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CA 2399378 C 2009/04/21

(11)(21) **2 399 378**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2001/01/09
(87) Date publication PCT/PCT Publication Date: 2001/08/16
(45) Date de délivrance/Issue Date: 2009/04/21
(85) Entrée phase nationale/National Entry: 2002/08/06
(86) N° demande PCT/PCT Application No.: JP 2001/000031
(87) N° publication PCT/PCT Publication No.: 2001/058944
(30) Priorité/Priority: 2000/02/07 (JP2000-28822)

(51) Cl.Int./Int.Cl. *C12N 15/12* (2006.01),
C07H 21/00 (2006.01), *C07K 14/47* (2006.01),
C07K 16/18 (2006.01), *C12N 15/63* (2006.01),
C12Q 1/02 (2006.01)

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(54) Titre : PETITS TRANSPORTEURS D'AMINOACIDE NEUTRE INDEPENDANTS DU SODIUM

(54) Title: SODIUM-INDEPENDENT SMALL NEUTRAL AMINO ACID TRANSPORTERS TRANSPORTING L- AND D-AMINO ACIDS AND GENES THEREOF

(57) **Abrégé/Abstract:**

Novel sodium-independent small neutral amino acid transporters which transport L- and D-amino acids. A protein comprising the amino acid sequence represented by SEQ ID NO:1 or 4 or an amino acid derived therefrom by deletion, substitution or addition of one or more amino acids and being capable of sodium-independently transporting L- and D-small neutral amino acids and analogs thereof; a gene encoding the above protein; a method of screening substances inhibiting or promoting the function of the above protein; an antibody against the above protein; and a method of regulating cell function by using the above antibody, function inhibitors, function promoters, etc.



Abstract

Novel sodium-independent small neutral amino acid transporters which transport L- and D-amino acids. A protein comprising the amino acid sequence represented by SEQ ID NO:1 or 4 or an amino acid derived therefrom by deletion, substitution or addition of one or more amino acids and being capable of sodium-independently transporting L- and D-small neutral amino acids and analogs thereof; a gene encoding the above protein; a method of screening substances inhibiting or promoting the function of the above protein; an antibody against the above protein; and a method of regulating cell function by using the above antibody, function inhibitors, function promoters, etc.

SODIUM-INDEPENDENT SMALL NEUTRAL AMINO ACID TRANSPORTERS
TRANSPORTING L- AND D-AMINO ACIDS AND GENES THEREOF

Technical Field

The present invention relates to a gene concerning a sodium-independent transport of a small neutral amino acid and analogs thereof, to protein encoding the genes and to an antibody against the protein. The present invention further relates to a method for screening the substance to be tested using the said protein.

Background Art

Cells are required that amino acids are to be always incorporated thereinto as nutrition and such a function is carried by an amino acid transporter which is a membrane protein existing in cell membrane. The amino acid transporter is aligned in a specific site in each tissue in multicellular living things and plays an important role in expression of specific functions in each tissue.

A transport system asc is an amino acid transport system which transports small neutral amino acids mostly alanine, serine and cysteine and, originally, it was found in erythrocyte membrane and mentioned in many literatures. After that, its existence was confirmed in cultured cells as well (Christensen, *Physiol. Rev.*, volume 70, page 43, 1990). The transport system

asc is a transporter which is sodium-independent or, in other words, it does not need sodium ion for its function. Its transport substrate selectivity and transport characteristic have been known to have some differences depending upon cells and animal species.

The transport system asc shows a high affinity to a transport substrate such as alanine, serine or cysteine and, as a transport system similar thereto, there is a transport system C where small neutral amino acid such as alanine, serine or cysteine is a transport substrate as well but affinity to a transport substrate is low (Young et al., *Biochem. J.*, volume 154, page 43, 1976; Young et al., *Biochem. J.*, volume 162, page 33, 1977). The transport system C is believed to be a subsystem for the transport system asc. Sheep where the transport system C is genetically deficient was found, reduction in glutathione content in its erythrocytes was shown and the importance of incorporation of cysteine mediated by cell membrane in the production of glutathione was proved (Young, et al., *Nature*, volume 254, page 156, 1975).

However, in the conventional methods, it is difficult to analyze the details of transport of amino acid and analogs thereof mediated by the amino acid transport system asc and the functional role *in vivo* and there has been a demand to isolate gene of neutral amino acid transporter carrying a function of the amino acid transport system asc so as to make the detailed

function analysis possible.

As to a small neutral amino acid transporter, there were cloned ASCT1 and ASCT2 (Kanai, *Curr. Opin. Cell Biol.*, volume 9, page 565, 1997). However, they are sodium-dependent transporters and are entirely different from a sodium-independent amino acid transport system asc. In the meanwhile, glycine transporter and proline transporter were cloned (Amara and Kuhar, *Annu. Rev. Neurosci.*, volume 16, page 73, 1993) but both of them transport only glycine and proline in a sodium-dependent manner and are different from the transport system asc.

cDNA of rBAT and 4F2hc which are type II membrane glycoproteins having only one transmembrane structure which is considered to be an activating factor of an amino acid transporter were cloned although they are not transporters *per se* and it was known that, when they were expressed in oocytes of *Xenopus*, incorporation of basic amino acid together with neutral amino acid was activated (Palacin, *J. Exp. Biol.*, volume 196, page 123, 1994).

With regard to a transporter which selectively transports neutral amino acid, there were cloned neutral amino acid transporter LAT1 (Kanai et al., *J. Biol. Chem.*, volume 273, pages 23629-23632, 1998) and LAT 2 (Segawa et al., *J. Biol. Chem.*, volume 274, pages 19745-19751, 1999) corresponding to a transport system L. It was further shown that LAT1 and LAT2

functioned only when they coexist together with a cofactor 4F2hc. Both are not dependent on Na^+ but LAT1 shows an exchange transport activity for transporting the large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophane, methionine and histidine and LAT2 has a wide substrate selectivity transporting the small neutral amino acids such as glycine, alanine, serine, cysteine and threonine in addition to large neutral amino acids. However, even they are different from the amino acid transport system asc in terms of substrate selectivity.

With regard to proteins analogous to the neutral amino acid transporters LAT1 and LAT2, there were cloned the above-mentioned $\text{y}^+\text{LAT1}$ and $\text{y}^+\text{LAT2}$ having a function of a transport system y^+L transporting the neutral amino acids and basic amino acids (Torrents et al., *J. Biol. Chem.*, volume 273, pages 32437-32445, 1998). It was also shown that both $\text{y}^+\text{LAT1}$ and $\text{y}^+\text{LAT2}$ functioned only when coexisted together with the cofactor 4F2hc. $\text{y}^+\text{LAT1}$ and $\text{y}^+\text{LAT2}$ mainly transport glutamine, leucine and isoleucine as neutral amino acids and are different from the amino acid transport system asc in terms of the substrate selectivity.

With regard to a transporter demanding the cofactor 4F2hc for the expression of the function, there was cloned xCT which is a protein analogous to the neutral amino acid transporters LAT1 and LAT2 (Sato et al., *J. Biol. Chem.*, 274: 11455-11458,

1999). The xCT transports cystine and glutamic acid and is different from the amino acid transport system asc in terms of the substrate selectivity.

With regard to a transporter demanding other cofactor rBAT having a structure analogous to 4F2hc for expressing the function, there was cloned BAT1 which is a protein analogous to the neutral amino acid transporters LAT1 and LAT2 (Chairoungdua et al., *J. Biol. Chem.*, 274: 28845-28848, 1999). BAT1 transports cystine, neutral amino acids and basic amino acids and is different from the amino acid transport system asc in terms of substrate selectivity.

As such, molecular substances of the transporters which function upon linking to 4F2hc and rBAT were made clear and it was clarified that there was a group of transporters which achieve a transporting function when a molecular complex is formed with the type II glycoprotein.

Disclosure of the Invention

An object of the present invention is to provide a gene of a transporter which transports small neutral amino acid in a sodium-independent manner and shows a function of transport system asc and to provide a sodium-independent small neutral amino acid transporter which is a polypeptide encoded by the gene. Other objects will be apparent from the following description.

The present inventors have checked the EST (expressed sequence tag) database using the base sequence of translated region of cDNA of LAT1 and identified a base sequence analogous to LAT1. They have prepared a probe corresponding to that, screened a cDNA library and cloned a gene coding for a novel protein. They have further expressed this genetic product in oocytes of *Xenopus* and made clear that 4F2hc is essential when the genetic product achieves the function and that, although the expressed function corresponds to the neutral amino acid transport system asc, it makes not only L-amino acids but also D-amino acids into high-affinity transport substrates unlike the conventionally mentioned property of the transport system asc whereupon the present invention has been achieved.

Thus, the present invention relates to a protein comprising an amino acid sequence represented by SEQ ID NO: 1 or 4 or an amino acid sequence where one or more amino acid(s) therein is/are deleted, substituted or added and being capable of transporting the small neutral amino acid and analogs thereof in a Na^+ -independent manner. The protein of the present invention is a protein having an ability of transporting the small neutral amino acids and analogs thereof in a sodium-independent manner when co-existed together with a protein having an amino acid sequence represented by SEQ ID NO: 3 or 6 or a protein comprising an amino acid sequence where one or more amino acid(s) is/are deleted, substituted or added.

The present invention further relates to a gene coding for the above-mentioned protein of the present invention. To be more specific, the present invention relates to a gene coding for a protein having an ability of transporting the small neutral amino acid and analogs thereof in a sodium-independent manner comprising a base sequence represented by SEQ ID NO: 2 or 5 or a base sequence being able to hybridize with DNA comprising the said base sequence under a stringent condition.

The novel protein of the present invention being capable of transporting small neutral amino acids and analogs thereof in a sodium-independent manner or an asc-type amino acid transporter 1 has an ability of transporting (incorporating) the small neutral amino acid such as glycine, L-alanine, L-serine, L-cysteine and L-threonine in a highly affinitive manner when co-existed together with an amino acid transport activating factor 4F2hc. It further transports L-valine, L-methionine, L-isoleucine, L-leucine, L-histidine and L-phenylalanine in a lowly affinitive manner. The asc-1 further transports D-alanine, D-serine, D-cysteine and D-threonine and particularly D-serine in a highly affinitive manner. In addition, the asc-1 transports the substances analogous to amino acid such as α -aminoisobutyric acid, β -alanine and alanine methyl ester.

Further, the sodium-independent small neutral amino acid transporter asc-1 of the present invention which transports

L- and D-amino acids is mostly expressed in brain, lung, small intestine and placenta *in vivo*. Particularly, asc-1 transports D-serine which is believed to be an endogenous function-modifying substance for an NMDA-type glutamic acid receptor and, therefore, there is a possibility that it participates in the kinetics of D-serine in brain and affects the functioning state of the NMDA receptor. Furthermore, since asc-1 transports cysteine, it is believed to be a factor regulating the production amount of glutathione produced from cysteine as a material.

Brief Description of the Drawings

Fig. 1 is a drawing which shows a comparison of amino acid sequence of mouse asc-1 with those of rat LAT2, rat LAT1, human γ^+ LAT1, human γ^+ LAT2 and mouse xCT. The presumed transmembrane sites are shown by the lines added.

Fig. 2 is a drawing which shows a comparison of amino acid sequences of mouse asc-1 and human asc-1.

