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ISOLATION OF *N*-ETHYLMALEIMIDE-LABELLED PHLORIZIN-SENSITIVE D-GLUCOSE BINDING PROTEIN OF BRUSH BORDER MEMBRANE FROM RAT KIDNEY CORTEX

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SUMMARY

A glucose receptor with high affinity for phlorizin from isolated brush border of rat kidney was labelled specifically with *N*-[^{14}C]ethylmaleimide and then extracted from the membranes.

After the solubilization of the brush borders with sodium dodecyl sulphate the *N*-[^{14}C]ethylmaleimide-labelled receptor protein was isolated and was found to have a molecular weight of approximately 30000 as determined by sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis. The receptor protein eluted from the sodium dodecyl sulphate-containing gels migrates as a single band on sodium dodecyl sulphate-free polyacrylamide gels.

The receptor protein can also be released from the brush borders with low concentrations of sodium deoxycholate. Under these conditions the molecular weight of the *N*-[^{14}C]ethylmaleimide–labelled receptor protein is approximately 60000 in contrast to the protein component solubilized with sodium dodecyl sulphate. Since this detergent is known to dissociate the brush border membrane into its protein components, our results suggest that the phlorizin-sensitive glucose receptor protein has a molecular weight of about 30000.

INTRODUCTION

The identification and isolation of substrate receptors which act as membrane carriers was initiated with the purification of membrane protein components from bacteria[1,2] and yeast cells[3] and with the isolation of proteins from mammalian plasma membranes and membranes from cell organelles[4–7].

Recently we isolated membrane proteins from purified brush borders of rat kidney cortex which contained the phlorizin-sensitive glucose receptor[8]. The brush border membranes were dissociated with detergents into protein components of different sizes and separated with polyacrylamide gel disc electrophoresis. The protein band containing the receptor protein was identified by determination of the glucose-sensitive phlorizin binding to the separated proteins. The identification of the receptor protein is based on the maintainance of its functional behaviour during the isolation procedure. In order to purify the receptor protein from associated membrane proteins and to determine its molecular weight, solubilization of the brush

border membranes must be achieved. This can only be done with sodium dodecyl sulphate[9,10]. During this procedure the receptor protein denatures and loses its binding behaviour (Thomas, L., unpublished results). Therefore the phlorizin-sensitive glucose receptor must be labelled irreversibly before the brush border membranes are solubilized.

Previous reports from this laboratory have described the irreversible labelling of the phlorizin-sensitive glucose receptor with radioactive *N*-ethylmaleimide[11].

This report describes the isolation of the radioactive *N*-ethylmaleimide-labelled phlorizin-sensitive glucose receptor from the brush border membranes and the determination of its molecular weight. The receptor sites were labelled with *N*-[^{14}C]ethylmaleimide; the other *N*-ethylmaleimide-sensitive sites on the membrane either with *N*-[^{3}H]ethylmaleimide or *N*-[^{14}C]ethylmaleimide. The membrane was then solubilized with sodium dodecyl sulphate and the proteins separated on polyacrylamide gel disc electrophoresis. The observed increase in the amount of *N*-[^{14}C]-ethylmaleimide relative to the amount of *N*-[^{3}H]ethylmaleimide in a certain protein band indicated that this band contained the receptor protein.

MATERIALS AND METHODS

Isolation of brush borders

The isolation of rat kidney cortex brush borders was achieved by differential centrifugation[12]. The enrichment of the membranes was monitored by the determination of marker enzymes such as alkaline phosphatase, aminopeptidase and glucose-6-phosphatase. The specific activity of the alkaline phosphatase was 8 times greater and that of the aminopeptidase 2.5 times higher than in the starting material. The activity of the glucose-6-phosphatase was reduced.

Labelling of the phlorizin-sensitive glucose receptor with N-[^{14}C]ethylmaleimide

The labelling of the phlorizin-sensitive glucose receptor with *N*-[^{14}C]ethylmaleimide was achieved as previously described[11] with the exception that triethanolamine–HCL–EDTA buffer (10 mM triethanolamine–HCl, 5 μM ethylenediaminetetraacetate (disodium salt), 150 mM NaCl, pH 7.6 at 20 °C) was used.

