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Comparison of the effects of certain thiol reagents on alanine transport in plasma membrane vesicles from rat liver and their use in identifying the alanine carrier

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The Na^+-dependent uptake of alanine into plasma membrane vesicles from rat liver was inhibited by *N*-ethylmaleimide (NEM) and by mersalyl. NEM did not inhibit alanine-independent Na^+ uptake and the inhibition of alanine transport by NEM was protected by pre-incubation with an excess of substrate. It was therefore concluded that NEM acted by binding to the alanine carrier. A protein of M_r 20000 was found to bind NEM with a concentration dependence parallel to the NEM inhibition of alanine transport. The inhibition of binding of [^{3}H]NEM to this protein by mersalyl had a concentration dependence similar to that of the inhibition of transport by mersalyl. Preincubation with L-alanine, but not with D-alanine, led to protection of the M_r 20000 protein from binding NEM. It is concluded that this protein is an essential component of the alanine transport system.

The uptake of amino acids into isolated rat hepatocytes has recently been reviewed by Kilberg (1982). The alanine carrier in the rat liver plasma membrane is of particular interest because alanine is an important substrate for gluconeogenesis in the liver. Sips *et al.* (1980*a*) concluded that transport of alanine into perifused hepatocytes is rate limiting for alanine metabolism. Furthermore, transport of alanine into isolated rat hepatocytes has been found to be stimulated by certain hormones, e.g. glucagon (Edmondson & Lumeng, 1980) and by starvation (Kilberg *et al.*, 1979; Hayes & McGivan, 1982).

Edmondson *et al.* (1977) have used artificial amino acid analogues to resolve hepatic alanine transport into uptake by systems similar to the A, ASC and L systems found in Ehrlich ascites cells. Some 60% of the total uptake of 1 mM-alanine into isolated hepatocytes occurs via the A system. Many studies have used 2-aminoisobutyrate as a non-metabolizable analogue for alanine (Le Cam & Freychet, 1977). Although transport of 2-aminoisobutyrate is not quantitatively comparable with that of alanine (Edmondson *et al.*, 1979), it is likely that many of the effects found on 2-aminoisobutyrate transport will also be found with alanine. For example, 2-aminoisobutyrate transport is also stimulated on starvation (Fehlmann *et al.*, 1979). Other hormonal effects have been reviewed by Kilberg (1982).

Abbreviations used: NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulphate.

The protein responsible for transporting alanine across the hepatocyte plasma membrane has not been identified. Nor it is certain whether the various transport systems observed in liver cells correspond to different proteins. Attempts to purify and reconstitute alanine or 2-aminobutyrate carrier proteins have so far been confined to bacteria and Ehrlich ascites cells. Hirata *et al.* (1977) observed two peaks on SDS/polyacrylamide gels of the partially purified and reconstituted alanine carrier from the thermophilic bacterium PS3. However, they did not determine whether both these peaks were essential components of the alanine carrier. Kusaka & Kanai (1978) obtained a single peak of M_r 7500 from the purified alanine carrier of *Bacillus subtilis*. Partial purification and reconstitution from Ehrlich cells of functional transport systems for both 2-aminoisobutyrate (Johnson & Johnstone, 1982) and alanine (Cecchini *et al.*, 1978) have been achieved. However, a suitable label has not been found to identify the protein responsible for transport.

NEM is known to bind covalently to thiol groups. It has also been shown to inhibit uptake of alanine into isolated hepatocytes (Kilberg *et al.*, 1980) and alanine-dependent Na^+ uptake into plasma membrane vesicles from rat liver (Sips & van Dam,

1981). NEM would therefore seem to be a possible label for the alanine carrier. In this paper, the effect of NEM and other thiol reagents on the uptake of alanine into rat liver plasma membrane vesicles is investigated. NEM is subsequently used in an attempt to identify the alanine carrier in this system.

Materials and methods

Plasma membrane vesicles were prepared from the livers of 200–300g female Wistar rats fed *ad libitum*. The method was basically the same as that of van Amelsvoort *et al.* (1978) except that the liver was homogenized in 25vol. (v/w) instead of 5vol. of homogenization buffer and was not subsequently diluted. The composition of the homogenization buffer was 0.25M-sucrose/0.2mM-$CaCl_2$/10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH (pH7.5) as used by van Amelsvoort *et al.* (1978) but sucrose gradients were those used by Samson & Fehlmann (1982).

