Monoclonal antibodies to the membrane domain of the human erythrocyte anion transport protein

Localization of the C-terminus of the protein to the cytoplasmic side of the red cell membrane and distribution of the protein in some human tissues

Shane D. WAINWRIGHT,* Michael J. A. TANNER,*§ Giles E. M. MARTIN,* Janet E. YENDLE† and Christopher HOLMES‡

*Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.,

†U.K. Transplant Service, Southmead, Bristol, BS10 5ND, U.K., and ‡Department of Obstetrics and Gynaecology, Bristol Maternity Hospital, Bristol BS2 8EG, U.K.

(1) We have prepared murine monoclonal antibodies to the membrane domain of the human erythrocyte anion transport protein (band 3). (2) All of these antibodies react with regions of the protein located at the cytoplasmic surface of the red cell. (3) One of the antibodies reacts with an epitope present on a cytoplasmic loop of the protein located between the C-terminus and a point 168 amino acids from the C-terminus. The other antibodies recognize different epitopes on the C-terminal tail of the protein and the sequences likely to be involved in these epitopes are defined. (4) Our results show that the C-terminus of the red-cell anion transport protein is located on the cytoplasmic side of the red-cell membrane. (5) None of the antibodies inhibited sulphate exchange transport when introduced into resealed red-cell membranes; however, the bivalent form of one of the antibodies reduced the inhibitory potency of 4-acetamido-4'-isothiocyanatostilbene disulphonate on sulphate exchange transport in resealed erythrocyte membranes. (6) Immunostaining of human kidney sections with the antibodies showed strong staining of the basolateral membrane of some but not all of the epithelial cells of distal tubules and the initial connecting segment of collecting tubules. With human liver, only the haematopoietic cells of fetal liver reacted with all the antibodies.

INTRODUCTION

The rapid obligatory exchange transport of anions across the red-cell membrane is carried out by an abundant 95 kDa integral membrane protein (band 3; see Jay & Cantley, 1986, for a recent review). The anion transport protein consists of two domains. A portion of the protein extending approximately 40 kDa from the N-terminus is located entirely in the red-cell cytoplasm and acts as an anchoring site for the red-cell skeleton and for several glycolytic enzymes. The C-terminal 55 kDa domain is membrane-bound and carries out the anion transport function of the protein. This region contains a single N-glycosidically linked oligosaccharide which is very heterogeneous in size and gives rise to the characteristic diffuse band for the protein on SDS/polyacrylamide-gel electrophoresis (SDS/PAGE). The complete sequence of the murine erythrocyte anion transport protein has been derived from the cDNA sequence (Kopito & Lodish, 1985). The characteristics of this sequence suggest that in the transport domain, the polypeptide chain may cross the membrane as many as 12 times. Protein modification studies on the human protein have provided experimental evidence for at least eight membrane-spanning regions in the protein (Williams *et al.*, 1979; Jennings, 1985; Jennings *et al.*, 1986); however there are still uncertainties about the

detailed topography of the protein especially towards the C-terminus.

Monoclonal antibodies are potentially useful tools in studying the structure and function of the anion transport protein. Thus far there has been only one report of monoclonal antibodies which react with the membrane domain of the human protein (Jennings *et al.*, 1986). We have prepared murine monoclonal antibodies to the membrane domain of the human anion transport protein. The locations of the epitopes binding these antibodies have been established. The results show that the C-terminus of the protein is located on the cytoplasmic side of the membrane. The antibodies have also been used to determine the distribution of the red-cell anion transport protein in the cells of human kidney and human liver.

MATERIALS AND METHODS

Proteases and protein molecular standards were obtained from Sigma Chemical Co. Nitrocellulose, prestained protein molecular mass standards and horseradish peroxidase-conjugated antibodies were from Bio-Rad Laboratories, St. Albans, Herts., U.K.

Protease digestion of membranes

Leaky human erythrocyte membranes were prepared

Abbreviations used: SITS, 4-acetamido-4'-isothiocyanatostilbene disulphonate; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (0.15 M-NaCl/310 mOsm-sodium phosphate buffer, pH 7.4, 9:1, v/v); PMSF, phenylmethanesulphonyl fluoride; endo F, endoglycosidase F; H₂DIDS, di-isothiocyanatodihydrostilbene disulphonate.

§ To whom correspondence should be addressed.

according to the method of Dodge *et al.* (1963). Trypsin digestion of leaky membranes was performed at an enzyme concentration of 0.2 mg/ml at 37 °C for 30 min in 0.15 M-KCl and 5 mM-magnesium acetate. The membranes were stripped of peripheral proteins by washing with ice-cold 0.1 M-NaOH and were then washed three times with 5 mM-sodium phosphate buffer, pH 8.0. Digestion of ghosts with trypsin under low ionic strength conditions was done using an enzyme concentration of 50 µg/ml for 1 h at 0 °C in 5 mM-sodium phosphate buffer, pH 8.0. The ghosts were stripped of peripheral proteins as outlined above. Chymotrypsin digestion of intact erythrocytes was done using an enzyme concentration of 1 mg/ml at 37 °C for 1 h on a 25% red-cell suspension in phosphate-buffered saline, pH 7.4. (PBS, containing 9 parts of 0.15 M-NaCl and 1 part of 310 mOsm-sodium phosphate buffer, pH 7.4, prepared as described by Dodge *et al.* (1963). The cells were washed three times with PBS containing 0.2% bovine serum albumin and 2 mM-phenylmethylsulphonyl fluoride (PMSF) and then with PBS. Membranes were prepared and then digested with trypsin and alkali-stripped as outlined above. SDS/PAGE was done using the method of Laemmli (1970) with gels containing 10% acrylamide.

Endoglycosidase F digestion of membranes

Membranes were deglycosylated with an endoglycosidase F (endo F) preparation as described by Tanner *et al.* (1988a). This preparation contained both peptidyl/*N*-glycosidase F and endo- β -*N*-glucosaminidase F activities. The ghosts (1 ml) were mixed with 0.8 ml of 50 mM-EDTA/112.5 mM-NaCl/25 mM-sodium phosphate, pH 5.8, and incubated for 18 h at 37 °C with the endo F preparation. The membranes were then washed twice with 5 mM-sodium phosphate buffer, pH 8.0.