At first non-radioactive *N*-ethylmaleimide was attached to the brush borders in the presence of phlorizin. Phlorizin at a concentration of 5 μM initially protects its specific binding site while other *N*-ethylmaleimide-sensitive groups (unspecific sites) are attacked with 0.5 mM *N*-ethylmaleimide. Subsequently the membranes were washed to remove the bound phlorizin and excess *N*-ethylmaleimide. Then the membrane fraction was divided into two aliquots. One was exposed to 0.5 mM *N*-[^{3}H]ethylmaleimide with a specific activity of 22 Ci/mole *N*-ethylmaleimide in the presence of 5 μM phlorizin. The other was exposed to 0.5 mM *N*-[^{14}C]ethylmaleimide with a specific activity of 9.8 Ci/mole ethylmaleimide in the absence of phlorizin. Excess phlorizin and *N*-ethylmaleimide were removed by washing and centrifugation and the ^{14}C and ^{3}H radioactivity in the pellets determined by liquid scintillation counting. Under these conditions 1 cpm of either ^{14}C or ^{3}H represents $3.2 \cdot 10^{-14}$ moles of *N*-ethylmaleimide attached to the membranes. Then both pellets were combined. In the combined fraction the phlorizin-sensitive glucose receptor was labelled with *N*-[^{14}C]ethylmaleimide and the unspecific sites were labelled with equal amounts of either *N*-[^{3}H]ethylmaleimide or *N*-[^{14}C]ethylmaleimide.

Papain digestion of labelled brush border fractions

The combined brush border fractions were digested with papain (18 units/27 mg membrane protein) for 10 min at 37 °C and then centrifuged at 35000 × *g* for 20 min. The sediment containing the microvillus matrix was resuspended in Tris–borate buffer (0.04 M Tris–boric acid buffer, pH 8.6, at 20 °C).

Dissociation of the microvillus matrix

Microvillus matrix preparations were dissolved in 0.5% sodium dodecyl sulfate (5.0 mg/mg membrane protein) or dissociated with 0.05% sodium deoxycholate (0.5 mg/mg membrane protein) for 30 min at 4 °C in Tris–borate buffer, pH 8.6. The solubilized portion of the deoxycholate dissociated microvillus matrix was separated from the non-solubilized portion by centrifugation at 35000 × *g* for 20 min.

Separation of microvillus matrix with polyacrylamide gel disc electrophoresis

In order to separate the phlorizin-sensitive glucose receptor from other proteins from the microvillus matrix, samples of the solubilized matrix protein were run on polyacrylamide gels in the presence and absence of sodium dodecyl sulphate. Separations in the presence of sodium dodecyl sulphate were done on 15% acrylamide gels (0.2% *N,N'*-methylenebisacrylamide) at pH 8.9 using the discontinuous buffer system of Davis[13]. The gels and the buffer in the upper reservoir contained 0.1% sodium dodecyl sulphate. Electrophoresis was carried out at 4 mA per gel and at room temperature.

Separations in the absence of sodium dodecyl sulphate were carried out on 15% acrylamide gels using the multiphasic borate–sulphate buffer system of Jovin *et al.*[14] with a running pH of 8.6. Electrophoresis was carried out at 4 mA per gel at 4 °C. Every electrophoretic separation was done in triplicate, one gel was stained with 0.125% Coomassie blue (w/v) in 12% trichloroacetic acid (w/v), 50% methanol (v/v) and the other two were used for radioactive determinations. In experiments where the separated proteins were recovered from the gel, slices containing the proteins were dispersed with a gel plunger into small beads. The proteins were then eluted from the gel beads with Tris–borate buffer for 12 h.

Molecular weight determinations were obtained using the sodium dodecyl sulphate–acrylamide gel system described above. There is a linear relationship between migration rate on 15% acrylamide gels and the logarithm of the molecular weight of standard proteins between 25000 and 70000 (Fig. 1). The following standard proteins of known molecular weight, *i.e.* chymotrypsinogen A 25000, ovalbumin 45000 and bovine serum albumin 67000 were used. All proteins were equilibrated with 0.1% sodium dodecyl sulphate for 10 min before they were subjected to electrophoresis.

Detection of radioactivity in polyacrylamide gels

The polyacrylamide gels which were 6 cm long were cut into 2-mm slices. The wet slices were packed in filter paper and combusted in a Packard Tri-Carb Sample Oxidizer, Model 305. The separated ^{3}H and ^{14}C were counted in a Packard Tri-Carb liquid Scintillation Counter Model 3380 using Instagel as scintillation fluid. Utilizing this method the background counts of radioactive-free combusted gel slices were

30 cpm for ^{3}H and 40 cpm for ^{14}C and were low enough to determine small amounts of radioactivity in the samples. Each sample was counted to a minimum of 2000 counts above background.