[^{3}H]Alanine transport was assayed as described by van Amelsvoort *et al.* (1978). An aliquot of vesicles containing approx. 100μg of protein was mixed with an equal volume of homogenization medium containing 10mM-$MgCl_2$, 0.4mM-[^{3}H]alanine (50–100Ci/mol) and 120mM-NaSCN. Incubations were performed at 20°C. The transport was quenched with 1.5ml of a stopping solution of ice-cold homogenization medium containing 100mM-NaCl. The diluted suspension was then passed through a Millipore filter (HAWP, 0.45μm) at a pressure of 400mmHg and washed with 3 × 1.5ml of stopping solution. The filter was placed in 5ml of scintillant and left for 30min before counting. The same method was used to measure uptake of $^{22}Na^+$. Vesicles were mixed with homogenization buffer containing 10mM-$MgCl_2$ and 0.4mM-^{22}NaSCN and quenched with ice cold homogenization buffer containing no NaCl.

For experiments on inhibition, the vesicles were pre-incubated with inhibitor for 10–15min before assaying alanine uptake. Proteins were separated on 18% SDS/polyacrylamide gels using the method of Laemmli (1970). The gels were stained in 0.1% Coomassie Blue. For labelling experiments, vesicles were preincubated for 10–15min with 0.1mM-[^{3}H]-NEM before separating the proteins. Labelled gels were cut into 2mm or 5mm strips and digested in a mixture of $HClO_4$ and H_2O_2 (Mahin & Lofberg, 1966). Samples were placed in 15ml of scintillant and left for 30min before counting. Protein concentrations were assayed as described by Lowry *et al.* (1951). Further experimental details are to be found in the legends to the Figures and Tables.

[^{3}H]NEM was obtained from New England Nuclear, and [^{3}H]alanine and ^{22}NaCl were from The Radiochemical Centre. The scintillant used was Unisolve E (Koch–Light).

Results

Inhibition of alanine transport by thiol reagents

Fig. 1 shows the effect of NEM on the time course for the uptake of 0.2mM-alanine into rat liver plasma membrane vesicles in the presence of 60mM-NaSCN. In the absence of inhibitor, there was a rapid uptake of alanine reaching a maximum after 40s. This maximum value was consistent with values obtained in previous studies (van Amelsvoort *et al.*, 1978; Samson & Fehlmann, 1982). The transient overshoot observed under control conditions was inhibited by about 50% with 1mM-NEM and completely eliminated with 10mM-NEM. The time course with 10mM-NEM was comparable with that obtained under Na^+-free conditions, i.e. with KSCN replacing NaSCN, in the absence of NEM (results not shown). From these results it follows that NEM inhibited the Na^+-dependent uptake of alanine. The titration of alanine transport against NEM concentration (Fig. 2*a*) showed half-maximal inhibition

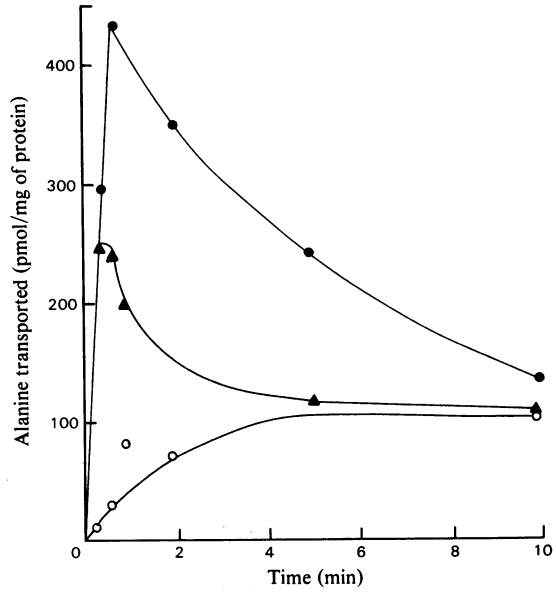

Fig. 1. *Effect of NEM on the time course of alanine uptake by plasma membrane vesicles from rat liver*
A representative experiment is shown. Transport was measured under control conditions as described in the text (●) and after pre-incubation for 10–15min with 1mM (▲) or 10mM (○) NEM. Mean alanine uptake (±S.E.M.) after 40s was 384 ± 25pmol/mg from 14 vesicle preparations. These values are representative of the control values in Figs. 2, 4 and 5.