Carboxypeptidase Y digestion of membranes

Erythrocyte ghosts and trypsin-digested ghosts were obtained as outlined above, except that after trypsin digestion the ghosts were treated with 0.4 mg/ml soybean trypsin inhibitor. The membranes were incubated in 2 mM-PMSF for 5 min at 0 °C and then alkali-stripped and washed as described above. The membranes were resuspended in 112.5 mM-NaCl/25 mM-sodium phosphate/50 mM-EDTA, pH 5.8, containing 25 µM-pepstatin A and 10 µg of soybean trypsin inhibitor/ml. The membranes were then digested with either 0.3% or 1% (w/w) (enzyme/band 3) carboxypeptidase Y. The digestion was done at 37 °C and samples were taken at 0, 10, 20 and 60 min, and 18 h. The reaction was halted by adding PMSF to give a final concentration of 2 mM, and the digested membranes were washed three times in 5 mM-sodium phosphate buffer, pH 8.

Purification of the 55 kDa tryptic membrane-bound domain of the anion transport protein

Ghosts which had been digested with trypsin alone or trypsin plus the endo F preparation were mixed with a small amount of ghosts from red cells labelled by ¹²⁵I-radioiodination using lactoperoxidase and which had been treated in a similar way. The samples were then separated by SDS/PAGE and the bands were revealed by autoradiography of the dried gel. The 55 kDa band

was excised from the gel and rehydrated in 0.1% sodium deoxycholate/0.1% SDS/2 mM-EDTA/30 mM-Tris/HCl, pH 8.0. The gel and the buffer were sealed in a dialysis membrane bag and this was placed between the electrodes of a Bio-Rad Transblot tank filled with the rehydration buffer. The protein was eluted from the gel by applying 20 V for 18 h at 4 °C. The eluted protein solution was dialysed extensively against PBS, and finally concentrated by pressure dialysis using an Amicon PM10 membrane.

Production of monoclonal antibodies

Balb/c mice were injected subcutaneously with either the untreated or the endo F-treated 55 kDa tryptic bound domain of band 3 (100 µg in Freund's complete adjuvant). The mice were boosted intraperitoneally after 14 and 28 days with 100 µg of the protein in Freund's incomplete adjuvant. Three days prior to the fusion the mice were boosted with 100 µg of the protein in PBS administered intraperitoneally. Hybridomas were produced by fusing NS-1 cells with spleen cells from the immunized mice at a ratio of 1:4 as described by Parsons *et al.* (1982). After 14 days the cell culture fluids were screened for the presence of antibodies which reacted with human erythrocytes or with the deglycosylated 55 kDa tryptic membrane domain of the anion transport protein. To screen for antibodies directed against human erythrocytes, the culture fluids were tested for the direct or indirect agglutination of human erythrocytes. The presence of antibodies directed against the deglycosylated membrane-bound domain was tested by using the culture fluids to probe nitrocellulose 'dot-blot' containing 0.5 µg of the purified deglycosylated membrane-bound domain of band 3. The nitrocellulose was blocked by washing for 30 min in PBS containing 5% bovine skimmed milk powder and 0.2% Tween 20. The dot-blot was incubated with the culture supernatants from the cell-fusion plates for 2 h at room temperature in a moisture chamber and then washed twice in PBS containing 0.2% Tween 20. This was followed by a 1 h incubation at room temperature with a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody in PBS containing 5% skimmed milk powder and 0.2% Tween 20. The washed dot-blot was developed in a solution containing 0.6 mg of diaminobenzidine/ml and 1 µl of 30% H₂O₂/ml. Cells from wells giving positive results were harvested and the cells were cloned twice by limiting dilution using the same screening method. BRIC 130 and BRIC 132 were found to be of the IgG1 subclass while BRIC 154 and BRIC 155 were of the IgG2a and IgG2b subclasses respectively (determined as described by McDougal *et al.*, 1983). The antibody samples used in the anion transport experiments were affinity-purified by absorption of 4 ml of ascites fluid onto alkali-stripped erythrocyte ghosts from 40 ml of red cells for 2 h on ice. The membranes were washed once with 5 mM-sodium phosphate buffer, pH 8.0, containing 0.05% digitonin, and three times with 5 mM-sodium phosphate buffer, pH 8.0. The antibodies were eluted from the membranes at 0 °C by extraction with 40 ml of 200 mM-glycine/HCl, pH 2.8, containing 150 mM-NaCl. Fab fragments of BRIC 155 were prepared by treatment of the affinity-purified antibodies with immobilized papain (Pierce) as recommended by the manufacturer. Fc fragments and uncleaved antibody were removed by passage through a column of protein A-Sepharose.

Immunoblotting

Immunoblots of SDS/polyacrylamide gels were performed using the method of Towbin *et al.* (1979), except that the nitrocellulose was blocked with PBS containing 5% skimmed milk powder and 0.2% Tween 20, and washes were in PBS containing 0.2% Tween 20.

Measurement of anion transport in resealed red-cell membranes

Anion transport was measured using the tracer equilibrium self-exchange of [35 S]sulphate (Gardos *et al.*, 1969; Lepke & Passow, 1976). Antibodies were resealed into membranes as follows. A 50% red cell suspension in 0.15 M-NaCl was made to contain 12 mg of antibody or bovine serum albumin/ml and cooled to 0 °C. The cells were lysed with 10 vol. of 4 mM-MgSO₄ containing 10 μ Ci of [35 S]sulphate/ml (Amersham International, Amersham, Bucks., U.K.) and incubated for 2 h on ice. The membrane suspension was made 20 mM-sodium phosphate buffer, pH 7.2/5 mM-Na₂SO₄/122.5 mM-NaCl. After 5 min on ice, the membranes were resealed by incubation at 37 °C for 1 h. At this point, a sample was taken for determination of the specific radioactivity of the solution. The membranes were then cooled to 0 °C and extracellular [35 S]sulphate was removed by three washes of the membranes in ice-cold sulphate transport buffer, pH 7.2 (20 mM-sodium phosphate/5 mM-Na₂SO₄/122.5 mM-NaCl) at 0 °C. The resealed membranes were made up to a concentration equivalent to a 5% suspension of the original red cells by adding sulphate transport buffer warmed to 37 °C, and the efflux of radioactive sulphate from the membranes into the medium was measured at 37 °C. Samples of the suspension were taken at different times, cooled on ice and then microcentrifuged at 4 °C for 3 min at 12000 *g*. A measured volume of the supernatant was taken and a half volume of 21% trichloroacetic acid was added at 0 °C. The precipitate was removed by centrifugation and a known volume of the supernatant was counted for radioactivity. An estimate of the radioactivity in the supernatant at equilibrium was obtained by taking a sample of the membrane suspension and lysing the membranes at 0 °C with a half volume of 21% trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant was counted for radioactivity. The number of resealed erythrocyte membranes in the 5% membrane suspension was determined using a haemocytometer. Changes in the internal volumes of the different resealed membrane preparations were corrected for by normalizing the transport data in terms of the sulphate self-exchange flux (*J*) as described by Funder & Weith (1976). Radioactivity was measured using the liquid scintillator Unisolve E (Koch-Light) and corrected for quenching using a quench curve and the external standard ratio method.