Materials

Phlorizin obtained from Roth Chemicals Karlsruhe (Germany) was repurified by thin-layer chromatography with the nonpolar silica gel system described by Diedrich[15]. *N*-Ethylmaleimide was purchased from Sigma Chemicals Company, *N*-[^{14}C]ethylmaleimide (spec. act. 9.8 Ci/mole) and *N*-[^{3}H]ethylmaleimide (spec. act. 74.8 Ci/mole) from NEN Chemicals Boston. The standard proteins were obtained from Serva Chemicals, Heidelberg (Germany).

RESULTS

Labelling of the phlorizin-sensitive glucose receptor with N-[^{14}C]ethylmaleimide

Brush border fractions labelled with *N*-[^{3}H]ethylmaleimide in the presence of phlorizin and fractions labelled with *N*-[^{14}C]ethylmaleimide in the absence of phlorizin, as described under methods, bind $27.3 \cdot 10^{-10} \pm 2.8$ moles *N*-[^{14}C]ethylmaleimide and $24.8 \cdot 10^{-10} \pm 3.5$ moles *N*-[^{3}H]ethylmaleimide per mg brush border protein (means of 12 experiments ±S.D.). This difference is statistically significant at a $P<0.05$. If equal amounts of a ^{3}H-labelled fraction and a ^{14}C-labelled fraction were combined, the phlorizin-sensitive glucose receptor sites were labelled with $2.5 \cdot 10^{-10}$ moles *N*-[^{14}C]ethylmaleimide per mg protein while the unspecific sites were labelled either with $24.8 \cdot 10^{-10}$ moles *N*-[^{3}H]ethylmaleimide or $24.8 \cdot 10^{-10}$ moles *N*-[^{14}C]ethylmaleimide per mg membrane protein. The *N*-[^{14}C]ethylmaleimide label on the phlorizin-sensitive glucose receptors represents 9±2% of the total *N*-[^{14}C]ethylmaleimide bound to the membrane fraction (mean of 12 experiments ±S.D.).

Treatment of the N-ethylmaleimide-labelled brush border membranes with papain

If a brush border fraction in which the phlorizin sensitive glucose receptors were labelled with *N*-[^{14}C]ethylmaleimide and the unspecific sites either with *N*-[^{3}H]-ethylmaleimide or *N*-[^{14}C]ethylmaleimide was digested with papain, 21±6% of the protein, 33±6% of the *N*-[^{3}H]ethylmaleimide and 31±8% of the membrane-bound *N*-[^{14}C]ethylmaleimide were removed (means of 8 experiments ±S.D.). The sedimented microvillus matrix after papain digestion contained more *N*-[^{14}C]ethylmaleimide per mg protein than the original brush border fraction. The *N*-[^{14}C]ethylmaleimide-labelled to the phlorizin-sensitive glucose receptors now represented 13±3% of the total membrane-bound *N*-[^{14}C]ethylmaleimide (mean of 8 experiments ±S.D.). Therefore only the microvillus matrix was utilized for further purification experiments.

Separation of the phlorizin-sensitive glucose receptor from sodium dodecyl sulphate-solubilized microvillus matrix by sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis

The microvillus matrix fraction in which the receptor was labelled with *N*-[^{14}C]ethylmaleimide and the unspecific sites either to equal amounts with *N*-[^{3}H]-

ethylmaleimide or with *N*-[^{14}C]ethylmaleimide was solubilized with sodium dodecyl sulphate and samples containing 20–60 μg of protein were layered on top of the gels. The amounts of *N*-[^{14}C]ethylmaleimide and *N*-[^{3}H]ethylmaleimide bound in $1 \cdot 10^{-14}$ moles to the separated proteins in the gels are shown in comparison to the Coomassie stained protein pattern in Fig. 2. Further the difference in moles *N*-[^{14}C]ethylmaleimide *minus* moles *N*-[^{3}H]ethylmaleimide of each gel slice is shown in the lower graph. An excess of *N*-[^{14}C]ethylmaleimide relative to *N*-[^{3}H]ethylmaleimide in a gel slice indicates the localization of *N*-[^{14}C]ethylmaleimide-labelled receptor protein. The amounts of *N*-ethylmaleimide bound to the protein bands were calculated from the ^{14}C and ^{3}H radioactivities in the gel slices under the condition that either 1 cpm ^{3}H or 1 cpm ^{14}C is equivalent to $3.2 \cdot 10^{-14}$ moles *N*-ethylmaleimide. The total radioactivity attached to 60 μg of protein separated on the gel shown in Fig. 2 was $1.7 \cdot 10^{-10}$ moles *N*-[^{14}C]ethylmaleimide and $1.5 \cdot 10^{-10}$ moles *N*-[^{3}H]ethylmaleimide. The excess *N*-[^{14}C]ethylmaleimide localized in slices 21 and 22 represents 14.5% of the *N*-[^{14}C]ethylmaleimide of the sample layered on top of the gel. Since in this sample 13% of the ^{14}C radioactivity was attached to the receptor sites these data suggest that all the glucose receptors are localized in slices 21 and 22. As can be seen from the *N*-[^{3}H]ethylmaleimide labelling, proteins with unspecific sites are also present in these same slices. The peak of the excess *N*-[^{14}C]ethylmaleimide corresponds to a strongly stained protein band.