Fig. 3 is a picture which is a substitute for a drawing which shows the result of analysis of expression of asc-1 gene mRNA in various organ tissues of mouse by a northern blotting.

Fig. 4 is a picture which is a substitute for a drawing which shows the result of by a western blotting analysis using mouse brain membrane sample conducted under a non-reductive condition (-) and a reductive condition (+) using anti-asc-1

antibody (left) and anti-4F2hc antibody (right).

Fig. 5 is a drawing which shows the result of an experiment for incorporation of alanine by oocytes into which cRNA of mouse asc-1 gene and/or mouse 4F2hc gene are/is injected.

Fig. 6 is a drawing which shows the result of checking the influence of salt added in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 7 is a drawing which shows the result of checking the influence of concentration of alanine substrate in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 8 is a drawing which shows the result of checking the influence of addition of various L-amino acids or analogous compounds thereof to the system in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 9 is a drawing which shows the result of checking the influence of addition of various D-amino acids to the system in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 10 is a drawing which shows the result of checking the influence of addition of alanine or analogous compounds

thereof to the system in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 11 is a drawing which shows the result of checking the incorporation of radiolabeled L-amino acid by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 12 is a drawing which shows the result of checking the incorporation of radiolabeled D-amino acid by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 13 is a drawing which shows the result of checking the incorporation of radiolabeled L-alanine or analogous compounds thereof by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 14 is a drawing which shows the result of checking the influence of pH in an experiment of incorporation of alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected.

Fig. 15 is a drawing which shows the result of checking the release of ^{14}C -alanine by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 16 is a drawing which shows the result of checking

the progress of release of ^{14}C -alanine with a lapse of time by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected. In the drawing, $\bigcirc$ is the case where, in the release of ^{14}C -alanine in the oocytes into which water is injected as a control instead of cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse, a Na^+ -free uptake solution to which no alanine is added is used; $\bullet$ is the case where, in the release of ^{14}C -alanine in the oocytes into which water is injected as a control instead of cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse, a Na^+ -free uptake solution to which alanine is added is used; $\square$ is the case where, in the release of ^{14}C -alanine in the oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected, a Na^+ -free uptake solution to which no alanine is added is used; and $\square$ is the case where, in the release of ^{14}C -alanine in the oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected, a Na^+ -free uptake solution to which alanine is added is used. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 17 is a drawing which shows the result of checking the progress of release of ^{14}C -leucine with a lapse of time by oocytes into which cRNA of LAT1 gene of rat and cRNA of 4F2hc gene of rat are injected. In the drawing, $\bigcirc$ is the case where, in the release of ^{14}C -leucine in the oocytes into which water

is injected as a control instead of cRNA of LAT1 gene of rat and cRNA of 4F2hc gene of rat, a Na^+ -free uptake solution to which no leucine is added is used; ● is the case where, in the release of ^{14}C -leucine in the oocytes into which water is injected as a control instead of cRNA of LAT1 gene of rat and cRNA of 4F2hc gene of rat, a Na^+ -free uptake solution to which leucine is added is used; □ is the case where, in the release of ^{14}C -leucine in the oocytes into which cRNA of LAT1 gene of rat and cRNA of 4F2hc gene of rat are injected, a Na^+ -free uptake solution to which no leucine is added is used; and ■ is the case where, in the release of ^{14}C -leucine in the oocytes into which cRNA of LAT1 gene of rat and cRNA of 4F2hc gene of rat are injected, a Na^+ -free uptake solution to which leucine is added is used. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 18 is a drawing which shows the result of checking the release of ^{14}C -alanine when various L-amino acids are added to a Na^+ -free uptake solution containing no sodium ion by oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected (black bars) or by oocytes into which water is injected instead of cRNA as a control (white bars). (-) shows the release of ^{14}C -alanine mediated by asc-1 of mouse when no amino acid is added to a Na^+ -free uptake solution. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 19 is a drawing which shows the result of checking the release of ^{14}C -alanine mediated by mouse asc-1 when various kinds of D-amino acids are added to a Na^+ -free uptake solution. (-) shows the release of ^{14}C -alanine mediated by asc-1 of mouse when no amino acid is added to a Na^+ -free uptake solution. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 20 is a drawing which shows the result of checking the release of ^{14}C -alanine mediated by asc-1 of mouse when various kinds of alanine-analogous compounds are added to a Na^+ -free uptake solution. (-) shows the release of ^{14}C -alanine mediated by asc-1 of mouse when no amino acid is added to a Na^+ -free uptake solution. The ordinate shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 21 is a drawing which shows the result of checking the release of the injected ^{14}C -amino acid from oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse are injected. Black bars show the case where alanine is added to a Na^+ -free uptake solution while bars with oblique lines show the case where alanine is not added to a Na^+ -free uptake solution.

Best Mode for Carrying Out the Invention

SEQ ID NO: 2 and NO: 1 in the Sequence Listing which will be mentioned later stand for a full-length cDNA base sequence

(about 1.6 kbp) of gene of sodium-independent small neutral amino acid transporter (asc-1 of mouse) transporting L- and D-amino acid derived from brain of mouse and an amino acid sequence (530 amino acids) of protein encoded in its translated region.

SEQ ID NO: 5 and NO: 4 in the Sequence Listing which will be mentioned later stand for a full-length cDNA base sequence (about 1.9 kbp) of gene of sodium-independent small neutral amino acid transporter (human asc-1) transporting L- and D-amino acid derived from human brain and an amino acid sequence (523 amino acids) of protein encoded in its translated region.

When the base sequence or the amino acid sequence represented by the above SEQ ID NO: 1 or 2 or SEQ ID NO: 4 or 5 was subjected to a homology check for all sequences contained in the known DNA databases (GenBankTM and EMBL) and protein databases (NBRF and SWISS-PROT), there was nothing which was identical whereby all those sequences are believed to be novel.

With regard to the protein of the present invention, that which has an amino acid sequence represented by SEQ ID NO: 1 or 4 and, in addition, protein having an amino acid sequence where one or more amino acid(s) is/are deficient, substituted or added in the amino acid represented by SEQ ID NO: 1 or 4 for example may be listed. Defect, substitution or addition of amino acid may be within such an extent that the neutral amino acid transport activity is not lost and that is usually

from 1 to about 106 or, preferably, from 1 to about 53. Like the amino acid sequence represented by SEQ ID NO: 1 or 4, such a protein has usually 1-80% or, preferably, 1-90% of homology of amino acid.

With regard to the gene of the present invention, that which contains DNA being hybridizable under a stringent condition with DNA comprising a base sequence represented by SEQ ID NO: 2 or 5 may be exemplified in addition to that having a base sequence represented by SEQ ID NO: 2 or 5. The DNA which is hybridizable as such may be in such an extent that the protein encoded by the DNA is capable of transporting the neutral amino acid. Such a DNA has usually not less than 70% or, preferably, not less than 80% of homology of a base sequence to the base sequence represented by SEQ ID NO: 2 or 5. Such a DNA includes mutant gene found in nature, artificially modified mutant and homologous gene derived from living things of difference species.

Hybridization under a stringent condition according to the present invention is usually carried out in such a manner that hybridization is carried out in a hybridization solution of 5×SSC or the equivalent salt concentration at the temperature condition of 37-42°C for about 12 hours, a preliminary washing is carried out upon necessity by a solution of 5 × SSC or the equivalent salt concentration and a washing is carried out in a solution of 1 × SSC or the equivalent salt concentration.

The sodium-independent small neutral amino acid transporter gene of the present invention which transports L- and D-amino acids can be isolated and obtained by carrying out a screening using appropriate mammalian tissues or cells as a gene source. With regard to mammals, human being may be listed in addition to non-human animals such as dog, cow, horse, goat, sheep, monkey, pig, rabbit, rat and mouse.

Screening and isolation of gene may be appropriately carried out by a homology cloning, etc.

For example, brain of mouse or human being is used as a gene source and mRNA (poly(A)⁺RNA) is prepared therefrom. A cDNA library is constructed therefrom and a screening is carried out for the cDNA library using a probe corresponding to LAT1-homologous sequence (such as GenBankTM/EBI/DDBJ accession No. N32639) obtained by checking an EST (expressed sequence tag) database whereupon clone containing cDNA of asc-1 gene is obtained.

With regard to the obtained cDNA, its base sequence is determined by a conventional method, the translated region is analyzed and an amino acid sequence of the protein encoded thereby, i.e. asc-1, can be determined.

The fact that the resulting cDNA is a cDNA of a sodium-independent small neutral amino acid transporter gene which transports L- and D-amino acids or, in other words, it is a genetic product encoded by cDNA is a sodium-independent

small neutral amino acid transporter which transports L- and D-amino acids is, for example, able to be tested as follows. Thus, RNA (cRNA) (a capped one) complementary thereto prepared from the resulting cDNA of asc-1 gene is expressed by introducing into oocytes together with cRNA having a base sequence of 4F2hc represented by SEQ ID NO: 3 or 6 and an ability of transporting (incorporating) a neutral amino acid into cells can be confirmed by measuring the incorporation of a substrate into the cells by means of a conventional incorporation test (Kanai and Hediger, *Nature*, volume 360, pages 467-471, 1992) where an appropriate neutral amino acid is a substrate.

An asc-1 protein is synthesized by means of an *in vitro* translation method (Hediger, et al., *Biochim. Biophys. Acta*, volume 1064, page 360, 1991) using RNA (cRNA) which is complementary thereto prepared from the resulting cDNA of asc-1 whereupon it is possible to check the size of protein, addition or non-addition of sugar, etc. by means of electrophoresis.

Since the cDNA of gene of 4F2hc was reported already (Broer, et al., *Biochem. J.*, volume 312, page 863, 1995), it is possible to prepare a gene of 4F2hc easily by means of PCR or the like. cRNA (a capped one) can be synthesized from the resulting cDNA of 4F2hc.

The same incorporation experiment is applied to expression cells whereupon it is possible to check the characteristics of the asc-1 such as a characteristic that asc-1

conducts transport of an exchange type of amino acid as well as substrate selectivity of asc-1 and pH-dependency.

When an appropriate genomic DNA library or cDNA library prepared from different gene source is screened using the resulting cDNA of asc-1 gene, it is possible to isolate homologous gene or chromosomal gene derived from different living things.

When a synthetic primer designed on the basis of information of the disclosed base sequence of gene of the present invention (a base sequence represented by SEQ ID NO: 2 or 5 or a part thereof) is used and a conventional PCR (polymerase chain reaction) is carried out, it is possible to isolate a gene from a cDNA library or a genomic DNA library.

DNA libraries such as a cDNA library and a genomic DNA library can be prepared by a method mentioned in, for example, "Molecular Cloning" (by Sambrook, J., Fritsh, E. F and Manitis, T.; published by Cold Spring Harbor Press). Alternatively, when a commercially available library is available, that may be used.

The sodium-independent small neutral amino acid transporter (asc-1) transporting D- and L-amino acids according to the present invention may be produced by, for example, a gene recombination technique using the cDNA coding therefor. For example, DNA (such as cDNA) coding for asc-1 is incorporated into an appropriate expression vector and the resulting

recombinant DNA can be introduced into an appropriate host cell. Examples of the expression system (host-vector system) for the production of polypeptide are expression systems of bacteria, yeasts, insect cells and mammalian cells. Among them, the use of insect cells and mammalian cells is preferred for the preparation of functional protein.