Binding of tritiated H₂-DIDS to resealed membranes

[3 H]Di-isothiocyanatodihydrostilbene disulphonate ([3 H]H₂DIDS) (202 mCi/mmol; HSC Research Development Corp., Toronto, Canada) was added to a 5% suspension of resealed membranes at a range of concentrations up to 3 μ M and samples were taken at different time intervals during incubation at 37 °C. The membranes were washed twice with PBS containing

0.2% bovine serum albumin and twice with PBS, and leaky membranes were prepared by lysis in 5 mM-sodium phosphate buffer, pH 8.0, and washed three times in the same buffer. The membrane proteins were separated by SDS/PAGE and the gels were stained with Coomassie Blue and then scanned on a densitometer. The band 3 region of the gel was excised and solubilized (Mahin & Lofberg, 1966) before being counted for radioactivity and corrected for quenching as above.

Examination of human kidney tissue

Renal tissue was obtained from perfused kidneys removed from cadaveric donors. The kidneys had been offered to the U.K. Transplant Service but were unsuitable for transplantation due to prolonged cold ischaemia time or anatomical abnormalities in the blood vessels supplying the kidneys. Blocks of kidney tissue were snap frozen and stored in liquid N₂.

Indirect immunoperoxidase staining of kidney sections was carried out using methods based on those of Evans *et al.* (1985) and Hancock *et al.* (1988). Cryostat sections (5 μ m) of kidney with normal morphology were air-dried and fixed for 10 min in acetone at room temperature and then incubated with murine monoclonal antibodies for 30 min at room temperature. Sections were washed for 30 min with three changes of PBS, pH 7.3, after each incubation. Affinity-purified rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:150 in PBS was applied to the sections for 30 min followed by 1:150 peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatts). Colour was developed for 3–5 min with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Isopac) in 0.2 M-Tris/HCl, pH 7.6, containing 0.01% H₂O₂. Sections were counterstained with Mayer's Haemalum (BDH), dehydrated and mounted in DPX (BDH).

In control experiments, primary antibody was replaced with PBS or murine monoclonal antibodies against erythrocyte antigens that did not stain kidney. A chequerboard experiment was carried out to determine optimal dilutions of secondary and tertiary antibody. Slides were examined to determine the location and intensity of the peroxidase staining.

Examination of human liver

Human liver was obtained either from elective termination of pregnancy at 6–16 weeks gestation (five first-trimester and three second-trimester specimens) or from adults within 4 h *post mortem* (two specimens). Blocks of tissue were placed in OCT embedding compound (Lab-tek Products, Naperville, IL, U.S.A.), frozen in liquid N₂-cooled isopentane (2-methylbutane) on small cork boards and stored in liquid N₂. Tissue sections were stained by an indirect immunoperoxidase technique exactly as described by Tavare & Holmes (1989).

RESULTS AND DISCUSSION

Production and characterization of anti-(human red-cell anion transporter) monoclonal antibodies

Murine monoclonal antibodies were produced against the 55 kDa tryptic fragment of the human erythrocyte anion transport protein which contains the membrane-bound domain. In addition, this fragment was also deglycosylated using an endo F preparation and used as

Fig. 1. Reactivity of antibodies on immunoblots of erythrocyte membranes and protease digests of erythrocyte membranes

Lanes *a-d*, untreated erythrocyte membranes probed with: *a*, BRIC 130; *b*, BRIC 132; *c*, BRIC 154; *d*, BRIC 155. Lanes *e-s*, protease digests of erythrocyte membranes probed with: *e-i*, BRIC 130; *j-n*, BRIC 132; *o-s*, BRIC 155. BRIC 154 gave the same results as BRIC 155. Samples *h*, *m*, *r* were erythrocyte ghosts digested with trypsin under isotonic ionic strength conditions and *g*, *l*, *q* were the same samples which were also digested with the endo F preparation. Samples *i*, *n*, *s* were erythrocyte membranes digested with trypsin under low ionic strength conditions. Samples *f*, *k*, *q* were trypsin-treated membranes from chymotrypsin-treated red cells and *e*, *j*, *o* were the same samples which were also treated with the endo F preparation.

an immunogen. Four stable antibody-secreting cell lines were produced (BRIC 130, 132, 154, 155). None of the antibodies agglutinated intact human erythrocytes directly, or indirectly with the addition of rabbit anti-mouse immunoglobulin, nor did they agglutinate chymotrypsin-, Pronase-, trypsin- or endo F-treated erythrocytes, but they all reacted with the purified deglycosylated 55 kDa tryptic fragment of the human anion transporter in the dot-blot assay. Immunoblots of SDS/PAGE-separated erythrocyte membranes confirmed that they all reacted with the anion transport protein (Fig. 1, lanes *a-d*). As expected, all the antibodies reacted with the diffuse 55 kDa tryptic fragment of the anion transport protein on immunoblots of trypsin-treated erythrocyte membranes (Fig. 1, lanes *h*, *m*, *r*). When trypsin-treated membranes were also treated with the endo F preparation, the antibodies reacted with the sharp 52 kDa band which corresponds to the deglycosylated tryptic membrane-bound fragment of band 3 (Fig. 1, lanes *g*, *l*, *q*), showing that the antibodies all react with polypeptide epitopes in the protein.