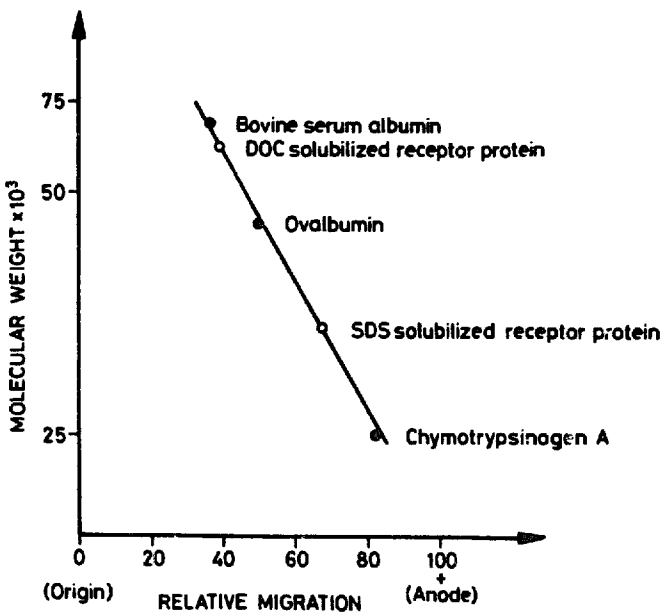

Fig. 1. Semilogarithmic relationship between molecular weight of the reference proteins and their relative migration rates in 15% acrylamide gels. Each point is the mean of 5 experiments. The apparent molecular weights of the deoxycholate(DOC)-solubilized and sodium dodecyl sulphate (SDS)solubilized receptor protein are marked with the circles.

As can be seen in Fig. 1 the migration rate of these proteins containing the excess *N*-[^{14}C]ethylmaleimide relative to the migration rate of the standard proteins indicates an apparent molecular weight for the receptor protein of 33000 ± 5000 (mean of 6 experiments $\pm$S.D.).