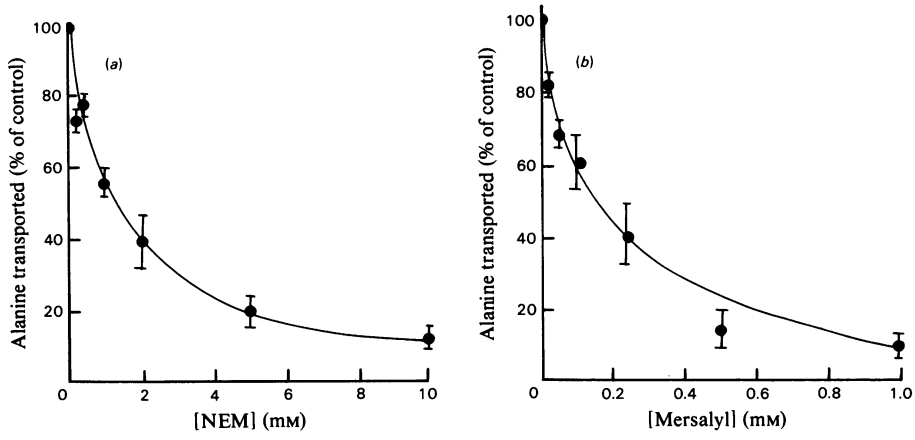

Fig. 2. *Titration of alanine uptake against (a) NEM and (b) mersalyl*
Vesicles were pre-incubated with inhibitor (NEM or sodium mersalyl) for 10–15 min and alanine uptake was measured after 40 s. Each point represents the mean (±S.E.M.) of transport from at least three vesicle preparations expressed as a percentage of transport in the absence of inhibitor.

at approximately 1 mM. Under Na^+-free conditions, alanine transport was not inhibited at concentrations of NEM up to 10 mM (results not shown).

Mersalyl inhibited alanine transport (Fig. 2*b*) with half-maximum inhibition at 0.2 mM. Iodoacetate was not an effective inhibitor of transport in the same concentration range as NEM or mersalyl. At 1 mM-iodoacetate (added as the K^+ salt) alanine transport was 90 ± 5.8% of the control and at 10 mM it was 73 ± 5.4% of the control (mean ± S.E.M. for three vesicle preparations). Sips & van Dam (1981) have previously shown that NEM and mersalyl, but not iodoacetate, were effective inhibitors of alanine-dependent Na^+ transport.

Before using NEM as a label to identify a carrier, it is important to ascertain whether the inhibitory action of NEM is by direct binding to the carrier or by some indirect action. For example, thiol reagents have been shown to affect the Na^+ permeability of certain membranes (Will & Hopfer, 1979; Biber & Hauser, 1979). To confirm the direct action of NEM on the carrier, experiments on Na^+ transport in the absence of alanine were carried out. It was shown that NEM had no effect on the uptake of $^{22}Na^+$ over the first 2 min of transport. Finally, if the inhibition of alanine transport was caused by NEM competing with alanine for a site on the carrier, it would be expected that pre-incubation of the vesicles with a large excess of substrate would protect the carrier from binding NEM. Table 1 shows that an excess of L-alanine partially protected alanine transport from inhibition by NEM. D-Alanine however exerted no protective effect. The results of Sips *et al.* (1980*b*), showing the uptake of alanine into plasma membrane vesicles to be completely stereospecific, have been confirmed in this laboratory.

Table 1. *Substrate protection of the inhibition of alanine uptake by NEM*
Vesicles were taken from the sucrose gradient interface and divided into four samples which were treated as follows: (i) untreated (control), (ii) incubated with 2 mM-NEM for 10–15 min, (iii) pre-incubated with 100 mM-L-alanine for 10–15 min and then incubated with 2 mM-NEM for a further 10–15 min, (iv) treated as (iii) but using D-alanine instead of L-alanine. Vesicles were kept on ice throughout. Cysteine (10 mM) was then added to all four tubes to remove excess NEM. The vesicles were then subjected to two washing cycles, in each of which they were spun down at 70000 *g* and resuspended in 10 ml of homogenization buffer. They were then finally spun down and resuspended to give a final concentration of 5–10 mg of protein/ml. Alanine uptake was measured after 40 s as described in the Materials and methods section. Results are given as % of transport in the control vesicles (control ± S.E.M. from five different vesicle preparations), which was 252 ± 33 pmol/mg from the five vesicle preparations. It is probable that the washing and spinning cycles used in this procedure have led to some reduction in the integrity of the vesicles.