The antibodies react with the cytoplasmic region of the anion transport protein

The observation that none of the antibodies agglutinated human erythrocytes when either the direct or indirect methods were used suggested that they reacted with epitopes at the cytoplasmic side of the membrane. This was confirmed by immunoprecipitation experiments in which the antibodies were incubated either with lactoperoxidase-radioiodinated erythrocytes or with leaky membranes from radioiodinated erythrocytes. The cytoplasmic surface of the membrane would be accessible to the antibodies in the latter preparation but not in the former. The results in Fig. 2 (lanes *a-i*) show that the anion transport protein was precipitated when the antibodies were added to leaky erythrocyte membranes but not when they were added to intact erythrocytes, demonstrating that they all react with the cytoplasmic region of

the anion transport protein. The more effective immunoprecipitation obtained with BRIC 154 and BRIC 155 is probably due to these antibodies being of subclass IgG2, which binds protein A efficiently, while the other antibodies were of subclass IgG1 and required the additional use of a polyclonal rabbit anti-mouse IgG antiserum. All the antibodies could be quantitatively purified by absorption onto erythrocyte membranes and elution, confirming their reactivity with the cytoplasmic side of the membrane. The strong band obtained in addition to band 3 when BRIC 130 was added to erythrocyte membranes (Fig. 2; lane *g*) comigrated with red-cell sialoglycoprotein- α (glycophorin A), a major labelled component in the preparation. The presence of this band is surprising since the other data in this paper clearly show that BRIC 130 specifically reacts with the anion transport protein. There is some evidence that band 3 and sialoglycoprotein- α are associated in the red-cell membrane (Nigg *et al.*, 1980). It is conceivable that interaction of BRIC 130 with the anion transport protein stabilized this association so that sialoglycoprotein- α was also coprecipitated.

Locations of the antibody epitopes in the anion transport protein sequence

The fragments of the anion transport protein resulting from treatment of erythrocytes or erythrocyte ghosts with proteases are well characterized (reviewed by Jennings, 1985; Jay & Cantley, 1986) and were used to determine the locations of the epitopes of the monoclonal anti-(band 3) antibodies. The antibodies were used to probe immunoblots of SDS/PAGE-separated protease digests of erythrocyte membranes. Trypsin treatment of membranes from chymotrypsin-treated erythrocytes yields a 17 kDa fragment and a diffuse 38 kDa fragment which represent the *N*-terminal and *C*-terminal portions respectively of the 55 kDa tryptic membrane-bound fragment of band 3. All the antibodies reacted with the 38 kDa fragment even when this was deglycosylated by

Fig. 2. Immunoprecipitation of band 3 and reactivity of antibodies with protease digests of erythrocyte membranes

Lanes *a-i*, autoradiographs of immunoprecipitates obtained when antibodies were added to intact red cells (*b, d, f, h*) or red-cell membranes (*c, e, g, i*) using BRIC 154 (*b, c*), BRIC 155 (*d, e*), BRIC 130 (*f, g*) or BRIC 132 (*h, i*). Lane *a* contained membranes from radioiodinated red cells. When the antibodies were added to intact red cells they were incubated with lactoperoxidase-radioiodinated red cells at 37 °C for 1 h. The cells were washed four times with PBS. When BRIC 130 and BRIC 132 were used the cells were then allowed to react with an equal volume of a 1:100 dilution of rabbit anti-mouse IgG (Dakopatts) at 37 °C for 1 h and then washed four times with PBS. Membranes were prepared and solubilized by incubation at room temperature for 15 min with 6 vol. of PBS containing 2% Triton X-100, 2 mM-EDTA and 2 mM-PMSF. After centrifugation at 40000 *g* for 40 min, the supernatant was shaken with 10 μ l/ml of a 50% suspension of protein A-Sepharose (Pharmacia, Milton Keynes, Bucks., U.K.) for 30 min. The protein A-Sepharose was washed five times with PBS containing 1% Triton X-100, 2 mM-EDTA and 2 mM-PMSF and once with water, and the proteins separated by SDS/PAGE. When the antibodies were added to red-cell ghosts they were incubated with membranes from lactoperoxidase-radioiodinated red cells as described above. The membranes were washed four times with 5 mM-sodium phosphate buffer, pH 8.0, and subsequently treated as described above. Lanes *j-q*, immunoblots of proteinase digests of erythrocyte membranes using BRIC 132 (*j-m*) and BRIC 130 (*n-q*). BRIC 154 and BRIC 155 gave the same results as BRIC 130. Lanes *j, n* contained membranes treated with 1 mg/ml trypsin at low ionic strength for 1 h at 37 °C, while lanes *k, o* contained membranes treated with 1 mg/ml chymotrypsin for 1 h at 37 °C, followed by one wash in 0.1 M-NaOH, two washes in 5 mM-sodium phosphate, pH 8, and digestion with 2 mg/ml chymotrypsin for 1 h at 37 °C. Lanes *l, p* contain membranes which had been washed once with 0.1 M-NaOH, twice with 5 mM-sodium phosphate, pH 8, and digested with 2 mg/ml elastase for 30 min at 37 °C. Lanes *m, q* were membranes treated with pepsin in 1 M-acetic acid as described by Tanner *et al.* (1979).

treating the membranes with the endo F preparation to yield a sharp 35 kDa band (Fig. 1).

Jennings *et al.* (1986) have shown that a minor product of tryptic cleavage, which is obtained in higher yield when the digestion is done at low ionic strength, results from cleavage by the enzyme at a point 168 amino acids from the C-terminus of the protein and migrates with an M_r of about 20000. The antibodies all reacted with this fragment (which migrated at 22.5 kDa in our system, Fig. 1) which was present in variable amounts in all the digests, but was obtained in higher yields when trypsin digestion was done under conditions of low ionic strength (Fig. 1, lanes *i, n, s*). All the antibodies therefore react with epitopes located within 168 residues from the C-terminus of the protein.

The smallest fragment in the digests reactive with BRIC 130, 154 and 155 was the 22.5 kDa fragment (Fig. 1). However, BRIC 132 also reacted with a series of smaller fragments which were most evident in membranes which had been treated with trypsin under low ionic strength conditions (Fig. 1, lane *n*). When erythrocyte ghosts were incubated at 37 °C with a high concentration of trypsin and at low ionic strength, the 55 kDa fragment was completely degraded. A number of smaller fragments which reacted with BRIC 132 were found, ranging in size from 22.5 kDa to approximately 8 kDa (Fig. 2, lane *j*). The most prominent of these was a 19.5 kDa fragment.