In order to determine whether the *N*-[^{14}C]ethylmaleimide-labelled receptor

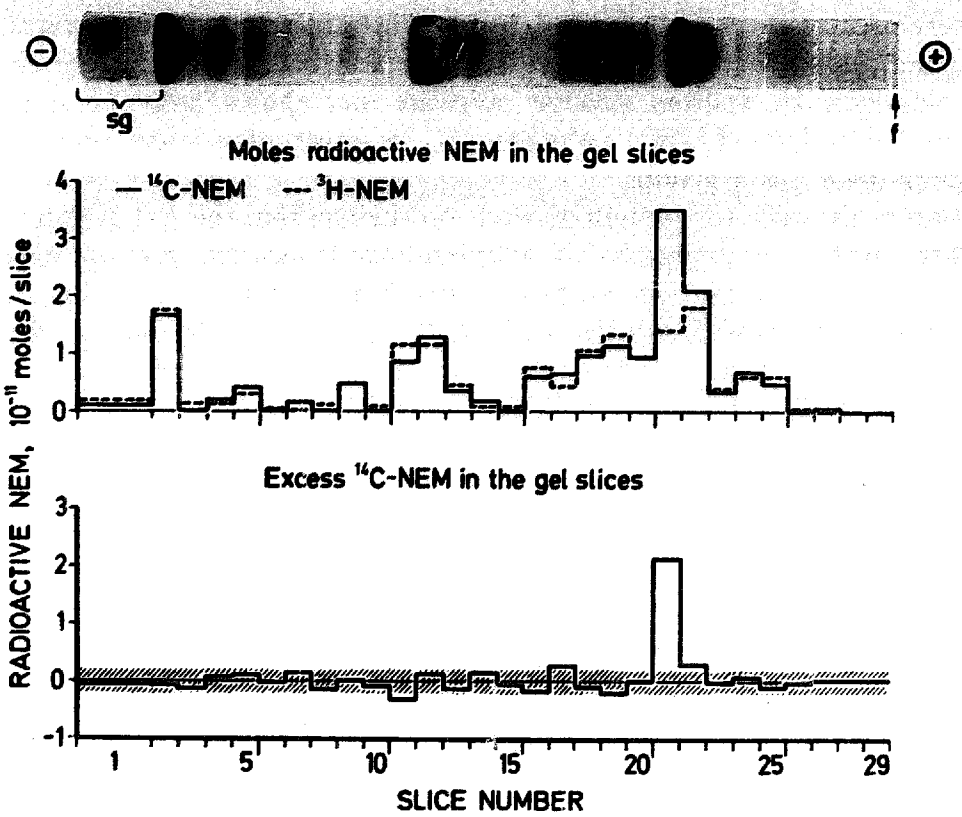

Fig. 2. Separation of 0.5% sodium dodecyl sulphate solubilized microvillus matrix proteins on sodium dodecyl sulphate-polyacrylamide gel disc electrophoresis. The phlorizin-sensitive glucose receptor was labelled with *N*-[^{14}C]ethylmaleimide (^{14}C-NEM), the *N*-ethylmaleimide-labelled unspecific sites with equal amounts of either *N*-[^{3}H]ethylmaleimide, (^{3}H-NEM), or *N*-[^{14}C]ethylmaleimide. The Coomassie blue-stained protein pattern of the gel is shown at the top, the amounts of *N*-[^{14}C]ethylmaleimide and *N*-[^{3}H]ethylmaleimide in the middle and the excess of *N*-[^{14}C]ethylmaleimide relative to *N*-[^{3}H]ethylmaleimide per slice at the bottom. The moles of radioactive *N*-ethylmaleimide bound to the proteins were calculated from the analyzed radioactivity in each gel slice. After subtraction of the background counts, the net counts per minute of each slice were multiplied with 3.2, since each 1 cpm ^{14}C or ^{3}H was equivalent to $3.2 \cdot 10^{-14}$ moles *N*-ethylmaleimide. The excess *N*-[^{14}C]ethylmaleimide representing the localization of the receptor protein was calculated by subtracting the moles of *N*-[^{3}H]ethylmaleimide from that of *N*-[^{14}C]-ethylmaleimide in each slice. The standard deviation of the excess *N*-[^{14}C]ethylmaleimide in the gel is indicated by the shadowed areas. The spacer gel is marked by sg, the bromothymol blue band by f.

protein is attached to the [^{3}H]ethylmaleimide-labelled proteins in the protein band with a molecular weight of about 30000 or if the receptor protein is solubilized and shows only the same migration rate as these proteins in the sodium dodecyl sulphate gels, the gel slices that contained the receptor protein were pooled. Then the proteins were eluted and re-electrophoresed on gels containing no sodium dodecyl sulphate. In Fig. 3 the protein pattern of such gels and the amounts of *N*-[^{14}C]ethylmaleimide and *N*-[^{3}H]ethylmaleimide bound to the separated proteins in the gels are shown. The localization of the excess *N*-[^{14}C]ethylmaleimide in a separate protein band shows that in the re-electrophoresis the *N*-[^{14}C]ethylmaleimide-labelled glucose receptor could be separated from other proteins with *N*-ethylmaleimide-sensitive groups. Only one protein band can be observed in the region where the peak of the *N*-[^{14}C]ethylmaleimide excess is located while most of the proteins containing the

unspecific sites are located in densely stained bands with different migration rates. This experiment indicates that the *N*-[^{14}C]ethylmaleimide-labelled receptor protein is completely solubilized by sodium dodecyl sulphate and shows only the same migration rate in the sodium dodecyl sulphate gels as other membrane proteins with *N*-ethylmaleimide-sensitive groups.

The isolation of the receptor protein depends on the fact that the *N*-[^{3}H]ethylmaleimide-labelled protein and the *N*-[^{14}C]ethylmaleimide-labelled protein were separated identically by the electrophoresis procedures. Experiments were performed in which the glucose receptors were labelled with *N*-[^{14}C]ethylmaleimide or *N*-[^{3}H]-ethylmaleimide and then solubilized. In both cases the protein pattern after the polyacrylamide gel electrophoresis was identical.