Incubation conditions	Alanine uptake after 40 s as % of control
(i) Control (i.e. no NEM)	100
(ii) NEM inhibited (no protection)	50 ± 6
(iii) Protected with L-alanine	71 ± 5
(iv) Protected with D-alanine	49 ± 3

Use of [^{3}H]NEM to identify the carrier protein

Rat liver plasma membrane vesicles were incubated wtih [^{3}H]NEM, dissolved in detergent and the proteins were separated by polyacrylamide-gel

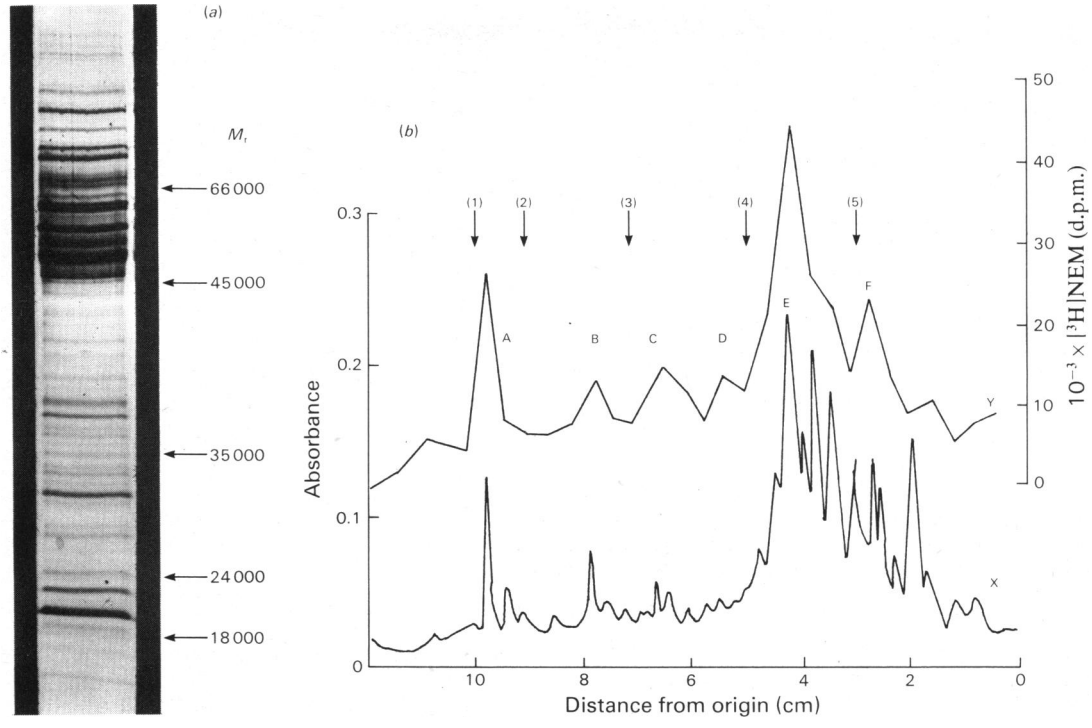

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of rat plasma membrane vesicles*

(*a*) A photograph of a representative gel is shown. The protein markers used (with M_r values in parentheses) were (1) β-lactoglobulin (18400), (2) trypsinogen (24000), (3) pepsin (34700), (4) egg albumin (45000) and (5) bovine albumin (66000). (*b*) Densitometric scan of the gel in (*a*) (X) and the binding of [^{3}H]NEM to plasma membrane vesicles (Y). Markers shown are as in (*a*).

electrophoresis. Fig. 3(*a*) shows a photograph of a representative gel. A densitometric scan of this gel together with the labelling pattern observed is shown in Fig. 3(*b*). Six major peaks (labelled A–F) were found to bind [^{3}H]NEM. This pattern of binding was consistently observed in several membrane preparations (Table 2). The most noticeable feature of the labelling pattern was a sharp, highly labelled peak of M_r approx. 20000 (peak A), which was well separated from the rest of the protein components. The major area of NEM binding was located in the M_r range 45000–70000, which corresponded to the major portion of the protein. The proteins involved in mitochondrial adenine nucleotide transport (see Klingenberg, 1979) and phosphate transport (Wohlrab, 1978; Kolbe *et al.*, 1981), and the phosphate carrier in chloroplasts (Flogge & Heldt, 1979) all exhibit M_r values in the range 20000–30000 when separated on SDS gels. For these reasons, attention was concentrated on peak A as a putative alanine-transporting protein.

