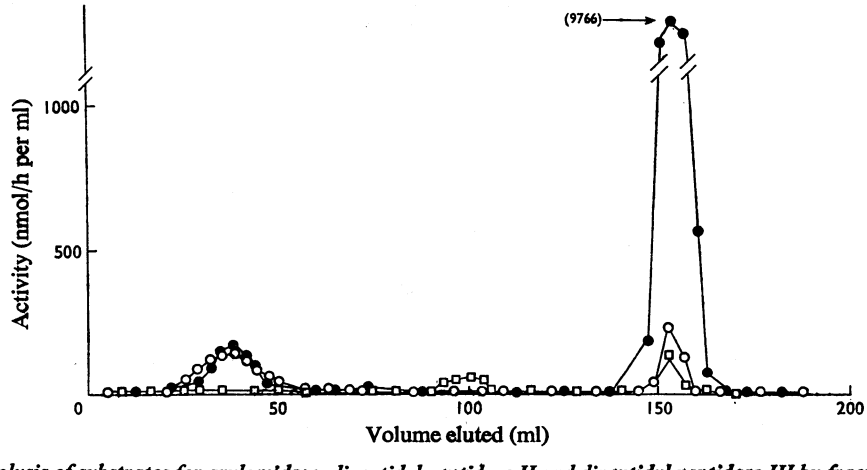


Fig. 2. *Hydrolysis of substrates for arylamidase, dipeptidyl peptidase II and dipeptidyl peptidase III by fractions from DEAE-Sephadex chromatography of rat muscle extract*

For experimental details see the text. ●, Arginine 2-naphthylamide; ○, lysyl-alanine 2-naphthylamide; □, arginyl-arginine 2-naphthylamide. The largest peak has been contracted and the number in parenthesis is the activity in the most active fraction.

Lysyl-alanine 2-naphthylamide and arginyl-arginine 2-naphthylamide, substrates for dipeptidyl peptidases II and III respectively (Fig. 2), were each hydrolysed by an enzyme from a separate peak, but, as for dipeptidyl peptidase I, activity appeared also in the arylamidase peak. The dipeptidyl peptidase II peak largely overlapped the smaller peak of activity towards arginine 2-naphthylamide. However, when the high-speed supernatant obtained from the re-extraction of the muscle with the solution containing Triton X-100 was chromatographed alone, the dipeptidyl peptidase II peak was of the usual size, but both peaks of activity towards arginine 2-naphthylamide had been largely lost.

The size of the dipeptidyl peptidase III peak showed considerable variation between experiments, suggesting that the enzyme may be unstable under the conditions used.

The activity for hydrolysis of leucine amide, the commonly used substrate for leucine aminopeptidase, was also not confined to a single peak (Fig. 3). Arylamidase appears to be responsible for much of the hydrolysis of this substrate. Presumably one or both of the two early peaks contains leucine aminopeptidase. Practically all of the activity on leucine 2-naphthylamide was due to arylamidase; a little activity was associated with the other two peaks of leucine amide hydrolysis.

In other experiments it was found that the enzyme from the large arylamidase peak hydrolysed leucine 4-nitroanilide; the amount hydrolysed was 58% of that obtained with arginine 2-naphthylamide. The hydrolysis of leucine 4-nitroanilide was inhibited almost completely (95%) by 0.1 mM-puromycin.

In view of the report (McDonald *et al.*, 1971) that purified cathepsin B1 hydrolyses glycyl-arginine 2-naphthylamide at 30% of the rate for benzoyl-arginine 2-naphthylamide, its usual substrate, the two first peaks of glycyl-arginine 2-naphthylamide-splitting activity were tested with benzoylarginine 2-naphthylamide. However, only slight activity was found.

Glycyl-arginine amide was hydrolysed by the dipeptidyl peptidase I peak enzyme to 53% of the extent obtained with the corresponding 2-naphthylamide. The amide, in contrast with the 2-naphthylamide, was not perceptibly hydrolysed by the major arylamidase component.