Separation of the phlorizin-sensitive glucose receptor from deoxycholate-dissociated microvillus matrix by sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis

In a previous study we demonstrated that the phlorizin-sensitive glucose receptor can be extracted from the microvillus matrix with the anionic detergent deoxycholate[8]. In order to release the receptor protein from the *N*-ethylmaleimide-labelled membranes, the microvillus matrix fraction which contained the *N*-[^{14}C]-ethylmaleimide-labelled glucose receptor sites and the unspecific sites labelled either with *N*-[^{3}H]ethylmaleimide or with *N*-[^{14}C]ethylmaleimide was dissociated with

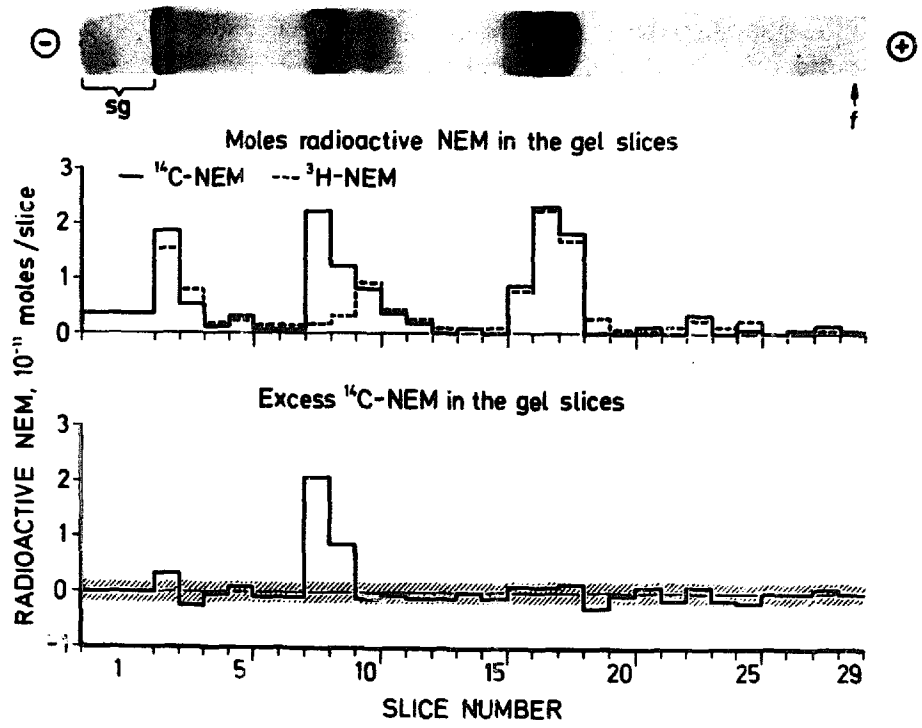

Fig. 3. Re-electrophoresis of membrane proteins containing the phlorizin-sensitive glucose receptor protein on polyacrylamide gels in the absence of sodium dodecyl sulphate. The protein band in the sodium dodecyl sulphate gels which contained the receptor protein was pooled and the proteins eluted. 60 μg of the eluted proteins were layered on top of 15% acrylamide gels and separated at pH 8.6 in the absence of sodium dodecyl sulphate. The protein pattern, the amount of *N*-[^{14}C]ethylmaleimide and *N*-[^{3}H]ethylmaleimide in each gel slice and the excess of *N*-[^{14}C]ethylmaleimide relative to *N*-[^{3}H]ethylmaleimide per slice are shown. The data are presented in the same fashion as in Fig. 2.

deoxycholate and centrifuged at 35000×*g*. 18±4% of the protein, 20±3% of the *N*-[^{14}C]ethylmaleimide and 19±5% of the *N*-[^{3}H]ethylmaleimide which were attached to the matrix fraction remained in the supernatant (means of 6 experiments ±S.D.). The protein remaining in the sediment can only be solubilized with 0.5% sodium dodecyl sulphate.

When the dialysed and concentrated supernatant was subjected to the sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis, the excess ^{14}C radioactivity in the gels, which represents the localization of the receptor protein, could be correlated with two protein bands. As shown in Fig. 4 the band near the cathode has only low contamination with *N*-[^{3}H]ethylmaleimide-labelled proteins and its migration rate relative to the standard proteins indicates an apparent molecular weight of 62000±8000 (Fig. 1) (mean of 5 experiments ±S.D.). If this protein band is eluted from the gel and treated with 0.5% sodium dodecyl sulphate and 5 mM dithiothreitol for 30 min the excess *N*-[^{14}C]ethylmaleimide could be only correlated to a protein band with a molecular weight of approx. 30000 in sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis (3 experiments).