The other antibodies only reacted with the small amounts of the 22.5 kDa band which remained in this digest (Fig. 2, lane *n*). When membranes were treated under conditions of low ionic strength with high concentrations of chymotrypsin or elastase, the anion transport protein was degraded to small fragments which ran close to the front of the gel. No reactivity with these fragments was observed using BRIC 130, 154 and 155 (Fig. 2, lanes *o, p*) but BRIC 132 reacted with a diffusely migrating band of approximately 9.5 kDa in the chymotrypsin digest and 8 kDa in the elastase digest (Fig. 2, lanes *k, l*). Pepsin digests of erythrocyte ghosts yielded a similar result, with BRIC 132 reacting with a band of approximately 7.5 kDa (Fig. 2, lane *m*) which did not react with BRIC 130, 154 or 155 (Fig. 2, lane *q*). These results show that the BRIC 132 epitope differs from those of the other antibodies.

In order to establish whether any of the antibodies reacted with the extreme C-terminus of the anion transport protein, immunoblots were done on erythrocyte ghosts and trypsin-treated erythrocyte ghosts which were alkali-stripped and then treated with carboxypeptidase Y (Figs. 3 and 4). The BRIC 130 epitope was found to be extremely sensitive to carboxypeptidase Y. Even at the lowest enzyme concentration tested, the band 3 in the sample taken immediately after carboxypeptidase Y was added at 0 °C showed a noticeable reduction in the

Fig. 3. Immunoblots of carboxypeptidase Y-treated erythrocyte membranes

Erythrocyte ghosts were treated with carboxypeptidase Y as described in the Materials and methods section and samples taken at the times indicated in the Figure were separated by SDS/PAGE. The carboxypeptidase Y (CPase Y) concentrations (% w/w, enzyme/band 3) used are shown in the Figure. The control samples were incubated under identical conditions but without added enzyme. Immunoblots were done using BRIC 130 (a-d), BRIC 155 (e-i) and BRIC 132 (j-n). BRIC 154 gave results very similar to those obtained with BRIC 155.

Fig. 4. Immunoblots of carboxypeptidase Y-treated trypsin-digested erythrocyte membranes

Trypsin-digested erythrocyte membranes were treated with carboxypeptidase Y as described in the Materials and methods section. Other details are as in the legend to Fig. 3. Immunoblots were done using BRIC 130 (a-d), BRIC 155 (e-l) and BRIC 132 (m-t). BRIC 154 gave results very similar to those obtained with BRIC 155.

binding of BRIC 130 (Fig. 3, lanes a, b). Under these conditions the binding of the other antibodies was not affected. After 10 min exposure to carboxypeptidase Y at 37 °C the anion transport protein did not show any reactivity with BRIC 130 while the binding of the other antibodies remained unchanged (Fig. 3, lanes c, d). After 60 min incubation with carboxypeptidase Y, there was a noticeable reduction in the binding of BRIC 154 and 155 and no binding of these antibodies was detected after 18 h incubation at 37 °C with the enzyme (Fig. 3, lanes e-i). However the binding of BRIC 132 remained unchanged under all these conditions of carboxypeptidase Y digestion. Even under the harshest conditions we employed, using 1% carboxypeptidase Y and

incubation for 18 h at 37 °C, the binding of BRIC 132 remained unaffected (Fig. 3, lanes j-n).

We conclude from these results that the epitope binding BRIC 130 is very close to the C-terminus of the anion transport protein while the epitopes for BRIC 154 and 155 are further from the C-terminus but still reside in the C-terminal tail of the protein, since they are not protected by the membrane against carboxypeptidase Y treatment. In contrast, the results suggest that the epitope for BRIC 132 is located in an internal cytoplasmic loop which is protected by the membrane from digestion with carboxypeptidase Y. Since BRIC 130, 154 and 155 all react only with the cytoplasmic surface of the membrane, these results clearly demonstrate that the C-terminus of

the anion transport protein is located on the cytoplasmic surface of the erythrocyte membrane. This is in agreement with the conclusion reached by Lieberman *et al.* (1987) based on the inability of carboxypeptidase Y to digest the protein in intact red cells and the accessibility of the anion transporter to digestion by the enzyme in red-cell membranes stripped of peripheral proteins by treatment at pH 12 or 1 M-KI. However, since the enzyme did not digest the anion transport protein in leaky unstripped membranes, they pointed out that it was possible that the alkali or high salt treatment caused an extracellular but cryptic C-terminus of the protein to become exposed to digestion by the enzyme. Our results are consistent with the alternative explanation for these observations suggested by Lieberman *et al.* (1987), that the C-terminal region of the anion transport protein interacts with peripheral proteins on the cytoplasmic surface of the erythrocyte membrane, and removal of these proteins by the stripping procedure allows access of the C-terminus to the carboxypeptidase.

Because of the diffuse band given by the anion transport protein and the 55 kDa tryptic fragment on SDS/PAGE, it was difficult to determine the changes in M_r of the protein caused by carboxypeptidase Y digestion. The changes in M_r of the minor C-terminal 22.5 kDa tryptic fragment could be estimated with more accuracy on immunoblots since this fragment migrates as a sharp band (Fig. 4). Carboxypeptidase Y treatment was carried out on alkali-stripped trypsin-treated membranes (Fig. 4). Immediately after the addition of carboxypeptidase Y at 0 °C, the amount of the 22.5 kDa fragment detected by BRIC 130 appeared to be reduced and no new fragments of lower M_r were detected (Fig. 4, lanes *a*, *b*). In the same sample, BRIC 155 detected both the original 22.5 kDa band and a 22 kDa band (Fig. 4, lanes *e*, *f*). After 10 min digestion with carboxypeptidase Y no immunoreactive material could be detected in the digest with BRIC 130 (Fig. 4, lanes *c*, *d*) and BRIC 155 detected only the 22 kDa band (Fig. 4, lanes *g*, *h*). BRIC 132 also detected the 22 kDa band at the early stages of digestion (Fig. 4, lanes *n*, *p*). The lack of reactivity of the 22 kDa band with BRIC 130 suggests that the BRIC 130 epitope is located within about 0.5 kDa of the C-terminus of the protein.