In order to establish that a particular protein is involved in the NEM-sensitive transport of alanine, it is necessary to establish first that the titration of NEM binding to this protein is parallel with the inhibition of transport by NEM. Secondly it must be demonstrated that the protein can be protected from binding NEM by preincubation with the transport substrate.

Table 2. *Binding of [^{3}H]NEM to rat liver plasma membranes*

Binding of [^{3}H]NEM (added at 0.1mM) to each peak is shown as % of the binding to peak A (0.74 ± 0.1nmol/mg of total protein from 11 vesicle preparations). Peaks correspond to those in Fig. 3(*b*). Each value represents the mean (± S.E.M.) from the number of vesicle preparations shown in parentheses.

Peak	Approx. M_r	Binding of 0.1mM-[^{3}H]NEM (as % of that to peak A)
A	20000	100
B	32000	45.3 ± 4.4 (11)
C	38000	51.7 ± 5.2 (10)
D	43000	61.8 ± 6.4 (8)
E	55000	222 ± 31 (7)
F	70000	108 ± 14 (7)

Fig. 4(*a*) shows that the binding of NEM to peak A was half-maximal at 1mM-NEM, in agreement with the inhibition of transport by this compound.

When NEM binding to peak A was plotted against alanine transport at the same concentration of NEM, a linear plot was obtained (Fig. 4*b*), indicating that the inhibition of transport was parallel with the binding of the inhibitor to this protein. Further, pre-incubation of the vesicles with mersalyl reduced the subsequent binding of [^{3}H]NEM binding to peak A (Fig. 5*a*). The effect of mersalyl in inhibiting NEM binding was paralleled by the effect of mersalyl in inhibiting alanine transport (Fig. 5*b*). Preincubation with 10mM-iodoacetate reduced the binding of [^{3}H]NEM to peak A by less than 30%

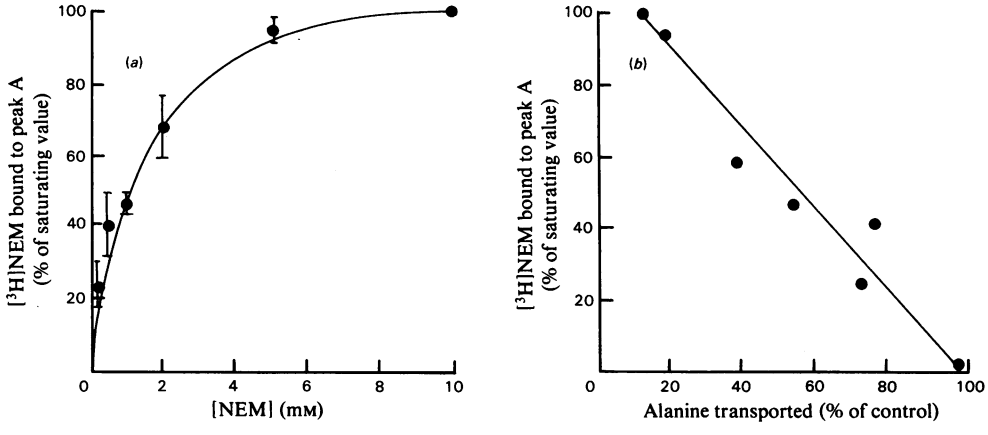

Fig. 4. *Concentration-dependence of binding of NEM to peak A*
Vesicles were incubated with different concentrations of [^{3}H]NEM for 10–15 min and the proteins were separated by SDS/polyacrylamide-gel electrophoresis. Peak A was cut out and digested for counting. Each point in (*a*) represents the mean (±S.E.M.) of experiments from three vesicle preparations as a percentage of saturation binding (2.42 ± 0.2 nmol of NEM/mg of total protein). The percentage of maximal NEM binding at each concentration was plotted against the percentage of control alanine transport after 40 s in the presence of the same concentration of NEM (*b*).