For example, in the case of expression of polypeptide in mammalian cells, DNA coding for a sodium-independent small neutral amino acid transporter asc-1 which transports the L- and D-amino acids is inserted into the downstream of an appropriate promoter (such as cytomegalovirus promoter, SV 40 promoter, LTR promoter or elongation 1a promoter) in an appropriate expression vector (such as vector of adenovirus type, vector of retrovirus type, papilloma virus vector, vaccinia virus vector or vector of SV 40 type) whereupon an expression vector is constructed. After that, an appropriate animal cell is transformed by the resulting expression vector and the transformant is incubated in an appropriate medium whereupon an aimed polypeptide is produced. Examples of the mammalian cell used as a host are cell strains such as simian COS-7 cell, CHO cell of Chinese hamster and human HeLa cell.

With regard to the DNA coding for a sodium-independent small amino acid transporter asc-1 which transports the L- and D-amino acids, the cDNA having a base sequence represented by SEQ ID NO: 2 or 5 may be used for example and, in addition,

it is not limited to the above-mentioned DNA sequence but DNA corresponding to amino acid may be designed and used as a DNA coding for the polypeptide. In that case, as to the codon for coding for one amino acid, from 1 to 6 kinds of codon(s) is/are known for each and, although the codon used may be selected freely, it is possible to design a sequence having higher expression efficiency by taking the frequency of use of codon of the host utilized for the expression into consideration. DNA having a designed base sequence can be prepared by chemical synthesis of DNA, by fragmentation of the above-mentioned cDNA and combination thereof, by a partial modification of a base sequence, etc. Artificial modification of a base sequence and introduction of variation can be carried out by means of a site-specific mutagenesis (Mars, D. F., et al., *Proceedings of National Academy of Sciences*, volume 81, page 5662, 1984), etc. utilizing a primer comprising a synthetic oligonucleotide coding for the desired modification.

The present invention further relates to nucleotide containing a partial sequence of continuous 14 or more bases, preferably 20 or more bases or, more preferably, 30 or more bases in the base sequence represented by SEQ ID NO: 2 or 5 of the Sequence Listing or a complementary sequence thereof. The nucleotide of the present invention may be used as a probe for the detection of gene coding for a protein being capable of transporting a sodium-independent small neutral amino acid

and analogs thereof.

When the sodium-independent small neutral amino acid transporter of the present invention transporting the L- and D-amino acids or a polypeptide having the immunological homology thereto is used, an antibody against that can be prepared. The antibody can be utilized for the detection or the purification of the sodium-independent small neutral amino acid transporter which transports the L- and D-amino acids. The antibody can be manufactured using the sodium-independent small neutral amino acid transporter of the present invention transporting the L- and D-amino acids, a fragment thereof, a synthetic peptide having a partial sequence thereof or the like as an antigen. A polyclonal antibody can be manufactured by a conventional method where antigen is inoculated to a host animal (such as rat or rabbit) and the immunized serum is recovered therefrom while a monoclonal antibody can be manufactured by a conventional technique such as a hybridoma method.

The sodium-independent small neutral amino acid transporter asc-1 of the present invention transporting the L- and D-amino acids, gene thereof and expression cell thereof can be used in an *in vitro* test for permeation efficiency at the cell membrane where asc-1 is present or at the site where asc-1 is presumed to be present.

Further, the sodium-independent small neutral amino acid transporter asc-1 of the present invention transporting the

L- and D-amino acids, gene thereof and expression cell thereof can be used in the development of compounds which efficiently permeate the cell membrane where asc-1 is present or at the site where asc-1 is presumed to be present. Furthermore, the sodium-independent small neutral amino acid transporter asc-1 of the present invention transporting the L- and D-amino acids, gene thereof and expression cell thereof can be used in an *in vitro* test for a drug interaction at the cell membrane where asc-1 is present or at the site where asc-1 is presumed to be present.

When the sodium-independent small neutral amino acid transporter asc-1 of the present invention which transports the L- and D-amino acids is inhibited, it is possible to limit the permeation of specific compounds at the cell membrane where asc-1 is expressed or at the site where asc-1 is presumed to be present. Further, the sodium-independent small neutral amino acid transporter asc-1 of the present invention which transports the L- and D-amino acids, gene thereof and expression cell thereof can be used in the development of drugs (such as specific inhibitor for asc-1) which limit the passing of the cell membrane or the permeation at the site where asc-1 is presumed to be present of a compound transported by asc-1.

Accordingly, the present invention provides a method for detection, identification or quantification of action as a substrate of a substance to be tested to the ability of the

present invention for transporting a small neutral amino acid and analogs thereof in a sodium-independent manner using the protein of the present protein. In accordance with the method of the present invention, it is possible to screen a substance which promotes the function of the protein of the present invention or a substance which inhibits that. When an uptake solution containing an amino acid which is labeled by radioactivity or by fluorescence such as ^{14}C -alanine is used and the amount of the said incorporated or released amino acid is measured in the presence of the substance to be tested, it is now possible to test the action of the said substance to be tested to the protein of the present invention.

The present invention further provides a method for controlling the resistance of cells to oxidative stress where the protein of the present invention, a specific antibody thereof, a substance for promoting the function thereof or a substance for inhibiting the function thereof is used to modulate an ability of transporting a small neutral amino acid of the said protein or analogs thereof.

The present invention furthermore provides a method for controlling the activity of a glutamic acid receptor of an NMDA type in a nervous system where the protein of the present invention, a specific antibody thereof, a substance for promoting the function thereof or a substance for inhibiting the function thereof is used to modulate an ability of

transporting a small neutral amino acid of the said protein or analogs thereof; a method for controlling the plasticity of synaptic transmission in which a glutamic acid receptor of an NMDA type is participated by means of the above-mentioned method; and a method for controlling the neuronal death in which a glutamic acid receptor of an NMDA type is participated by means of the above-mentioned method.

The present invention provides a method for controlling such as inhibition or promotion of growth of cells where the protein of the present invention, a specific antibody thereof, a substance for promoting the function thereof or a substance for inhibiting the function thereof is used to modulate an ability of transporting a small neutral amino acid of the said protein or analogs thereof.

The present invention provides a method for changing the fate of a drug transported by the protein *in vivo* where the protein of the present invention, a specific antibody thereof, a substance for promoting the function thereof or a substance for inhibiting the function thereof is used to modulate an ability of transporting a neutral amino acid of the said protein or analogs thereof.

The present invention provides a method for changing the fate of a toxin or an exogenous substance transported by the protein *in vivo* where the protein of the present invention, a specific antibody thereof, a substance for promoting the

function thereof or a substance for inhibiting the function thereof is used to modulate an ability of transporting a neutral amino acid of the said protein or analogs thereof.

The present invention will now be further illustrated by way of the Examples although the present invention is not limited thereto.

In the following Examples, each of the operation was carried out, unless otherwise clearly mentioned, by a method described in "Molecular Cloning" (by Sambrook, J., Fritsh, E. F. and Manitis, T.; published by Cold Spring Harbor Press in 1989) or, when a commercially available reagent or kit is used, it was carried out according to the direction for use of the said commercially available product.

Examples

Example 1: Mouse and human cDNA cloning of sodium-independent small neutral amino acid transporter which transports L- and D-amino acids

(1) Isolation of cDNA of 4F2hc of mouse and human being and preparation of cRNA

A cDNA library was prepared from poly(A)⁺ RNA purified from brain of mouse or from poly(A)⁺ RNA derived from human placenta (purchased from Clontec) using a kit for the synthesis of cDNA (trade name: Superscript Choice System; manufactured by Gibco) and was integrated into a site cleaved by a restriction

enzyme EcoRI of phage vector λ ZipLox (manufactured by Gibco). A segment which corresponds to the bases of from 135th to 580th bases of 4F2hc gene of rat (Broer, et al., *Biochem. J.*, volume 312, page 863, 1995) was amplified and labeled with ^{32}P -dCTP and the resulting one was used as a probe whereby a cDNA library of brain of mouse and a cDNA library of human placenta were screened. Hybridization was carried out for one night in a solution for hybridization and a filter membrane was washed at 37°C with 0.1 $\times$ SSC/0.1% SDS. As to the solution for hybridization, there was used a buffer of pH 6.5 containing 5 $\times$ SSC, 3 $\times$ Denhard's solution, 0.2% SDS, 10% dextran sulfate, 50% formamide, 0.01% Abtiform B (trade name; Sigma) (antifoaming agent), 0.2 mg/ml salmon sperm modified DNA, 2.5 mM sodium pyrophosphate and 25 mM-MES. A cDNA moiety of λ ZipLox phage into which cDNA was integrated was integrated into a plasmid pZL1. In cDNA of human 4F2hc, a cDNA moiety of λ ZipLox phage into which cDNA was integrated was recombined to a plasmid pZL1.

The resulting clone or the clone containing cDNA of 4F2hc of mouse and human being was subjected to a base sequence determination of cDNA by a dterminator cycle sequencing method (Applied Biosystems) using a synthetic primer for determination of base sequence. As a result, the cloned cDNA was confirmed to be that of gene of 4F2hc of mouse or human being. The base sequences of the resulting 4F2hc are represented by SEQ ID NO: 3 and 6 of the Sequence Listing which will be mentioned later.

From the plasmid containing cDNA of 4F2hc of mouse and human being prepared as above, cRNA (RNA complementary to cDNA) was prepared using a T7RNA polymerase.

(2) Isolation of cDNA of mouse of sodium-independent small neutral amino acid transporter asc-1 which transports L- and D-amino acids and preparation of cRNA

A sense primer (5'-CTCTTCACATGCATCTCCAC-3') corresponding to 35-54 bp of LAT1-analogous sequence GenBankTM/EBI/DDBI accession No. N32639 obtained by retrieval of EST (expressed sequence tag) database using a base sequence of translated region of LAT1, an antisense primer (5'-GGTACACGACCACACACATC-3') corresponding to 397-416 bp thereof and an IMAGE (Integrated and Molecular Analysis of Genomes and their Expression) cDNA clone No. 267666 were used as templates whereby DNA fragment was amplified by PCR. The resulting DNA fragment was labeled with ³²P-dCTP and was used as a probe to screen a cDNA library of brain of mouse.

The cDNA library was prepared from poly(A)⁺RNA derived from brain of mouse using a kit for the synthesis of cDNA (trade name: Superscript Choice System; manufactured by Gibco) and was integrated into a site of phage vector λ ZipLox (Gibco) cleaved by a restriction enzyme EcoRI. Hybridization by a probe labeled with ³²P-dCTP was carried out for one night in a solution of hybridization at 37°C and a filter membrane was washed at 37°C with 0.1 × SSC/0.1% SDS. As to the solution for hybridization,

there was used a buffer of pH 6.5 containing $5 \times$ SSC, $3 \times$ Denhard's solution, 0.2% SDS, 10% dextran sulfate, 50% formamide, 0.01% Abtiform B (trade name; Sigma) (antifoaming agent), 0.2 mg/ml salmon sperm modified DNA, 2.5 mM sodium pyrophosphate and 25 mM-MES. A cDNA moiety of λ ZipLox phage into which cDNA was integrated was integrated into a plasmid pZL1 and was further subcloned to a plasmid pBluescript II SK⁻ (manufactured by Stratagene).