## Discussion

The results of the chromatography of rat muscle are generally similar to those reported with autopsied human muscle (Bury & Pennington, 1973, 1975). No dipeptidyl peptidase III was detected in human muscle, but this may have been lost as a result of post-mortem changes. Definite conclusions about similarity or otherwise of the major peak of arylamidase with such enzymes reported in other tissues are not possible. If hydrolysis of all the substrates by

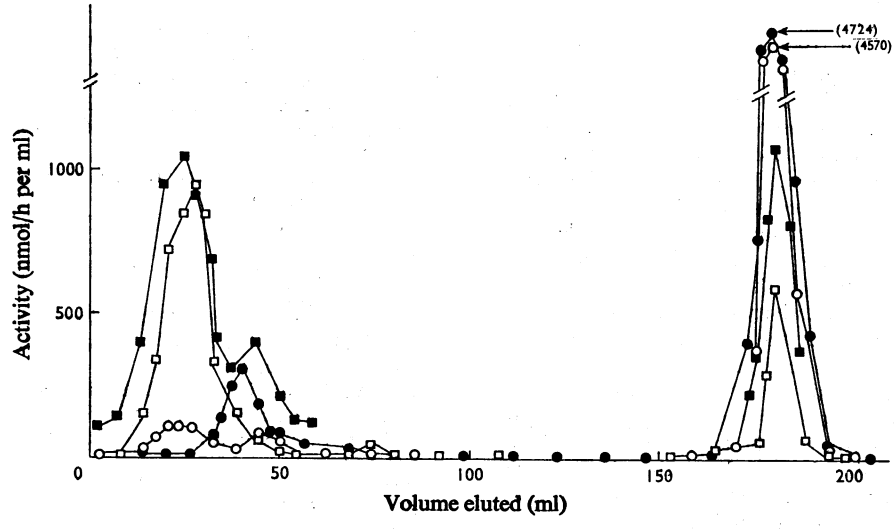


Fig. 3. *Hydrolysis of substrates for arylamidase, leucine aminopeptidase and dipeptidyl peptidase I by fractions from DEAE-Sephadex chromatography of rat muscle extract*

For experimental details see the text. ●, Arginine 2-naphthylamide; ○, leucine 2-naphthylamide; □, glycyl-arginine 2-naphthylamide; ■, leucine amide. The two largest peaks have been contracted and the numbers in parentheses are the activities in the most active fractions.

this peak is due to a single enzyme, it has a broader specificity than the enzyme from liver (Hopsu *et al.*, 1966); unlike the enzyme from bovine brain (Suszkiw & Brecher, 1970) it can hydrolyse simple amides as well as 2-naphthylamides. The presence of two enzymes capable of hydrolysing arginine 2-naphthylamide (Fig. 1) was noted also in bovine pituitary (Ellis & Perry, 1966).

Clearly the hydrolysis of the dipeptide 2-naphthylamides by arylamidase precludes their use as substrates for the dipeptidyl peptidases in crude muscle preparations unless, for example, selective inhibitors can be used. Puromycin may be used to inhibit arylamidase, but has a stimulatory effect on dipeptidyl peptidase I, as shown by McDonald *et al.* (1965) and attributed to its acting as an acceptor for the transpeptidase activity of the enzyme. As shown above, muscle dipeptidyl peptidase I is also stimulated by puromycin; there may be conditions, however, where this stimulation does not occur. Puromycin also causes some inhibition of dipeptidyl peptidase II from pituitary (McDonald *et al.*, 1968). Glycyl-phenylalanine 2-naphthylamide, which has been used for the assay of dipeptidyl peptidase I, is a particularly unsuitable substrate for use with muscle, since dipeptidyl peptidase I makes only a minor contribution to its hydrolysis. The alternative substrate, glycyl-arginine amide, appears to be practically specific, however.