After more prolonged digestion (60 min) with carboxypeptidase Y, the immunoreactivity of the digest with BRIC 155 was markedly reduced and no small fragments were detectable (Fig. 4, lanes *i*, *j*). BRIC 132 detected a new band of 21 kDa in this sample (Fig. 4, lanes *q*, *r*). This suggests that the BRIC 155 epitope is located in a region between 1.5 kDa and 0.5 kDa from the C-terminus. After 18 h exposure to carboxypeptidase Y, no immunoreactive bands were detected in the digest using BRIC 155 (Fig. 4, lanes *k*, *l*), but bands of 20 kDa and 19 kDa were observed using BRIC 132 (Fig. 4, lanes *s*, *t*). The smallest of these bands probably represents the limit of carboxypeptidase Y digestion under these conditions. The above experiments were also done using BRIC 154 (results not shown) and the results obtained were very similar to those observed for BRIC 155, suggesting that the epitopes for these two antibodies are located very close to each other.

It is clear from these results that the 22.5 kDa tryptic fragment extends to the C-terminus of the protein. The other smaller tryptic fragments which bind BRIC 132 (Fig. 2, lane *j*) do not bind BRIC 130 or BRIC 155 and

therefore result from additional cleavages on the N-terminal side of the BRIC 155 epitope at the C-terminus.

Reaction with the anion transport protein of red blood cells of other species

The reactivity of the antibodies with the anion transport protein of red blood cells from different species was examined to give further information on the epitopes for these antibodies. The reactions obtained with the anion transporter band on immunoblots of SDS gels of erythrocyte membranes from different species are shown in Table 1. All the antibodies reacted with the anion transport protein of rabbit erythrocytes and to a greater or lesser extent with that of porcine erythrocytes. BRIC 132, 154 and 155 reacted with the protein in murine and guinea pig red cells while BRIC 130 did not. In contrast BRIC 130 reacted with the anion transport protein of bovine erythrocytes while the other antibodies did not. BRIC 154 and BRIC 155 showed a different degree of reactivity with the mouse protein suggesting that they do not recognize identical epitopes. It is interesting that autoimmunity appears to have been induced in the cases of the antibodies which react with the murine protein.

Fig. 5 shows the C-terminal portion of the amino acid sequence of murine band 3 (Kopito & Lodish, 1985) together with the corresponding portion of human band 3 derived from the cDNA sequence (Tanner *et al.*, 1988b), and by direct protein sequencing (Kawano *et al.*, 1988), and the sequence of the chicken protein (Cox & Lazarides, 1988). Acidic residues predominate at the C-terminus of all the proteins, and in the human protein eight of the sixteen C-terminal residues are aspartic acid or glutamic acid. Lieberman *et al.* (1987) have studied the release of amino acids from the C-terminus of the anion transporter by carboxypeptidase Y. Amino acids corresponding to the six C-terminal residues of the

Table 1. Reactivity of antibodies with band 3 in SDS/PAGE separated membrane proteins of red cells from different species

+ indicates strong reactivity, w indicates weak reactivity and – indicates no detectable reactivity on immunoblots.

Antibody	Rabbit	Pig	Cow	Mouse	Guinea pig	Chicken
BRIC 130	+	+	+	–	–	–
BRIC 132	+	+	–	+	+	–
BRIC 154	+	w	–	+	+	–
BRIC 155	+	w	–	w	+	–

Fig. 5. C-terminal amino acid sequences of red-cell anion transport proteins from different species

Portions of the C-terminal amino acid sequences of the chicken (CHK B3), mouse (MUR B3) and human (HUM B3) red-cell anion transport proteins. Amino acids conserved with the human protein are boxed. The numbering of the residues is that of the human protein.

human protein were released most readily (including the alanine residue located four residues from the C-terminus). The susceptibility of the BRIC 130 epitope to carboxypeptidase Y treatment suggests it is located within this region and this is consistent with the change in M_r at the C-terminus of the anion transport protein concomitant with the loss of the BRIC 130 epitope. Further the lack of reactivity of BRIC 130 with the mouse and chicken proteins, in which the alanine residue four amino acids from the C-terminus of the human protein is substituted by proline and glutamine residues respectively, suggests that this alanine residue forms part of the epitope for BRIC 130.

Carboxypeptidase Y cleavage of residues penultimate to glycine is slow, and at pH 5.8 the digestion of acidic residues by the enzyme is also slow (Hayashi, 1977). The clusters of acidic residues and the presence of a glycine residue between six and sixteen amino acids from the C-terminus would impede digestion by the enzyme, as noted by Leiberman *et al.* (1987), and give rise to the discrete bands we observe during digestion by carboxypeptidase Y. The changes in M_r on SDS/PAGE of the 22.5 kDa tryptic fragment associated with the loss of the BRIC 154 and 155 epitopes during carboxypeptidase Y digestion suggest that these epitopes are located in the region 0.5–1.5 kDa from the C-terminus. Although these values have to be treated with caution because of possible anomalous mobility effects on SDS/PAGE resulting from the loss of a large number of negatively charged residues, they suggest that the epitopes for these antibodies are located in the region containing the clusters of acidic residues. Since these antibodies react with the mouse but not the chicken protein, their epitopes probably lie in one of the three regions of sequence conservation between the human and mouse proteins within residues 889–907 (Fig. 5) and contain one of the residues in these regions which is altered in the chicken protein.

A band 3.5 kDa smaller than the undigested 22.5 kDa fragment was obtained at the limit of carboxypeptidase Y digestion. Any anomalous mobility effects on SDS/PAGE due to the loss of the clustered acidic residues at the C-terminus would tend to underestimate the real change in mass of the fragment. The amino acid sequence of the protein contains a hydrophobic domain on the N-terminal side of the pair of arginine residues 41 and 42 amino acids from the C-terminus (residues 870, 871; Fig. 5). The limiting digestion product of carboxypeptidase Y treatment may result from protection of the anion transport protein polypeptide because it enters the membrane around this point. Although this conclusion is attractive, it must be regarded as tentative because some other feature of the structure or amino acid sequence at the C-terminus of the anion transport protein may limit further carboxypeptidase Y digestion.

Effects of the antibodies on anion transport

The effect of the antibodies on anion transport was measured after introducing them into resealed human erythrocyte ghosts and measuring the equilibrium exchange efflux of [35 S]sulphate from the resealed ghosts. The incorporation of the antibodies into the ghosts was confirmed by lysis of a sample of the resealed ghosts followed by SDS/PAGE. The heavy and light chains of the antibodies gave bands of the intensity expected for the incorporation of amounts of the antibodies

approximately stoichiometric with that of band 3. Control experiments using equivalent concentrations of bovine serum albumin or a murine monoclonal antibody (BRIC 163) which reacted with the cytoplasmic domain of red-cell membrane sialoglycoprotein- α showed that these had no effect on transport compared to membranes which had no exogenous protein incorporated. Neither BRIC 130 nor BRIC 132 (at a 2-fold excess of antibody to band 3) had any effect on the rate of sulphate exchange or on the ability of 4-acetamido-4'-isothiocyanatostilbene disulphonate (SITS) to inhibit anion transport in the resealed ghosts.