The resulting clone or the clone containing cDNA of asc-1 of mouse was subjected to a base sequence determination of cDNA by a dterminator cycle sequencing method (Applied Biosystems) using a synthetic primer for determination of base sequence.

As a result, a base sequence of asc-1 gene of mouse was obtained. Further, a base sequence of cDNA was analyzed by a conventional method to determine a translated region of cDNA and an amino acid sequence of asc-1 encoded there.

Those sequences are represented by SEQ ID NO: 1 (amino acid sequence) and 2 (base sequence) in the Sequence Listing which will be mentioned later.

The asc-1 had a homology of 45% in terms of amino acid sequence to a rat transporter LAT1 corresponding to a neutral amino acid transport system ⁻L and the homology of 65% to LAT2. Further, the asc-1 had a homology of 45% to a human transporter y⁺LAT1 corresponding to a neutral and basic amino acid transport system y⁻L and the homology of 45% to y⁺LAT2. Furthermore, the

asc-1 had a homology of 45% to a transporter xCT of mouse corresponding to cystine and an acidic amino acid transport system x⁻c and a homology of 44% in terms of amino acid sequence to a transporter BAT1 of rat corresponding to cystine and a neutral and basic amino acid transport system b⁰⁺.

Comparison of asc-1 with LAT2 of rat, LAT1 of rat, human y⁺LAT1, human y⁺LAT2 and xCT of mouse in terms of amino acid sequence is shown in Fig. 1.

As a result of analysis of an amino acid sequence of asc-1 by an SOSUI algorithm (Hirokawa, T. et al., *Bioinformatics*, volume 14, page 378 (1998)), 12 membrane-spanning domains were expected as shown by the lines in Fig. 1. There were also the sites which were believed to be tyrosine phosphorylated site in the second hydrophilic loop, protein kinase C-dependent phosphorylated site in N-terminal intracellular region, the eighth hydrophilic loop and C-terminal intracellular region and cAMP-dependent phosphorylated site in N-terminal intracellular region.

(3) Isolation of human cDNA of sodium-independent small neutral amino acid transporter asc-1 which transports L- and D-amino acids and preparation of cRNA.

Fragment cleaved by NcoI of asc-1 cDNA of mouse (corresponding to 523-1366 bp of asc-1 cDNA of mouse) was labeled with ³²P-dCTP and this was used as a probe for screening a human brain cDNA library.

The cDNA library was prepared from poly(A)⁺RNA (purchased from Clontech) derived from human brain using a kit for the synthesis of cDNA (trade name: Superscript Choice System; manufactured by Gibco) and integrated into a site of phage vector λ ZipLox (manufactured by Gibco) cleaved by a restriction enzyme EcoRI. Hybridization by a probe labeled with ³²P-dCTP was carried out at 37°C in a solution for hybridization for one night and a filter membrane was washed at 37°C with 0.1 × SSC/0.1% SDS. As to the solution for hybridization, there was used a buffer of pH 6.5 containing 5 × SSC, 3 × Denhard's solution, 0.2% SDS, 10% dextran sulfate, 50% formamide, 0.01% Abtiform B (trade name; Sigma) (antifoaming agent), 0.2 mg/ml salmon sperm modified DNA, 2.5 mM sodium pyrophosphate and 25 mM-MES. A cDNA moiety of λ ZipLox phage into which cDNA was integrated was integrated into a plasmid pZL1.

The resulting clone or the clone containing cDNA of human asc-1 was subjected to a base sequence determination of cDNA by a dterminator cycle sequencing method (Applied Biosystems) using a synthetic primer for determination of base sequence.

As a result, a base sequence of human asc-1 gene was obtained. Further, a base sequence of cDNA was analyzed by a conventional method to determine a translated region of cDNA and an amino acid sequence of asc-1 encoded there.

Those sequences are represented by SEQ ID NO: 4 (amino acid sequence) and 5 (base sequence) in the Sequence Listing

which will be mentioned later.

Comparison of the expected amino acid sequence of human asc-1 and asc-1 of rat is shown in Fig. 2.

(4) Expression of asc-1 gene in various tissues of mouse (analysis by a northern blotting)

cDNA fragment corresponding to 1-512 bases of asc-1 gene was excised by restriction enzymes EcoRI and XhoI and labeled with ^{32}P -dCTP and the resulting one is used as a probe for conducting a northern blotting as follows to RNA extracted from various tissues of mouse. Thus, 3 μg of poly(A)⁺RNA were subjected to electrophoresis with 1% agarose/formaldehyde gel and transferred to a nitrocellulose filter. This filter was subjected to hybridization for one night using a hybridization solution containing asc-1 cDNA fragment labeled with ^{32}P -dCTP. The filter was washed at 65°C with 0.1 × SSC containing 0.1% SDS.

Result of the northern blotting is shown in Fig. 3 by a picture which is a substitute for a drawing. As a result, a band was detected near 1.9 kb in brain, lung and placenta. In addition, a band was detected at about 4.4 kb in small intestine.

(5) Expression of asc-1 and 4F2hc protein in brain of mouse

Specific antibody to a synthetic oligopeptide [PSPLPITDKPLKTQC] corresponding to 517-530 amino acid residues

of asc-1 of mouse and to a synthetic oligopeptide [CEGLLLQFPFVA] (cysteine residue of C-terminal or N-terminal was introduced for a conjugation with KLH (keyhole limpet hemocyanine)) corresponding to 516-526 amino acid residue of 4F2hc of mouse was prepared according to a method of Altman, et al. (Altman, et al., *Proc. Natl. Acad. Sci. USA*, volume 81, pages 2176-2180, 1984).

Fraction of cerebral membrane of mouse was prepared according to a method of Thorens, et al. (Thorens, et al., *Cell*, volume 55, pages 281-290, 1988). A protein sample was treated at 100°C for 5 minutes in the presence (under reducing condition) or absence (under non-reducing condition) of 5% 2-mercaptoethanol, subjected to electrophoresis by SDS-polyacrylamide gel, subjected to blotting to a Hybond-P PVDV transfer membrane and treated with an anti-asc-1 antiserum (1:10,000) or an anti-4F2hc antiserum (1:10,000).

The result is shown in a picture of Fig. 4 as a substitute for a drawing. Left side of Fig. 4 is for the anti-asc-1 antibody while right side thereof is for the anti-4F2hc antibody. They were carried out under non-reducing condition (-) and reducing condition (+), respectively.

As shown in Fig. 4, in the anti-asc-1 antiserum, a band of 118 kDa observed under a non-reducing condition disappeared under a reducing condition and transferred to a band of 33 kDa. In the anti-4F2hc antiserum, a band of 118 kDa observed under

a non-reducing condition disappeared under a reducing condition and a band of 85 kDa appeared. Those results suggest that asc-1 and 4F2hc were connected by a disulfide bond to form a heterodimer.

Example 2: Characterization of sodium-independent small neutral amino acid transporter asc-1 which transports L- and D-amino acids

(1) Role of 4F2hc in transport activity of asc-1

Incorporation of alanine when asc-1 gene cRNA of mouse was solely expressed in oocytes of *Xenopus* and when asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were expressed in oocytes of *Xenopus* together was compared.

Into oocytes were injected 12 ng of asc-1 gene cRNA of mouse, 13 ng of 4F2hc gene cRNA of mouse or 12 ng asc-1 gene cRNA of mouse/13 ng of 4F2hc gene cRNA of mouse to express followed by incubating for 3 days. With regard to the oocytes in which asc-1 gene cRNA, 4F2hc gene cRNA or asc-1 gene cRNA/4F2hc gene cRNA was injected, there was carried out an experiment for incorporation of the substrate according to a method by Kanai, et al. (Kanai and Hediger, *Nature*, volume 360, pages 467-471, 1992) using alanine as a substrate as follows. Thus, the oocytes were allowed to stand for 30 minutes in a Na⁺-free uptake solution [100 mM choline chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 5 mM HEPES; pH 7.4] containing ¹⁴C-alanine (100 μM) as a substrate and the rate of the substrate

incorporated into the cells was measured by way of counting the radioactivity incorporated thereinto.

The result is shown in Fig. 5. In the oocytes where only asc-1 was expressed, incorporation of alanine was in the same level as in the case of the oocytes into which water was injected as a control while, in the oocytes where both asc-1 and 4F2hc were expressed together, a big incorporation of alanine was shown whereby it is believed that 4F2hc is necessary for asc-1 to achieve its function.

(2) Dependency of transport activity asc-1 on salt

In an experiment of incorporation of alanine by oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together, influence of the salt added to the medium was investigated.

The experiment of incorporation of alanine was carried out in accordance with the method mentioned in the above Example 2(1) using oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together. When the influence of sodium ion was checked however, a standard uptake solution (where 100 mM choline chloride was substituted with 100 mM sodium chloride) was used in place of the Na⁺-free uptake solution as the uptake solution. When the influence of chlorine ion was checked, a gluconic acid uptake solution (where 100 mM sodium chloride was substituted with 100 mM sodium gluconate) was used in place of the standard uptake solution.

The result is shown in Fig. 6. Even when choline outside the cells was changed to sodium or even when chlorine ion outside the cells was changed to gluconic acid ion, that does not affect the incorporation of alanine at all. From those, it is shown that asc-1 is a transporter which acts independently of sodium ion and chlorine ion.

(3) A Michaelis-Menten kinetic test for asc-1

A Michaelis-Menten kinetic test was carried out for a sodium-independent small neutral amino acid transporter asc-1 which transports L- and D-amino acids. By checking the changes in the rate of incorporation of alanine due to the difference in concentration of the alanine substrate, the Michaelis-Menten kinetic test of asc-1 was carried out.

An experiment for the incorporation of alanine was carried out according to the method mentioned in the above Example 2(1) using the oocytes into which both asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were injected. The result is shown in Fig. 7. The result was that the K_m value was 23.0 ± 5.1 μM (mean value $\pm$ standard error; $n = 4$).

With regard to amino acids other than alanine which were used as substrates, a Michaelis-Menten kinetic was carried out as well in the same manner whereupon K_m values and V_{max} values were calculated. The result is shown in the following Table 1. Each V_{max} value in Table 1 was shown in terms of the ratio when the V_{max} value of alanine was defined as 1.00.

Table 1

Km Values and Vmax values of Amino Acids Used as Substrates

Amino Acid	Km	Vmax ^a
	μM	
L-Alanine	23.0	(1.00)
Glycine	7.8	0.89
L-Serine	11.3	1.02
L-Threonine	19.3	0.86
L-Cysteine	23.7	0.82
L-Valine	112	1.17
L-Methionine	139	1.15
L-Isoleucine	160	1.33
L-Leucine	245	0.58
L-Histidine	368	0.79
L-Phenylalanine	464	1.09
AIB	22.7	0.81
D-Alanine	100	0.86
D-Serine	52.0	1.22
β -Alanine	281	0.92

a: The Vmax value for each amino acid was shown in terms of the ratio to the Vmax value for alanine

(4) Substrate selectivity of asc-1 (Experiment for inhibition by addition of amino acid and analogs thereof)

In an experiment of incorporation of alanine by oocytes into which both asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were injected, influence of addition of various amino acid and analogs thereof was investigated.

In an experiment of incorporation of alanine, a method according to that mentioned in the above Example 2(1) was carried out using oocytes into which both asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were injected. Here, a Na^+ -free uptake

solution was used and incorporation of ^{14}C -alanine (50 μM) was measured in the presence and absence of 5mM of various compounds (non-labeled).