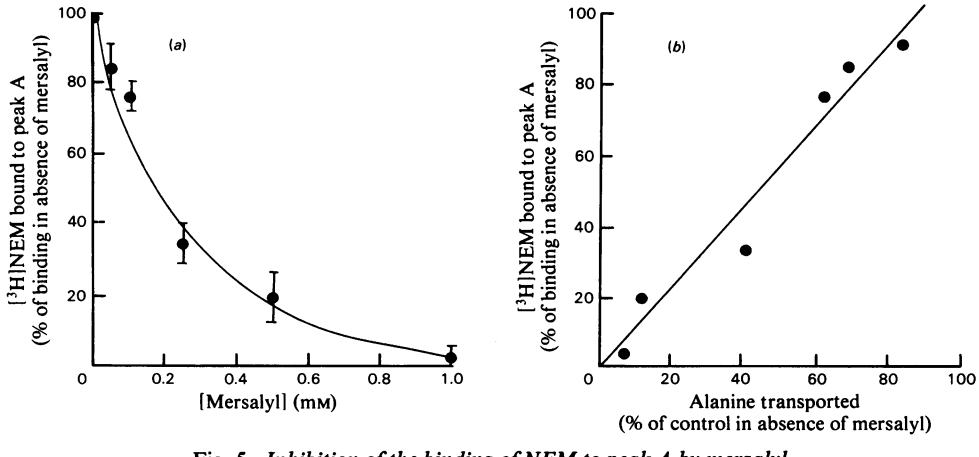

Fig. 5. *Inhibition of the binding of NEM to peak A by mersalyl*
Vesicles were pre-incubated with different concentrations of sodium mersalyl for 10–15 min and then incubated with 0.1 mM-[^{3}H]NEM for a further 10–15 min. Each point in (*a*) represents the mean (±S.E.M.) of experiments from three vesicle preparations as a percentage of binding in the absence of mersalyl (0.74 ± 0.1 nmol of NEM/mg of total protein from 11 vesicle preparations). In (*b*) the percentage of control NEM binding at each mersalyl concentration was plotted against the percentage of control alanine transport after 40 s after pre-incubation with the same concentration of mersalyl.

(results not shown) and this is consistent with the inhibition of transport by iodoacetate described earlier.

In experiments similar to that in Table 1, vesicles were pre-incubated with L-alanine or D-alanine before incubating with 1 mM unlabelled NEM. After removing the excess alanine and NEM the vesicles were incubated with [^{3}H]NEM and the proteins separated by gel electrophoresis. The result of a representative experiment is shown in Fig. 6. There was a clear protection of peak A by L-alanine as opposed to D-alanine. In eight separate vesicle preparations, the radioactivity associated with peak A was 3.0 ± 0.43 times higher in the vesicles preincubated with L-alanine than in those pre-incubated with D-alanine. In no case was protection of peak B observed under these conditions. The results of this particular experiment indicate an apparent protection of peak C and peak F. However, the labelling in the region of peak C was variable. Assessment of the apparent protection in the region of peak F presented technical difficulties due to the poor separation of protein bands in this region. These results are taken to establish that peak A is a protein involved in NEM-sensitive alanine transport in plasma membrane vesicles. They are not however adequate to eliminate the possibility of the involvement of other proteins in this process.

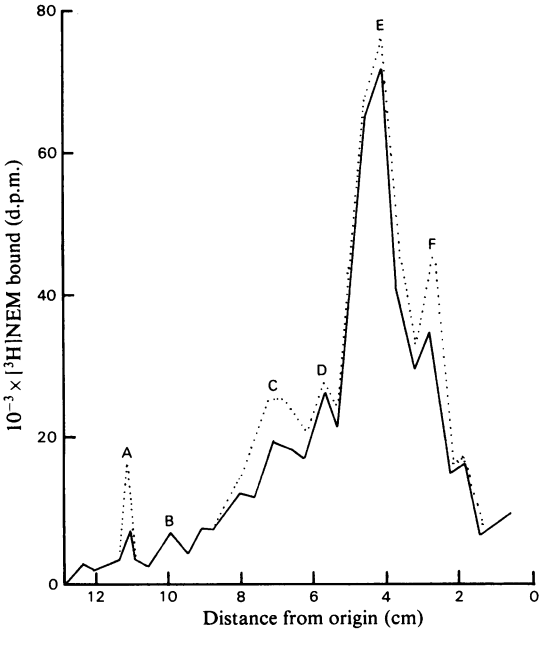

Fig. 6. *Protection of peak A by* L-*alanine*
Vesicles were treated with L-alanine (·····) or D-alanine (——) as described in the legend to Table 1 except that incubation was with 1 mM-NEM for 2 min. Vesicles were then incubated with 0.1 mM-[^{3}H]NEM for 10 min before electrophoresis. Where only one line is shown, the points were too close to resolve.