Unlike BRIC 130 and BRIC 132, BRIC 155 caused an increase in the intracellular volume of the ghosts as assessed by the amount of trapped [35 S]sulphate in the resealed ghosts containing BRIC 155 compared with the control resealed ghosts. This volume change was approximately 2-fold. Similar values were obtained from measurements of the internal volume of the membranes using $^3\text{H}_2\text{O}$ and [^{14}C]sucrose as described by Halestrap & Denton (1974). Since the measured transport rates are dependent on the internal volume of the membranes, the data was corrected for the differences in internal volume of the control and BRIC 155-containing membranes as described by Funder & Weith (1976). The BRIC 155-containing membranes gave the same value for the sulphate self-exchange flux as the control membranes, suggesting that this antibody has no effect on anion transport. However the presence of the antibody reduced the ability of SITS to inhibit sulphate exchange in BRIC 155-containing ghosts compared to control ghosts (Fig. 6). Since the antibody binds at the cytoplasmic surface of the anion transporter whereas SITS is known to bind to the extracellular surface of the protein, this does not result from the direct blocking of the inhibitor binding site by the antibody. To test if this

Fig. 6. Effect of BRIC 155 on inhibition of anion transport by SITS

Sulphate self-exchange fluxes (J) were calculated from the measured transport rates as described by Funder & Weith (1976) and used to derive the percentage inhibition by SITS of sulphate efflux from the resealed membranes. +, Control resealed membranes containing bovine serum albumin; ○, resealed membranes containing intact BRIC 155 (at a 2-fold molar excess over band 3); ●, resealed membranes containing the same concentration of Fab fragments of BRIC 155. Other details are given in the Materials and methods section.

effect was due to cross-linking of the anion transport protein by the antibody, Fab fragments of the antibody were prepared. When these were incorporated into the resealed ghosts there was no change in the internal volume of the resealed ghosts nor was there any effect on anion transport or on the inhibitory potency of SITS (Fig. 6). These observations suggest that the SITS effect results from the cross-linking of band 3 molecules by BRIC 155. In attempts to further study the effect of BRIC 155 on stilbene disulphonate inhibition of anion transport, the binding of [^3H]H₂DIDS to resealed ghosts containing BRIC 155 was examined. No difference was observed in the time-dependent covalent binding of H₂-DIDS to band 3 in the antibody-containing and control membranes at concentrations of inhibitor up to 3 μM . Higher concentrations of the inhibitor appeared to cause lysis of the resealed membranes.

The anion transport protein is present as dimers and possibly higher oligomers in the red-cell membrane (reviewed by Jay & Cantley, 1986). Although each monomer binds a stilbene disulphonate molecule, dimerization appears to be necessary for the binding of these inhibitors, suggesting that the formation of the binding sites requires the association of the monomers (Booth & Reithmeier, 1984). Other evidence suggests that the stilbene disulphonate binding sites are located close together on adjacent monomers (Macara & Cantley, 1981). A possible explanation of our results is that crosslinking of band 3 by the bivalent form of BRIC 155 causes a change in the interaction of the subunits of the

band 3 dimer which modifies the stilbene disulphonate binding sites. This effect may be associated with changes in the organization of the red-cell skeleton induced by BRIC 155. This is suggested by the alteration in internal volume of resealed ghosts in the presence of this antibody, since the shape of the red-cell membrane is known to be influenced by the cytoplasmic skeletal protein complex. Lieberman *et al.* (1987) have suggested that the C-terminus of band 3 interacts with peripheral membrane proteins and it is possible that this region (like the N-terminus of the protein) may interact with a component of the red cell skeleton.

Reaction of human kidney sections with the antibodies

The antibodies were used on sections from six normal human kidneys. The distribution and intensity of staining with each antibody are summarized in Table 2. BRIC 132 differed from the other antibodies in that it stained intensely the glomerular connective tissue matrix and arterial smooth muscle. A small proportion of glomerular cells was stained with antibodies BRIC 130, BRIC 154 and BRIC 155 but these may have been macrophages infiltrating the glomeruli, phagocytosing remnants of red blood cells. BRIC 130, BRIC 154 and BRIC 155 exhibited the same distinctive pattern of kidney tubular epithelial staining, varying only in the extent and intensity (Fig. 7a). A small amount of tubular staining was observed with BRIC 132, but it was much weaker. Staining was confined to a proportion of cells in distal tubules and the initial connecting segment of collecting tubules. Groups

Table 2. Distribution of staining with anti-band 3 antibodies on human kidney sections

— Indicates no reactivity; +/— indicates that different cells in the tissue were either positive or negative.

Antibody	Glomeruli	Peri-tubular capillaries	Veins	Arteries	Proximal tubules	Distal/collecting tubules
BRIC 130	+ / —	—	—	—	—	+ + / —
BRIC 132	+++	—	—	+++	—	+ / —
BRIC 154	+ / —	—	—	—	—	+ + + / —
BRIC 155	+ / —	—	—	—	—	+ + + / —

Fig. 7. Staining of human kidney sections with anti-(band 3) antibodies

(a) Staining of distal tubules with BRIC 155. G indicates a glomerulus (magnification $\times 145$). (b) Staining of distal tubule epithelial cells with BRIC 155. Arrow shows staining of the basolateral membrane (magnification $\times 362$).

of tubular epithelial cells or single cells separated by one or more nonreactive cells were observed to be strongly stained with anti-band 3 antibodies. More tubular cells were stained with BRIC 154 and BRIC 155 (at least half) than with BRIC 130 (about a third). The plasma membrane of the stained cells was strongly positive, particularly at the basolateral membrane of the cells, and the cytoplasm was generally also stained, but less intensely (Fig. 7*b*), suggesting that band 3 is located predominantly in the basolateral membrane of these cells.