The result in the presence and absence (-) of various L-amino acids or analogous compounds thereof is shown in Fig. 8. The result in the presence and absence (-) of various D-amino acids is shown in Fig. 9. The result in the presence and absence (-) of alanine or analogous compounds thereof is shown in Fig. 10.

In various neutral L-amino acids, a cis-inhibiting effect was observed. Glycine, alanine, serine, threonine and cysteine particularly strongly inhibited the incorporation of ^{14}C -alanine mediated by asc-1 (refer to Fig. 8).

Among the D-amino acids, D-alanine and D-serine strongly inhibited the incorporation of ^{14}C -alanine mediated by asc-1. D-Threonine and D-cysteine mediumly inhibited the incorporation of ^{14}C -alanine mediated by asc-1 (refer to Fig. 9).

Even in the case of the substances other than standard amino acids, β -alanine, alanine methyl ester and α -aminoisobutyric acid (α -methylalanine) also inhibited the incorporation of ^{14}C -alanine mediated by asc-1 (refer to Fig. 10). Acidic amino acids, basic amino acids, transport system L-specific inhibitor 2-amino-2-norbornane-carboxylic acid (BCH), γ -aminoisobutyric acid and N-methylamino acids

(N-methylalanine, α -aminomethylisobutyric acid and sarcosine) did not affect the incorporation of ^{14}C -alanine mediated by asc-1 (refer to Fig. 8 and Fig. 10).

(5) Substrate selectivity of asc-1 (Test for incorporation using various amino acids and analogs as substrate)

Incorporation by asc-1 was investigated using various amino acids and analogs thereof as substrates. Experiment for incorporation of various amino acids and analogs thereof was carried out according to the method mentioned in the above Example 2(1) using oocytes into which asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were injected together. With regard to the substrates however, various compounds labeled with radioactivity were used in place of ^{14}C -alanine.

Result of incorporation of the radiolabeled L-amino acids is shown in Fig. 11. Result of incorporation of the radiolabeled D-amino acids is shown in Fig. 12. Result of incorporation of the radiolabeled L-alanine or analogous compound thereof is shown in Fig. 13.

As a result, a big incorporation into the oocytes was noted when glycine (a ^{14}C compound), L-alanine (a ^{14}C compound), L-serine (a ^{14}C compound), L-threonine (a ^{14}C compound), L-cysteine (a ^{14}C compound) (for those, refer to Fig. 11), D-alanine (a ^{14}C compound), D-serine (a ^{14}C compound) (for those, refer to Fig. 12), β -alanine (a ^{14}C compound) and

α -aminoisobutyric acid (a ^{14}C compound) (for those, refer to Fig. 13) were used as substrates.

(6) pH-Dependency of transport activity of asc-1

Influence of pH was checked in an experiment for incorporation of alanine by oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together. With regard to an experiment for incorporation of alanine, the method according to that mentioned in the above Example 2(1) was carried out using oocytes into which both asc-1 gene cRNA of mouse and 4F2hc gene cRNA of mouse were injected together.

Result where the influence of pH in the experiment for incorporation of alanine was checked is shown in Fig. 14. As a result thereof, there was no significant dependency on pH in the incorporation of alanine (refer to Fig. 14).

(7) Test of release of amino acid mediated by asc-1

Release of a preloaded ^{14}C -alanine mediated by asc-1 was checked in the oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together. ^{14}C -Alanine (-3nCi) of $100\text{ }\mu\text{M}$ in an amount of 100 nl was injected into the oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together, washed with an ice-cooled Na^+ -free uptake solution containing no alanine and transferred to a Na^+ -free uptake solution to which alanine ($100\text{ }\mu\text{M}$) was added or not added at room temperature (18°C - 22°C) and the amount of ^{14}C -alanine released outside the cells was measured.

Further, ^{14}C -leucine was similarly injected into oocytes into which LAT1 gene cRNA and 4F2hc gene cRNA of rat were injected together (Kanai et al., *J. Biol. Chem.*, volume 273, page 23629, 1988), washed with an ice-cooled Na^+ -free uptake solution containing no leucine and transferred to a Na^+ -free uptake solution to which leucine (100 μM) was added or not added at room temperature (18°C-22°C) whereupon the amount of ^{14}C -leucine released outside the cells was measured.

Those results are shown in Fig. 15, Fig. 16 and Fig. 17.

Fig. 15 shows the result of checking the release of ^{14}C -alanine from the oocytes into which cRNA of asc-1 gene of mouse and cRNA of 4F2hc gene of mouse were injected and the ordinate in the drawing shows a rate (%) of the released radioactivity to the radioactivity injected into the oocytes. Left side of Fig. 15 shows the case in the absence of Na (-) while right side thereof shows that in the presence of Na (+) and, in each graph, L-Ala(-) shows the case where L-alanine was not added while L-Ala(+) shows the case where L-alanine was added.

Fig. 16 shows the result of checking the progress of release of ^{14}C -alanine with a lapse of time by oocytes into which cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene are injected. In the drawing, ○ is the case where, in the release of ^{14}C -alanine in the oocytes into which water is injected as a control instead of cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene, a

Na⁺-free uptake solution to which no alanine is added is used; ● is the case where, in the release of ¹⁴C-alanine in the oocytes into which water is injected as a control instead of cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene, a Na⁺-free uptake solution to which alanine is added is used; □ is the case where, in the release of ¹⁴C-alanine in the oocytes into which cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene are injected, a Na⁺-free uptake solution to which no alanine is added is used; and ■ is the case where, in the release of ¹⁴C-alanine in the oocytes into which cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene are injected, a Na⁺-free uptake solution to which alanine is added is used. The ordinate in the drawing shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

Fig. 17 shows the result of checking the progress of release of ¹⁴C-leucine with a lapse of time by oocytes into which cRNA of rat LAT1 gene and cRNA of rat 4F2hc gene are injected. In the drawing, ○ is the case where, in the release of ¹⁴C-leucine in the oocytes into which water is injected as a control instead of cRNA of rat LAT1 gene and cRNA of rat 4F2hc gene, a Na⁺-free uptake solution to which no leucine is added is used; ● is the case where, in the release of ¹⁴C-leucine in the oocytes into which water is injected as a control instead of cRNA of rat LAT1 gene and cRNA of rat 4F2hc gene, a Na⁺-free uptake solution to which leucine is added is used; □ is the case where, in the

release of ^{14}C -leucine in the oocytes into which cRNA of rat LAT1 gene and cRNA of rat 4F2hc gene are injected, a Na^+ -free uptake solution to which no leucine is added is used; and ■ is the case where, in the release of ^{14}C -leucine in the oocytes into which cRNA of rat LAT1 gene and cRNA of rat 4F2hc gene are injected, a Na^+ -free uptake solution to which leucine is added is used. The ordinate in the drawing shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

As a result, even when alanine was not added to the outside of the cells, a significant release of ^{14}C -alanine was observed in the case of asc-1 and such a release significantly increased by addition of alanine to the outside of the cells (refer to Fig. 15 and Fig. 16). On the other hand, in the case of LAT1 which is a complete exchange transport mediating the forced exchange, release of leucine was observed only when leucine was added to the outside of the cells (refer to Fig. 17). Accordingly, although asc-1 is mostly in an exchange transport mode, it was found to be a transporter where a transport mode of a promotion diffusion type is mixed as well.

(8) Investigation of substrate selectivity of asc-1 utilizing the release test of amino acids

In oocytes into which cRNA of asc-1 gene and cRNA of 4F2hc gene of mouse were injected together, release of preloaded ^{14}C -alanine mediated by asc-1 was investigated whereupon it was

checked whether the compound which inhibited the incorporation of ^{14}C -alanine mediated by asc-1 was a substrate of asc-1.

^{14}C -Alanine ($\sim 3\text{nCi}$) of $100\ \mu\text{M}$ in an amount of $100\ \text{nl}$ was injected into the oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together, washed with an ice-cooled Na^+ -free uptake solution containing no alanine and transferred to a Na^+ -free uptake solution to which amino acid or amino acid analog ($100\ \mu\text{M}$) was added or not added at room temperature (18°C - 22°C) and the amount of ^{14}C -alanine released outside the cells was measured.

The result is shown in Fig. 18. Black bars in Fig. 18 are the case where there were used oocytes into which cRNA of mouse asc-1 gene and cRNA of mouse 4F2hc gene were injected while white bars are the case where there were used oocytes into which water was injected instead of cRNA as a control. (-) shows the case where no amino acid was added to a Na^+ -free uptake solution. The ordinate in Fig. 18 shows the rate (%) of the released radioactivity to the radioactivity injected into the oocytes.

As a result, a high increase in the release of ^{14}C -alanine was observed in glycine, alanine, serine and threonine and a medium increase therein was observed in methionine and valine (refer to Fig. 18). Such a result coincides with the result of the test for incorporation of amino acids (refer to Fig. 11) and the test for release of amino acids was shown to be

able to be used for determination of substrate selectivity of asc-1.

The result where further investigation was conducted for D-amino acids and amino acid analogs using the said method is shown in Fig. 19 and Fig. 20. With regard to D-amino acids, D-alanine, D-serine, D-threonine and D-cysteine resulted in a significant increase in the release of ^{14}C -alanine (refer to Fig. 19). With regard to amino acid analogs, β -alanine, alanine methyl ester and α -aminoisobutyric acid (AIB) resulted in a significant increase in the release of ^{14}C -alanine (refer to Fig. 20). It was therefore found that D-threonine, D-cysteine and alanine methyl ester which have been unable to be subjected to an incorporation experiment using radiolabeled ones because of unavailability of radiolabeled compounds are now able to be substrates for asc-1. As such, when an amino acid releasing test is used, it is now possible to screen whether a compound can be a substrate for asc-1 or, in other words, whether it can be transported by asc-1 even in the case of the compound where no radiolabeled one is available for the investigation.

(9) Investigation of substrate selectivity of intracellular substrate-binding site of asc-1 utilizing the release test of amino acids

In oocytes into which cRNA of asc-1 gene and cRNA of 4F2hc gene of mouse were injected together, release of preloaded ^{14}C -amino acid mediated by asc-1 was checked whereby the

substrate selectivity of intracellular substrate-binding site of asc-1 was checked.

^{14}C -Amino acid ($\sim 3\text{nCi}$) of $100\text{ }\mu\text{M}$ in an amount of 100 nl was injected into the oocytes into which both asc-1 gene cRNA and 4F2hc gene cRNA of mouse were injected together, washed with an ice-cooled Na^+ -free uptake solution containing no alanine and transferred to a Na^+ -free uptake solution to which alanine ($100\text{ }\mu\text{M}$) was added or not added at room temperature (18°C - 22°C) and the amount of ^{14}C -amino acid released outside the cells was measured.

The result is shown in Fig. 21. Black bars in Fig. 21 show the case where alanine was added to a Na^+ -free uptake solution while bars with oblique lines show the case where alanine was not added to a Na^+ -free uptake solution. The ordinate of Fig. 21 shows the rate (%) of the release radioactivity to the radioactivity injected into oocytes.

As a result, an increase in release of ^{14}C -labeled glycine, alanine, serine, threonine and cysteine injected into the cells by extracellular alanine was observed. Therefore, it was shown that the intracellular substrate-binding site shows a substrate selectivity for receiving small neutral amino acid such as glycine, alanine, serine, threonine and cysteine as same as in the extracellular case.