It appears likely from the results in Table 1 that arylamidase is largely or wholly present in the cytoplasm of the cells. Previous work with other tissues has indicated a diversity in the subcellular distribution of arylamidases. Both soluble and bound forms occur in brain (Marks *et al.*, 1968); two separate enzymes in the pituitary had a predominantly cytoplasmic and microsomal distribution respectively (Ellis & Perry, 1966), and activity was associated both with soluble and particulate fractions from ascites cells (Patterson *et al.*, 1963). Kidwai *et al.* (1971) reported that arylamidase was concentrated in the plasma-membrane fraction from rat myometrium, a smooth muscle. The distribution of activity towards glycyl-arginine 2-naphthylamide indicates that most or all of the dipeptidyl peptidase I activity is extracted only in the presence of Triton, since the activity occurring in the first supernatant would be largely or wholly accounted for by arylamidase. This is confirmed by the failure of the first supernatant to hydrolyse glycyl-phenylalanine amide. Moreover, the lack of activity of the homogenate on this substrate shows that this enzyme has a high degree of latency. These results also demonstrate that glycyl-phenylalanine amide, like glycyl-arginine amide, is not attacked by muscle arylamidase. Dipeptidyl peptidase I is considered to have a lysosomal origin in liver (Bouma & Gruber, 1966) and pituitary (McDonald *et al.*, 1971). It would appear that the greater part of the leucine aminopeptidase activity and virtually all of the dipeptidyl peptidase III is extracted in the first supernatant, even when allowance is made for the action of arylamidase on their respective substrates. In several other tissues 60–80% of leucine aminopeptidase activity has been recovered in the supernatant fraction (de Duve *et al.*, 1962). Dipeptidyl peptidase III is localized in the cell sap of the pituitary (McDonald *et al.*, 1971). Dipeptidyl peptidase II appears to be more evenly distributed between the supernatants from the first and second extractions, although with relatively more activity in the first. This enzyme has a lysosomal localization in the pituitary (McDonald *et al.*, 1971).

The possible contribution made by these enzymes to the turnover and pathological breakdown of muscle proteins cannot be assessed until their activity on a range of peptides of differing chain length has been determined. It will be necessary to show also by histochemical techniques or other suitable methods whether they are located in the muscle fibres or in other cell types, such as fibroblasts, present in the muscle.

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## References

Behal, F. J., Asserson, B., Dawson, F. & Hardman, J. (1965) *Arch. Biochem. Biophys.* **111**, 335–344

Böhlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220

Bouma, J. M. W. & Gruber, M. (1966) *Biochim. Biophys. Acta* **113**, 350–358

Bury, A. F. & Pennington, R. J. (1973) *Biochem. Soc. Trans.* **1**, 1306–1307

Bury, A. F. & Pennington, R. J. (1975) *Biochem. J.* **145**, 413–416

de Duve, C., Wattiaux, R. & Baudhuin, P. (1962) *Adv. Enzymol.* **24**, 291–358

Ellis, S. & Perry, M. (1966) *J. Biol. Chem.* **241**, 3679–3686

Felicetti, D., Grundig, C. A. & Hanson, H. (1971) *Acta Biol. Med. Germ.* **26**, 475–482

Hopsu, V. K., Mäkinen, K. K. & Glenner, G. G. (1966) *Arch. Biochem. Biophys.* **114**, 567–575

Joseph, R. L. & Sanders, W. J. (1966) *Biochem. J.* **100**, 827–832

Kidwai, A. M., Radcliffe, M. A. & Daniel, E. E. (1971) *Biochim. Biophys. Acta* **233**, 538–549

Lundgren, E. & Roos, G. (1974) *Biochim. Biophys. Acta* **358**, 208–218

Marks, N., Datta, R. K. & Lajtha, A. (1968) *J. Biol. Chem.* **243**, 2882–2889

McDonald, J. K., Reilly, T. J. & Ellis, S. (1965) *Life Sci.* **4**, 1665–1668

McDonald, J. K., Ellis, S. & Reilly, T. J. (1966) *J. Biol. Chem.* **241**, 1494–1501

McDonald, J. K., Reilly, T. J., Zeitman, B. B. & Ellis, S. (1968) *J. Biol. Chem.* **243**, 2028–2037

McDonald, J. K., Callahan, P. X., Ellis, S. & Smith, R. E. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 69–107, North-Holland Publishing Co., Amsterdam

O'Donovan, D. J. (1971) *Clin. Chim. Acta* **32**, 59–61

Patterson, E. K., Hsiao, S.-H. & Keppel, A. (1963) *J. Biol. Chem.* **238**, 3611–3620

Seligson, D. & Seligson, H. (1951) *J. Lab. Clin. Med.* **38**, 324–330

Suszkiw, J. B. & Brecher, A. S. (1970) *Biochemistry* **9**, 4008–4017

Taylor, S., Ninjoor, V., Dowd, D. M. & Tappel, A. L. (1974) *Anal. Biochem.* **60**, 153–162

Vanha-Perttula, T., Hopsu, K., Sonninen, V. & Glenner, G. G. (1965) *Histochemie* **5**, 170–181