The band near the anode contains the *N*-[^{14}C]ethylmaleimide-labelled receptor sites and also *N*-[^{3}H]ethylmaleimide-labelled proteins. After the elution of the proteins from this band the receptor protein could be removed from the proteins with unspecific *N*-ethylmaleimide-sensitive sites by electrophoresis on sodium dodecyl sulphate-free gels. The protein thus purified has a molecular weight of 31000±5000

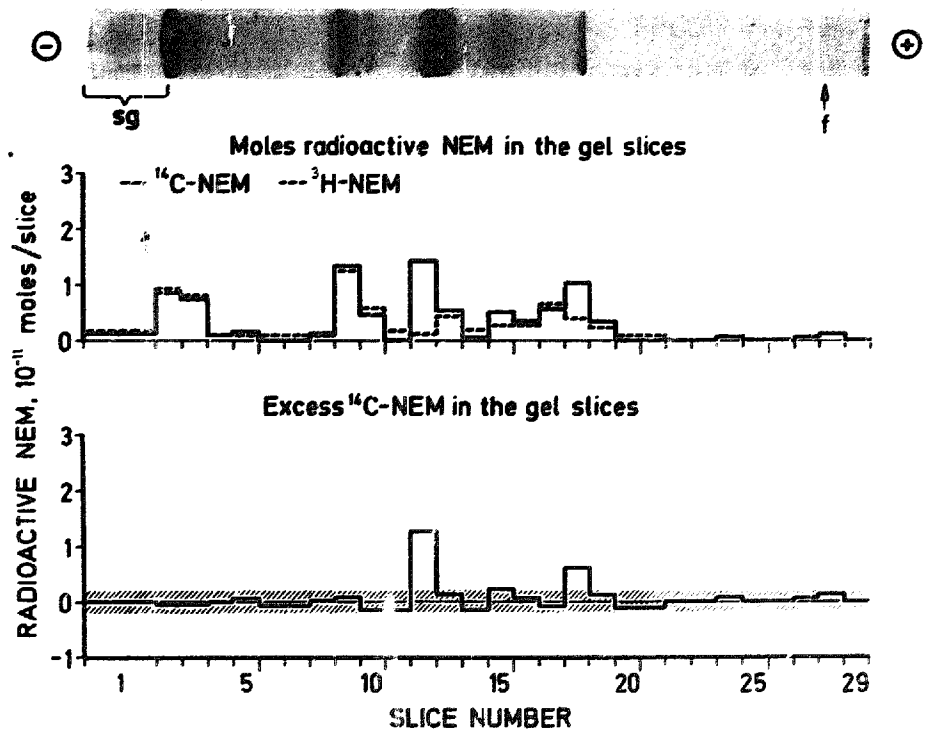

Fig. 4. Separation of deoxycholate-solubilized microvillus matrix proteins on sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis. The microvillus matrix was dissociated with 0.05% deoxycholate and the solubilized proteins separated from the non-solubilized ones by centrifugation at 35000×*g*. 30 μg of protein were layered on top of the gels. The stained protein pattern of the gel, the amounts of *N*-[^{14}C]ethylmaleimide and *N*-[^{3}H]ethylmaleimide in the gel slices and the excess of *N*-[^{14}C]ethylmaleimide relative to *N*-[^{3}H]ethylmaleimide per slice are shown. The data are presented in the same fashion as in Fig. 2.

on sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis (mean of 5 experiments ±S.D.).

Treatment of the microvillus matrix preparation with deoxycholate does not release all *N*-[^{14}C]ethylmaleimide-labelled receptor protein in the supernatant. If the remaining sediment was further solubilized with 0.5% sodium dodecyl sulphate and then separated on sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis, excess *N*-[^{14}C]ethylmaleimide could be located in a protein band with an apparent molecular weight of about 30000 (3 experiments).

DISCUSSION

In a previous report we have described a method for the purification of the phlorizin-sensitive glucose receptor from rat kidney cortex brush borders[8,16]. The membranes were digested with papain and the microvillus matrix containing the phlorizin-sensitive glucose receptor was dissociated with 0.05% deoxycholate. The solubilized proteins were separated with polyacrylamide gel disc electrophoresis and the receptor protein identified by determining the glucose-sensitive phlorizin binding of the various protein bands in the gel.

In this report we have described the purification of the phlorizin-sensitive glucose receptor which was specifically labelled with *N*-ethylmaleimide according to the method described by Fox and Kennedy[17] before the isolation from the brush borders. Since a high affinity phlorizin receptor (K_{phl} $0.26 \cdot 10^{-6}$ M/l) and low affinity ones ($K_{phl} \approx 10^{-4}$ M/l) have been found in the brush borders, low concentrations of phlorizin (5 μM) were used to protect the binding sites in the labelling experiments with *N*-ethylmaleimide. Therefore only the high affinity receptor was labelled. As shown in previous studies D-glucose like phlorizin protects this high affinity receptor from the reaction with *N*-ethylmaleimide[11], indicating that the receptor isolated is not only a phlorizin receptor but also a glucose binding protein of the brush border membrane.