(10) Confirmation of human asc-1

From a plasmid containing cDNA of human asc-1 obtained

in Example 1(3), cRNA (RNA complementary to cDNA) was prepared using a T7 RNA polymerase. Comparison was made between incorporation of ^{14}C -alanine in the case where human asc-1 gene cRNA was solely expressed in oocytes with that in the case where both human asc-1 gene cRNA and human 4F2hc gene cRNA were expressed in oocytes.

Expression was carried out by injection of 12.5 ng of human asc-1 gene cRNA, 12.5 ng of human 4F2hc gene cRNA or 12.5 ng of human asc-1 gene cRNA/12.5 ng of human 4F2hc gene cRNA into oocytes and incubation was conducted for three days. With regard to the oocytes into which human asc-1 gene cRNA, 4F2hc gene cRNA or human asc-1 gene cRNA/4F2hc gene cRNA was injected, an experiment for incorporation of substrate was carried out according to Example 2(1) using alanine as a substrate.

The result is as follows. Like in the case of asc-1 of mouse, the oocytes where only asc-1 was expressed showed incorporation of alanine in the same level as in the case of oocytes into which water was injected as a control while, in the oocytes where both asc-1 and 4F2hc were expressed together, a big incorporation of alanine was observed. Accordingly, like asc-1 of mouse, human asc-1 was also shown to achieve the function only when it was present together with 4F2hc. It is also noted that the human asc-1 shows the same property as the above-mentioned asc-1 of mouse.

Industrial Applicability

The sodium-independent small neutral amino acid transporter according to the present invention which transports L- and D-amino acids and gene thereof makes it possible to conduct an *in vitro* investigation of transport of small neutral amino acids of L- and D-forms and amino acid analogous compounds including exogenous matters at the expressed part of the said transporter and also to conduct an *in vitro* presumption of fate of those compounds *in vivo* on the basis of the above. Further, that is useful for the development of medicaments which efficiently permeate the expressed part of the said transporter and the present invention provides novel amino acid transporters. Furthermore, as a result of modulation of an ability of the said transporter for transporting the small neutral L- and D-amino acids and analogs thereof, it is useful as a method for controlling the resistance of cells to oxidative stress, a method for controlling the activity of glutamic acid receptor of an NMDA type in nervous system, a method for controlling the cell growth and a method for screening the medicament having such activities.

SEQUENCE LISTING

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TRANSPORTING L- AND D-AMINO ACIDS AND GENES THEREOF

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<140> CA 2,399,378

<141> 2001-01-09

<150> PCT/JP01/00031

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						Met Ser
						1
cag gac acc gag gtg gat atg aag gag gtg gag ctg aat gag tta gag						165
Gln Asp Thr Glu Val Asp Met Lys Glu Val Glu Leu Asn Glu Leu Glu						
5 10 15						
ccc gag aag cag ccg atg aac gcg gcg tct ggg gcg gcc atg tcc ctg						213
Pro Glu Lys Gln Pro Met Asn Ala Ala Ser Gly Ala Ala Met Ser Leu						
20 25 30						
gcg gga gcc gag aag aat ggt ctg gtg aag atc aag gtg gcg gaa gac						261
Ala Gly Ala Glu Lys Asn Gly Leu Val Lys Ile Lys Val Ala Glu Asp						

35	40	45	50	
gag gcg gag gcg gca gcc gcg gct aag ttc acg ggc ctg tcc aag gag				309
Glu Ala Glu Ala Ala Ala Ala Lys Phe Thr Gly Leu Ser Lys Glu	55	60	65	
gag ctg ctg aag gtg gca ggc agc ccc ggc tgg gta cgc acc cgc tgg				357
Glu Leu Leu Lys Val Ala Gly Ser Pro Gly Trp Val Arg Thr Arg Trp	70	75	80	
gca ctg ctg ctg ctc ttc tgg ctc ggc tgg ctc ggc atg ctt gct ggt				405
Ala Leu Leu Leu Leu Phe Trp Leu Gly Trp Leu Gly Met Leu Ala Gly	85	90	95	
gcc gtg gtc ata atc gtg cga gcg ccg cgt tgt cgc gag cta ccg gcg				453
Ala Val Val Ile Ile Val Arg Ala Pro Arg Cys Arg Glu Leu Pro Ala	100	105	110	
cag aag tgg tgg cac acg ggc gcc ctc tac cgc atc ggc gac ctt cag				501
Gln Lys Trp Trp His Thr Gly Ala Leu Tyr Arg Ile Gly Asp Leu Gln	115	120	125	130
gcc ttc cag ggc cac ggc gcg ggc aac ctg gcg ggt ctg aag ggg cgt				549
Ala Phe Gln Gly His Gly Ala Gly Asn Leu Ala Gly Leu Lys Gly Arg	135	140	145	
ctc gat tac ctg agc tct ctg aag gtg aag ggc ctt gtg ctg ggt cca				597
Leu Asp Tyr Leu Ser Ser Leu Lys Val Lys Gly Leu Val Leu Gly Pro	150	155	160	
att cac aag aac cag aag gat gat gtc gct cag act gac ttg ctg cag				645
Ile His Lys Asn Gln Lys Asp Asp Val Ala Gln Thr Asp Leu Leu Gln	165	170	175	
atc gac ccc aat ttt ggc tcc aag gaa gat ttt gac agt ctc ttg caa				693
Ile Asp Pro Asn Phe Gly Ser Lys Glu Asp Phe Asp Ser Leu Leu Gln	180	185	190	
tcg gct aaa aaa aag agc atc cgt gtc att ctg gac ctt act ccc aac				741
Ser Ala Lys Lys Lys Ser Ile Arg Val Ile Leu Asp Leu Thr Pro Asn	195	200	205	210
tac cgg ggt gag aac tcg tgg ttc tcc act cag gtt gac act gtg gcc				789
Tyr Arg Gly Glu Asn Ser Trp Phe Ser Thr Gln Val Asp Thr Val Ala	215	220	225	
acc aag gtg aag gat gct ctg gag ttt tgg ctg caa gct ggc gtg gat				837
Thr Lys Val Lys Asp Ala Leu Glu Phe Trp Leu Gln Ala Gly Val Asp	230	235	240	
ggg ttc cag gtt cgg gac ata gag aat ctg aag gat gca tcc tca ttc				885
Gly Phe Gln Val Arg Asp Ile Glu Asn Leu Lys Asp Ala Ser Ser Phe	245	250	255	
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Leu Ala Glu Trp Gln Asn Ile Thr Lys Gly Phe Ser Glu Asp Arg Leu	260	265	270	
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Leu Ile Ala Gly Thr Asn Ser Ser Asp Leu Gln Gln Ile Leu Ser Leu				

275	280	285	295	
ctc gaa tcc aac aaa gac ttg ctg ttg act agc tca tac ctg tct gat				1029
Leu Glu Ser Asn Lys Asp Leu Leu Leu Thr Ser Ser Tyr Leu Ser Asp				
	300	305	310	
tct ggt tct act ggg gag cat aca aaa tcc cta gtc aca cag tat ttg				1077
Ser Gly Ser Thr Gly Glu His Thr Lys Ser Leu Val Thr Gln Tyr Leu				
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aat gcc act ggc aat cgc tgg tgc agc tgg agt ttg tct cag gca agg				1125
Asn Ala Thr Gly Asn Arg Trp Cys Ser Trp Ser Leu Ser Gln Ala Arg				
	330	335	340	
ctc ctg act tcc ttc ttg ccg gct caa ctt ctc cga ctc tac cag ctg				1173
Leu Leu Thr Ser Phe Leu Pro Ala Gln Leu Leu Arg Leu Tyr Gln Leu				
	345	350	355	
atg ctc ttc acc ctg cca ggg acc cct gtt ttc agc tac ggg gat gag				1221
Met Leu Phe Thr Leu Pro Gly Thr Pro Val Phe Ser Tyr Gly Asp Glu				
	360	365	370	375
att ggc ctg gat gca gct gcc ctt cct gga cag cct atg gag gct cca				1269
Ile Gly Leu Asp Ala Ala Ala Leu Pro Gly Gln Pro Met Glu Ala Pro				
	380	385	390	
gtc atg ctg tgg gat gag tcc agc ttc cct gac atc cca ggg gct gta				1317
Val Met Leu Trp Asp Glu Ser Ser Phe Pro Asp Ile Pro Gly Ala Val				
	395	400	405	
agt gcc aac atg act gtg aag ggc cag agt gaa gac cct ggc tcc ctc				1365
Ser Ala Asn Met Thr Val Lys Gly Gln Ser Glu Asp Pro Gly Ser Leu				
	410	415	420	
ctt tcc ttg ttc cgg cgg ctg agt gac cag cgg agt aag gag cgc tcc				1413
Leu Ser Leu Phe Arg Arg Leu Ser Asp Gln Arg Ser Lys Glu Arg Ser				
	425	430	435	
cta ctg cat ggg gac ttc cac gcg ttc tcc gct ggg cct gga ctc ttc				1461
Leu Leu His Gly Asp Phe His Ala Phe Ser Ala Gly Pro Gly Leu Phe				
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tcc tat atc cgc cac tgg gac cag aat gag cgt ttt ctg gta gtg ctt				1509
Ser Tyr Ile Arg His Trp Asp Gln Asn Glu Arg Phe Leu Val Val Leu				
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Asn Phe Gly Asp Val Gly Leu Ser Ala Gly Leu Gln Ala Ser Asp Leu				
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cct gcc agc gcc agc ctg cca gcc aag gct gac ctc ctg ctc agc acc				1605
Pro Ala Ser Ala Ser Leu Pro Ala Lys Ala Asp Leu Leu Leu Ser Thr				
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Gln Pro Gly Arg Glu Glu Gly Ser Pro Leu Glu Leu Glu Arg Leu Lys				
	505	510	515	
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Leu Glu Pro His Glu Gly Leu Leu Leu Arg Phe Pro Tyr Ala Ala				

520	525	530	
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aaaaaaaaaa	aaaaaa		1897

CLAIMS

1. A protein comprising the amino acid sequence of SEQ ID No. 1 or SEQ ID No. 4.
2. A polynucleotide encoding the protein of claim 1.
3. A polynucleotide comprising a base sequence of SEQ ID No. 2 or SEQ ID No. 5.
4. A plasmid containing the polynucleotide of claim 3.
5. The plasmid according to claim 4, wherein the plasmid is an expression plasmid.
6. A host cell which is transformed by the plasmid of claim 4 or 5.
7. An antisense nucleic acid which modulates the expression of a polynucleotide of claim 2.
8. An antibody against the protein of claim 1.
9. A method of screening a substance which promotes or inhibits the activity of the protein of claim 1 which transports a small amino acid in a sodium-independent manner, which comprises (a) providing a substance, (b) incubating cell expressing the protein, the small amino acid and the compound, and (c) detecting the transporting activity of the protein by measuring the amount of the transported amino acid in the cell.