The cellular distribution of band 3 observed in these experiments resembles that found in collecting tubules of human kidney from normal and cystic fibrosis patients (Hazen-Martin *et al.*, 1987). These workers used polyclonal antibodies directed at the *N*-terminal cytoplasmic domain of the protein. Band 3 staining has been observed to be associated with the intercalating cells of the kidney collecting tubules of other mammals (Drenckhahn *et al.*, 1985; Schuster *et al.*, 1986; Holthofer *et al.*, 1987), where it has been suggested to have a role in transcellular Cl⁻ movement and acid secretion (Greger, 1988).

Antibody reactivity during human liver development

The reactivity of all four antibodies was assessed on frozen sections of human fetal and adult liver. The human liver is a major site of haemopoiesis during embryonic development and therefore provided an opportunity to examine the expression of the red-cell anion transporter during development of the erythroid lineage. All four antibodies stained nests of haemopoietic cells in first and second trimester human fetal liver specimens but there was no detectable reactivity with developing hepatocytes, bile duct or sinusoidal cells (including endothelial cells). Occasionally, dense aggregates of antibody-reactive material, which probably represented enucleated red cells, were observed within the lumens of developing portal tracts. In the adult liver, staining by BRIC 130, BRIC 154 and BRIC 155 was confined to sinusoidal spaces within the parenchyme and probably reflected the presence of red cells in the sinusoids. A similar pattern was observed with BRIC 132 but, in addition, this antibody also showed intense staining of the smooth muscle of hepatic arteries within portal tracts as was also found with kidney tissue. The significance of this latter observation is unclear.

After the completion of this work and during the final preparation of this paper, Lieberman & Reithmeier (1988) published the results of a study which showed that the C-terminus of mouse red cell band 3 is located on the cytoplasmic side of the red-cell membrane. These workers used polyclonal antibodies raised against a synthetic peptide containing the twelve C-terminal amino acids of the mouse protein to probe the location of the C-terminus of the mouse protein. Our results with the human protein, based on the use of monoclonal antibodies, are entirely consistent with their conclusion.

This work was supported by grants from the Wellcome Trust (M.J.A.T.) and the Cancer Research Campaign (C.H.). J.E.Y. was supported by DHSS NHS Procurement Directorate grant RDV/44/75/01. We are grateful to S. Parsons and Dr. D. J. Anstee for their help in the production of monoclonal antibodies and to Dr. A. G. MacIver for assistance with the interpretation of the kidney staining results.

REFERENCES

- Boodhoo, A. & Reithmeier, R. A. F. (1984) *J. Biol. Chem.* **259**, 785–790
- Cox, J. V. & Lazarides, E. (1988) *Mol. Cell. Biol.* **8**, 1327–1335
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Drenckhahn, D., Schluter, K., Allen, D. P. & Bennett, V. (1985) *Science* **230**, 1287–1289
- Evans, P. R., Trickett, L. P., Smith, J. L., MacIver, A. G., Tate, D. & Slapak, M. (1985) *Br. J. Exp. Pathol.* **66**, 79–87
- Funder, J. & Weith, J. O. (1976) *J. Physiol. (London)* **262**, 679–698
- Gardos, G., Hoffman, J. F. & Passow, H. (1969) in *Laboratory Techniques in Membrane Biophysics* (Passow, H. & Stampfli, R., eds.), pp. 9–20, Springer-Verlag, Berlin
- Greger, R. (1988) *Annu. Rev. Physiol.* **50**, 111–122
- Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **138**, 313–316
- Hancock, R. J. T., Harvey, J., Evans, P. R., Hodges, E., Molnar, J., Martin, A., Lewis, L., Cohen, B., Laundy, G., MacIver, A. G. & Bradley, B. A. (1988) *Tissue Antigens* **31**, 165–173
- Hayashi, R. (1977) *Methods Enzymol.* **47**, 84–93
- Hazen-Martin, D. J., Pasternack, G., Hennigar, R. A., Spicer, S. S. & Sens, D. A. (1987) *Pediatr. Res.* **21**, 235–237
- Holthofer, H., Schulte, B. A., Pasternack, G., Siegel, G. J. & Spicer, S. S. (1987) *Am. J. Physiol.* **253**, C323–C328
- Jay, D. & Cantley, L. (1986) *Annu. Rev. Biochem.* **55**, 511–538
- Jennings, M. L. (1985) *Annu. Rev. Physiol.* **47**, 519–533
- Jennings, M. L., Anderson, M. P. & Monaghan, R. (1986) *J. Biol. Chem.* **261**, 9002–9010
- Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S. & Hamasaki, N. (1988) *J. Biol. Chem.* **263**, 8232–8238
- Kopito, R. R. & Lodish, H. F. (1985) *Nature (London)* **316**, 234–238
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–681
- Lepke, S. & Passow, H. (1976) *Biochim. Biophys. Acta* **455**, 353–370
- Lieberman, D. M. & Reithmeier, R. A. F. (1988) *J. Biol. Chem.* **263**, 10022–10028
- Lieberman, D. M., Nattriss, M. & Reithmeier, R. A. F. (1987) *Biochim. Biophys. Acta* **903**, 37–47
- Macara, I. G. & Cantley, L. C. (1981) *Biochemistry* **20**, 5095–5105
- Mahin, D. T. & Lofberg, R. T. (1966) *Anal. Biochem.* **16**, 500–509
- McDougal, J. S., Browning, S. W., Kennedy, S. & Moore, D. D. (1983) *J. Immunol. Methods* **63**, 281–290
- Nigg, E. A., Bron, C., Girardet, M. & Cherry, R. J. (1980) *Biochemistry* **19**, 1887–1893
- Parsons, S., Judson, P. & Anstee, D. J. (1982) *J. Immunogenet.* **9**, 377–380
- Schuster, V. L., Bonsib, S. M. & Jennings, M. L. (1986) *Am. J. Physiol.* **251**, C347–C355
- Tanner, M. J. A., Williams, D. G. & Kyle, D. (1979) *Biochem. J.* **183**, 417–427
- Tanner, M. J. A., Anstee, D. J., Mallinson, G., Ridgwell, K., Martin, P. G., Avent, N. D. & Parsons, S. F. (1988*a*) *Carbohydr. Res.* **178**, 203–212
- Tanner, M. J. A., Martin, P. G. & High, S. (1988*b*) *Biochem. J.* **256**, 703–712
- Tavare, J. M. & Holmes, C. H. (1989) *Cell. Signalling* **1**, 54–64
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Williams, D. G., Jenkins, R. E. & Tanner, M. J. A. (1979) *Biochem. J.* **181**, 477–493