Fox and Kennedy could relate about 50% of all *N*-ethylmaleimide-labelled groups to the transport sites in bacterial membrane. On brush border membrane of rat kidney cortex only 9% of the *N*-ethylmaleimide-sensitive groups could be associated with the phlorizin-sensitive glucose receptor. Therefore a removal of unspecific *N*-ethylmaleimide-sensitive sites from the brush borders prior to the solubilization of the membrane structure was an important condition for the isolation of the phlorizin-sensitive glucose receptor. For that reason the brush border membranes were digested with papain, which is known to remove the surface coat and surface particles which contain the aminopeptidase from the microvilli[10,12]. By this treatment radioactive *N*-ethylmaleimide-labelled unspecific sites could be removed with the proteins of the surface coat, while the *N*-[^{14}C]ethylmaleimide-labelled receptor remained bound to the sedimentable microvillus matrix. This result is in accord with earlier findings where we could show that the number and affinity of the phlorizin-sensitive glucose receptors from the microvillus matrix are identical with those of the brush border membrane[16].

The anionic detergent sodium dodecyl sulphate is superior to other detergents such as deoxycholate, Triton X-100 or hexadecyltrimethylammoniumbromide in its ability to solubilize the brush borders[10]. However, if the membranes are previously

treated with *N*-ethylmaleimide they tended to aggregate irreversibly. No aggregation occurred when the glycoprotein-containing surface coat was removed and then the microvillus matrix solubilized.

Polyacrylamide gel disc electrophoresis was utilized in this study since in previous attempts it was not possible to separate a protein which contained the glucose receptor with gel filtration in the presence or absence of sodium dodecyl sulphate on Sephadex (Thomas, L., unpublished observation). Even with sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis it was not possible to separate completely the glucose receptor from other *N*-ethylmaleimide-labelled proteins. This may be due to the fact that in sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis proteins are separated according to molecular sieving rather than surface charge.

If the protein is removed from the sodium dodecyl sulphate gels and then re-electrophoresed in the absence of sodium dodecyl sulphate, the receptor can be separated from the proteins with *N*-ethylmaleimide-sensitive unspecific sites based on surface charge. However, even this protein cannot be considered pure since it may well be contaminated by other unlabelled proteins with similar characteristics.

With this in mind the receptor protein which was separated from sodium dodecyl sulphate-solubilized microvillus matrix and identified as a protein band which contained excess *N*-[^{14}C]ethylmaleimide relative to the other protein bands in the gel, has a molecular weight of approx. 30000 in sodium dodecyl sulphate–polyacrylamide gel disc electrophoresis.

However if the solubilized proteins of deoxycholate-dissociated microvillus matrix were separated the excess *N*-[^{14}C]ethylmaleimide could also be correlated to a protein band with a molecular weight of about 60000.

This suggests that the deoxycholate-solubilized receptor protein might be a dimer made up of two units each of 30000 size. Support for this idea comes from the finding that after the treatment of the deoxycholate-solubilized membrane proteins with sodium dodecyl sulphate and dithiothreitol only the *N*-[^{14}C]ethylmaleimide-labelled component with a molecular weight of 30000 appeared in the sodium dodecyl sulphate gels. No definite statement can be made as to which of the two forms is the actual phlorizin-sensitive glucose receptor. It is possible that both forms exist in the membrane or that these proteins are only components of the actual receptor. It is interesting to note that the sugar binding proteins which have been purified from bacterial cells also have molecular weight of approx. 30000[1,18,19]. The enzyme mutarotase which is thought to participate in some way in the transport of sugars in kidney also has a molecular weight in this order of magnitude[20,21].

Although no binding studies with phlorizin and glucose on the isolated receptor proteins with molecular weights of approximately 60000 and 30000 have been done, there is some evidence that we have actually isolated proteins which contain the phlorizin-sensitive glucose receptor. Previously we could extract protein components from non *N*-ethylmaleimide-labelled brush border membranes treated with papain and deoxycholate as described under methods. These proteins which are localized in a doublet of protein bands after electrophoretical separation and show glucose-sensitive phlorizin binding[8] have equal migration rates in the same polyacrylamide gel disc electrophoresis as the *N*-[^{14}C]ethylmaleimide-labelled receptor protein shown in Fig. 3.

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