Application number / numéro de demande: JP01/00031

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Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10^{ème} étage)

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Figure 3

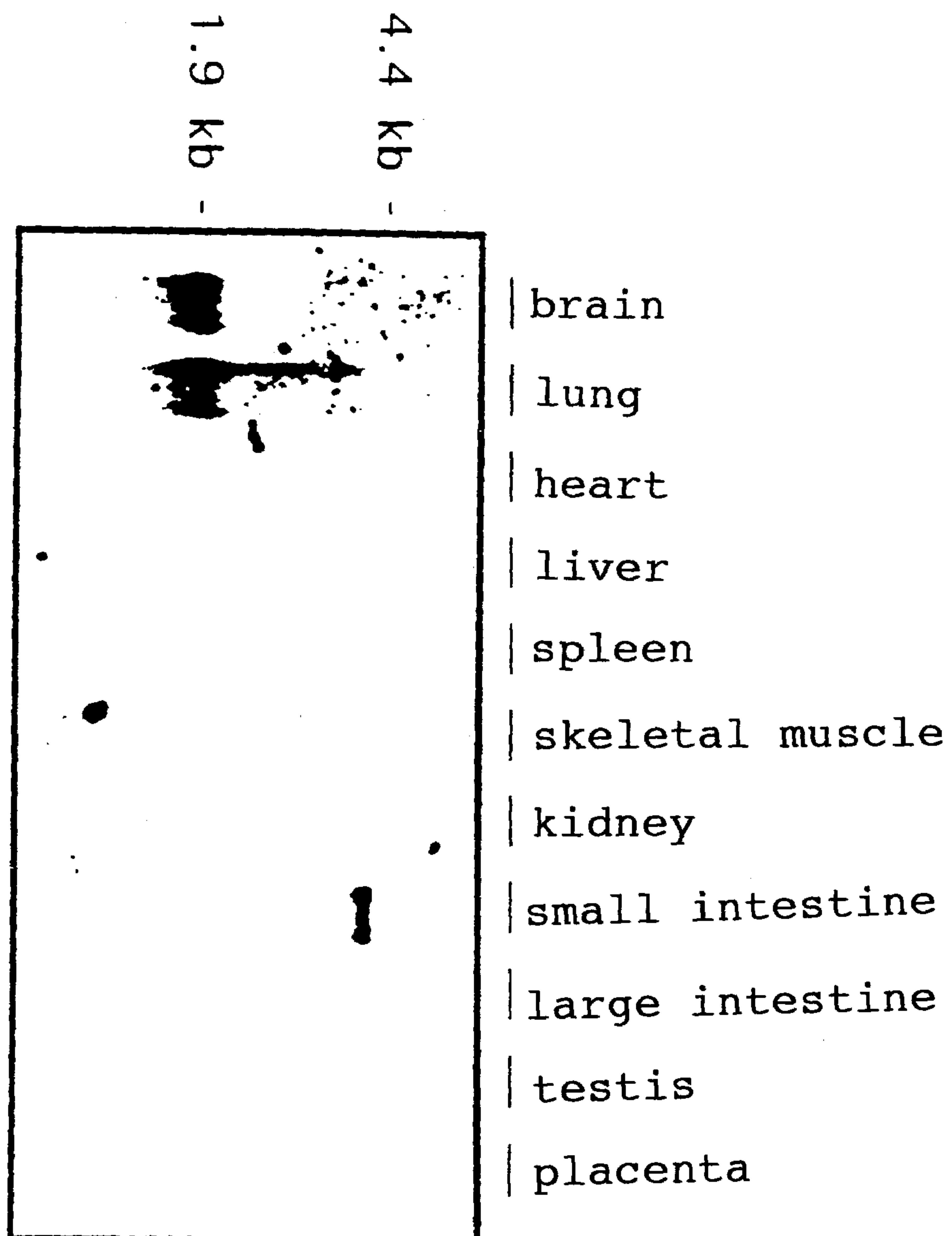