The saturating level of NEM binding to peak A (2.42 ± 0.2 nmol/mg of protein) together with the M_r of this protein (taken as 20000) were used to calculate the maximum proportion of the peak A protein in the vesicle preparation. On the assumption that 1 mol of protein binds 1 mol of NEM, a value for peak A of 4.8% of the total protein was obtained. The area of peak A as a proportion of the total area of the densitometric scans of five gels each of a different vesicle preparation was found to be 5.15 ± 0.73%.

Discussion

The results in this paper show that alanine transport in liver plasma membrane vesicles is inhibited by NEM and mersalyl. The lack of effect of NEM on non-specific Na^+ permeability in these vesicles, together with the protection of transport observed by pre-incubation of the vesicles with L-alanine (but not with D-alanine) before adding NEM shows that NEM inhibits alanine transport by binding to the transport protein itself. The particular thiol group involved in alanine transport appears to have a greater accessibility to NEM and mersalyl than to iodoacetate.

NEM has been used previously to identify the proteins involved in mitochondrial phosphate transport (Wohlrab, 1978; Kolbe *et al.*, 1981), phosphate transport in chloroplasts (Flogge & Heldt, 1979) and D-glucose transport in kidney brush border membranes (Poiree *et al.*, 1979). Since NEM is a non-specific inhibitor, identification of the carrier protein in each case has required the demonstration of the protection of a particular protein from binding NEM in the presence of the transport substrate. In this paper, using a similar approach, a protein (peak A) of M_r 20000 which is necessary for alanine transport in liver membranes has been identified. The peak A protein comprises approx. 5% of the membrane protein as measured from the densitometric scans of SDS/polyacrylamide gels. A similar value is calculated from the measurements of the saturating binding of NEM on the assumption that 1 mol of protein binds 1 mol of NEM. The present results do not show whether this protein is sufficient for alanine transport and it is possible that it is a subunit or a proteolytic fragment of the original carrier molecule. The band on the gel could also represent more than one protein with similar M_r values, only one of which may be the alanine carrier. Resolution of these uncertainties will require purification of the protein and its reconstitution with phospholipid to form artificial vesicles capable of

transporting alanine. Nevertheless, the close correspondence between the membrane content of peak A calculated by two different methods suggests that this band may represent a relatively homogeneous fraction comprised mostly of the carrier protein containing one reactive thiol group per monomer.

A systematic study of marker enzymes in plasma membrane vesicles prepared by the method used in this paper has been carried out by other workers (van Amelsvoort *et al.*, 1978; Samson & Fehlmann, 1982). Sips *et al.* (1982) have concluded on the basis of such studies that this vesicle preparation consists of 38–44% plasma membrane and 56–62% endoplasmic reticulum on a protein basis. The peak A protein may therefore constitute as much as 11–13% of the plasma membrane fraction. This would be consistent with the major metabolic role of this carrier. It is of interest that the adenine nucleotide carrier constitutes 12% of the total protein of heart mitochondria (see Klingenberg, 1979), the phosphate carrier makes up 20% of the chloroplast envelope protein (Flogge & Heldt, 1979) and the recently isolated uncoupling protein (Lin & Klingenberg, 1982) represents 14% of the inner membrane in brown adipose tissue mitochondria.

Use of NEM to label other amino acid transporting systems in liver may prove difficult, as NEM does not inhibit transport of amino acids entering through other systems to such a great extent as it does alanine transport (Kilberg *et al.*, 1980). The glutamine carrier (system N) seems the most likely possibility. If this could be labelled as a protein distinct from the alanine carrier, it would be interesting to compare the proteins in hepatocytes with those in, e.g., intestinal epithelial cells, which do not have separate systems for transporting alanine and glutamine (Bradford & McGivan, 1982).

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