Figure 4

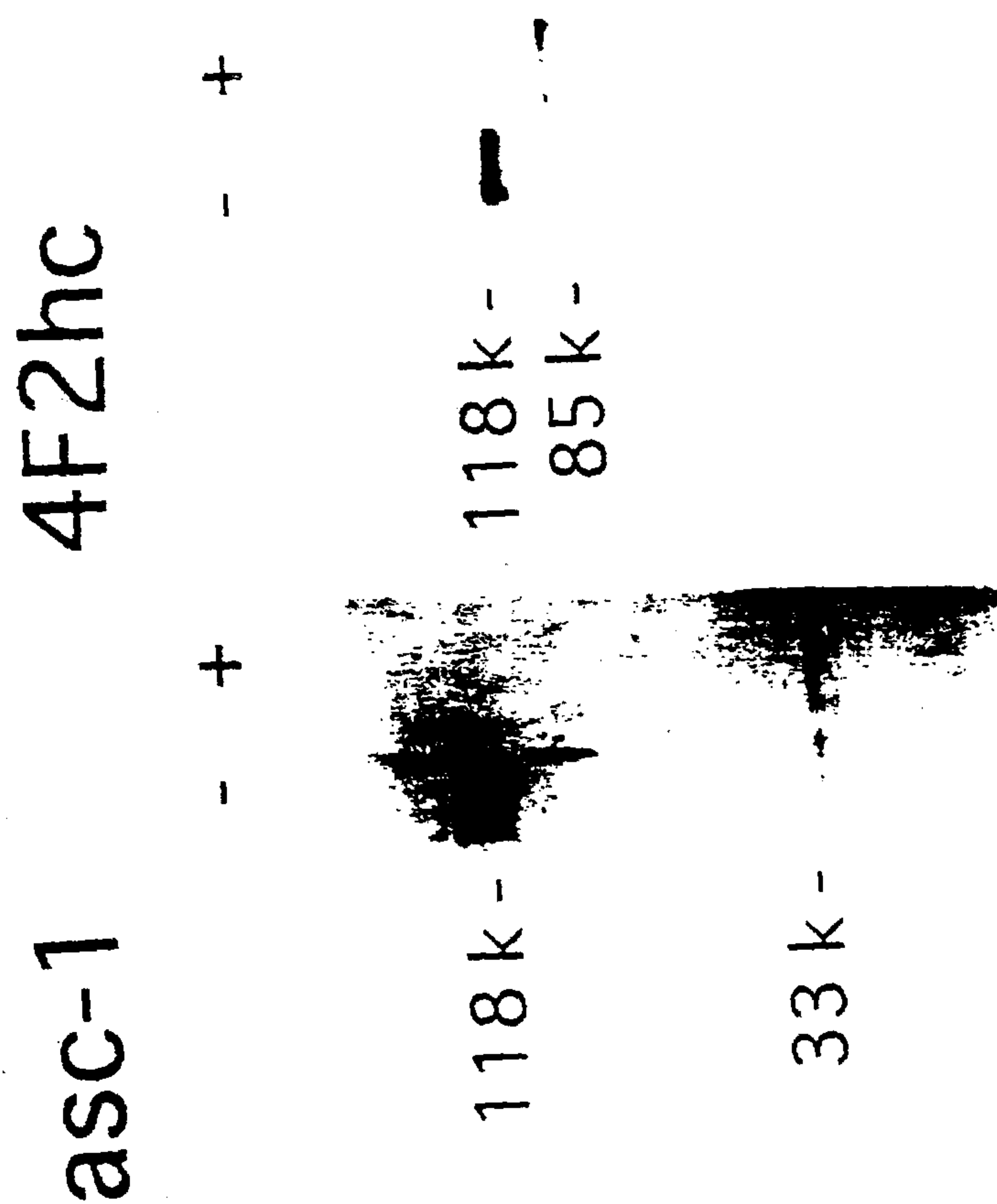


Figure 7

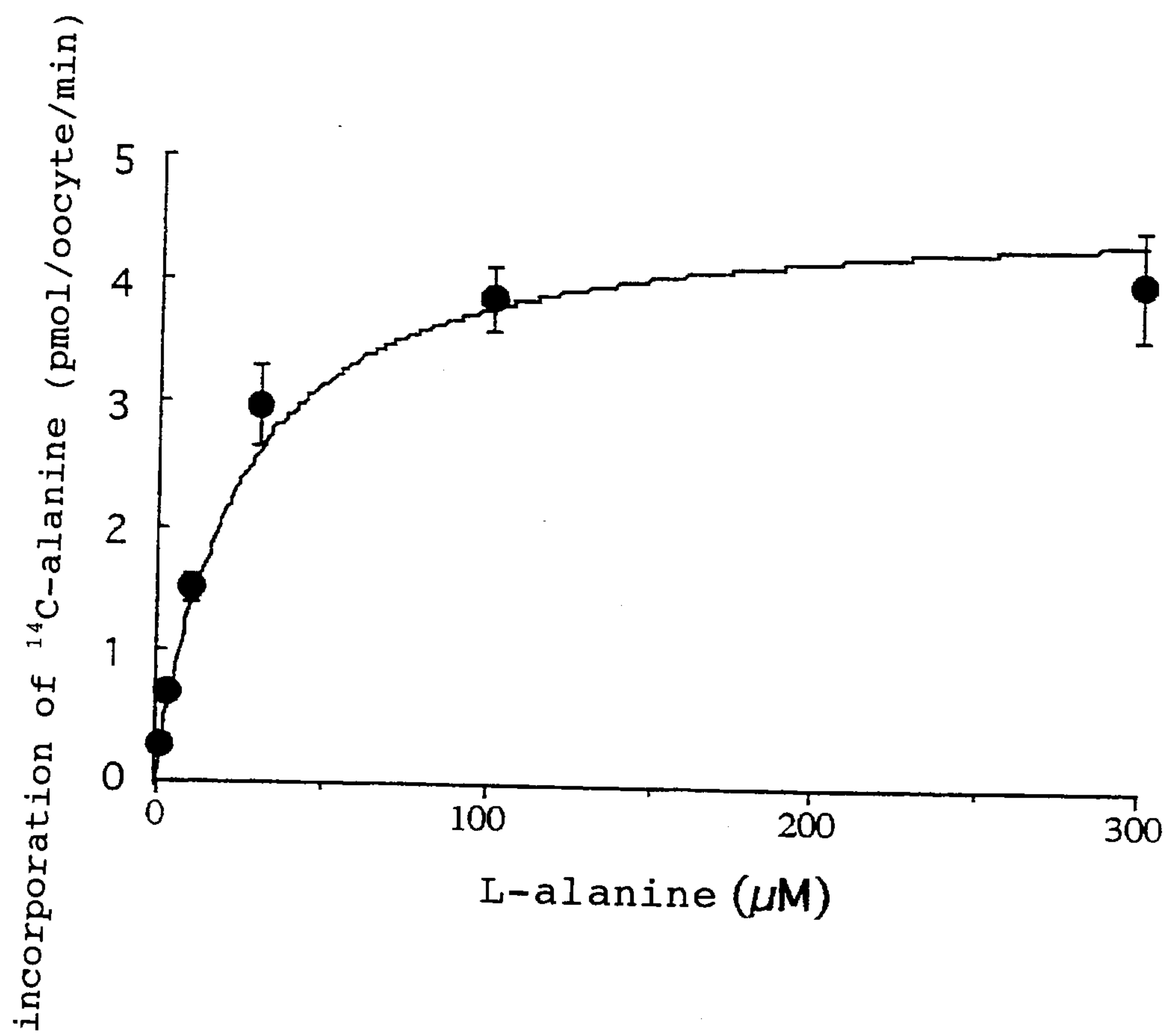


Figure 14

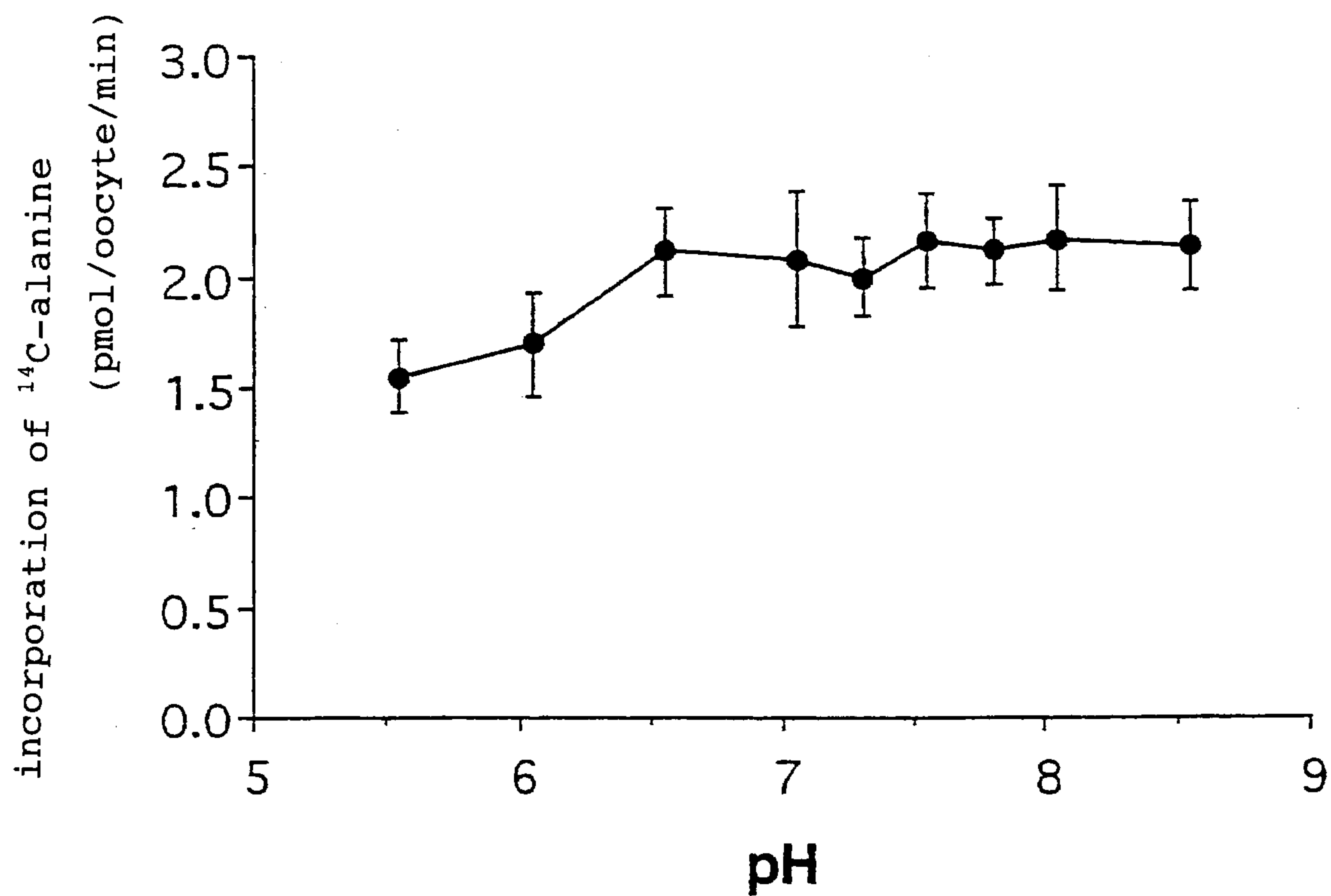


Figure 15

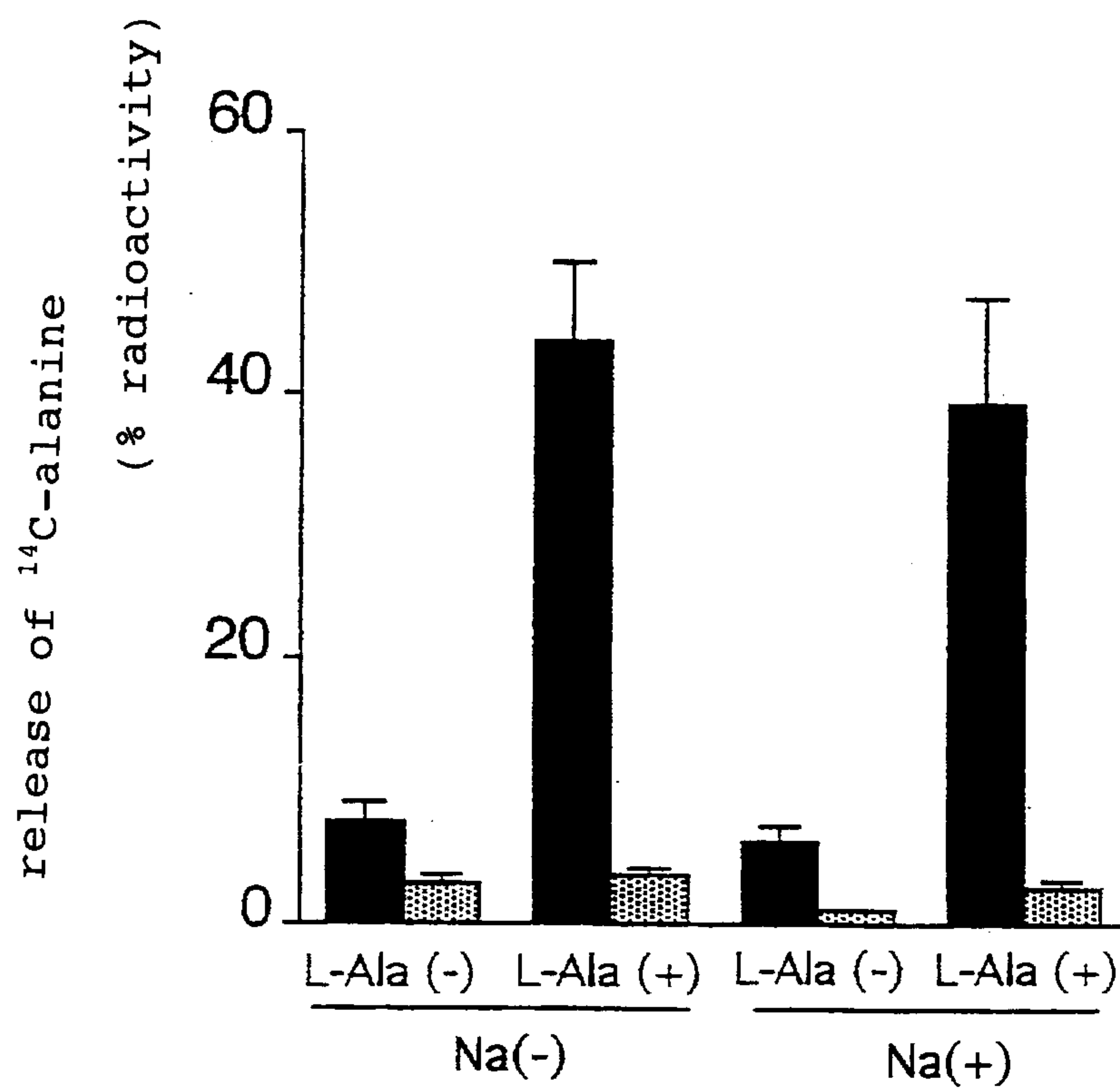


Figure 16

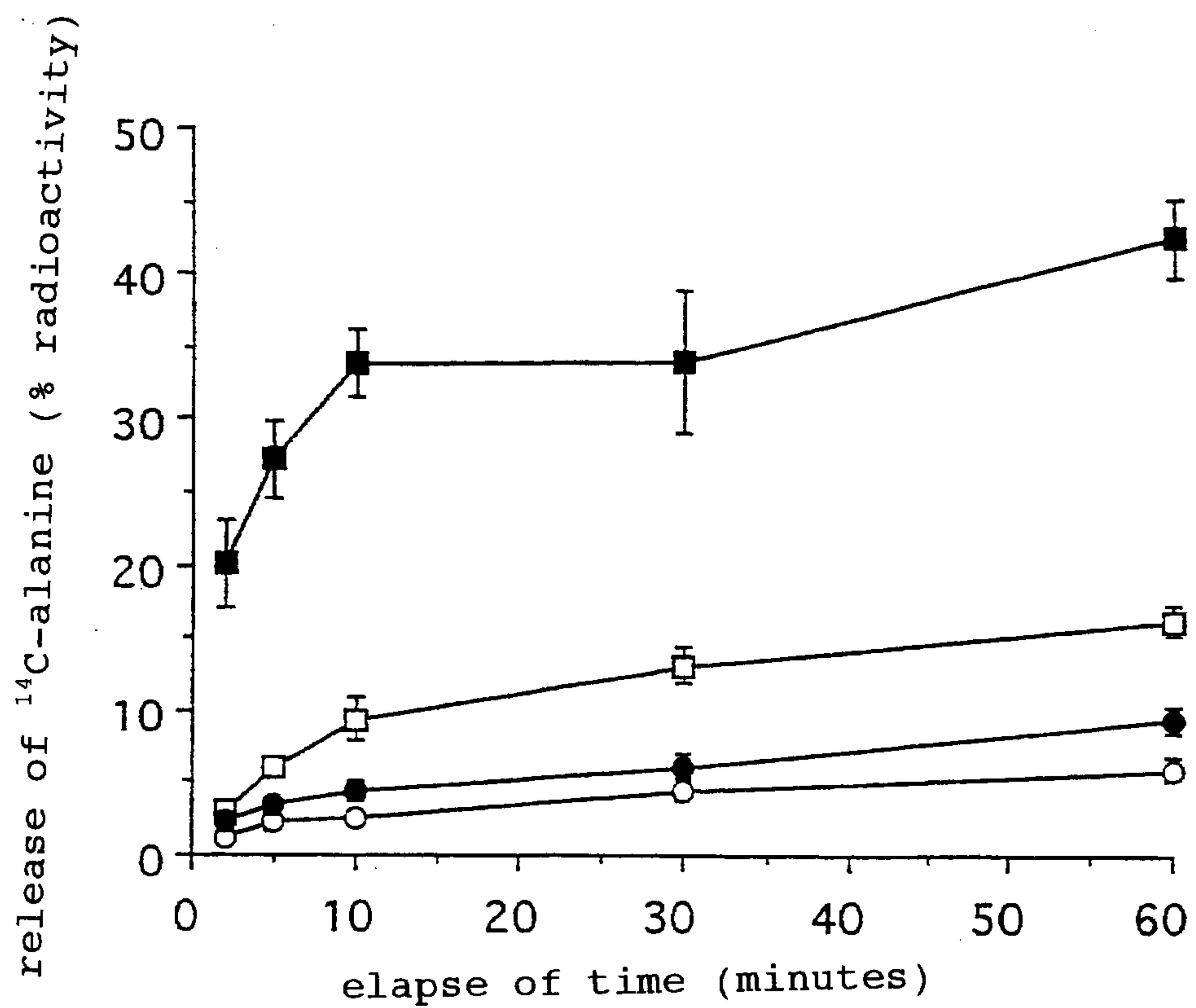


Figure 17

