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Basic proteins and basic membranes Adjusting blotting and staining conditions to Immobilon CD

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Abstract

Transfer efficiency from polyacrylamide gels and binding to Immobilon P and CD were tested in different buffers with ^{125}I-labelled proteins. With a derivatized poly(vinylidene difluoride) membrane, a pH 8 medium was found to be superior to a more alkaline solution, for both acidic and basic proteins. New staining protocols were tried on Immobilon CD. Toluidine Blue and iodine vapour gave a negative and a positive stain, respectively, with a fair band-to-background contrast. Protein sequencing after both stains was not impaired by interfering peaks. Biuret solution stained the protein bands pale pink but, even after copper removal with a chelating agent, it completely prevented Edman degradation. The first two procedures compare favourably with a commercial kit for protein detection, Quick Stain, that provides comparable sensitivity but results in several spurious peaks on protein sequencing.

1. Introduction

This work was intended to assess transfer conditions and to compare standard and new staining protocols for a recently marketed blotting membrane, specially devised for in situ protein fragmentation.

When sequencing by Edman degradation is attempted on proteins after one- or two-dimensional electrophoresis and blotting [1–6], various components show a blocked $-NH_2$ terminus. This usually results from co- and post-translational covalent processing of the peptides [7–10], although in a few instances it may be traced to artefactual chemical modifications on extraction and fractionation. No general protocol exists for $-NH_2$ terminus deblocking; instead, a few steps may be tried in sequence: formate and acetate moieties (if present on Ser and Thr) may be removed by chemical hydrolysis and pyroglutamate rings may be opened by enzymatic treatment [11]. Usually, however, it is only possible to digest the protein of interest and to fractionate, and eventually sequence, the internal fragments [12].

While $-NH_2$ sequencing of proteins is feasible both from dried polyacrylamide (PAA) gels and from immobilizing membranes [13,14], peptide recovery after in situ digestion is very poor from either source [15,16]. This latter observation has led to the recent development of a new blotting material, namely a basic poly(vinyledene diflu-

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oride) (PVDF) derivative. After experiments by Patterson et al. [17], Millipore marketed this product under the proprietary name Immobilon CD (Cat. No. ICDM 15150). On protein digestion, proteolytic peptides may be released either by the addition of trifluoroacetic acid (TFA), prior to HPLC separation [18,19]), or by extraction with sodium dodecyl sulfate (SDS), prior to electrophoresis (see below and Ref. [17]).

Owing to its positive charges, the new basic membrane is incompatible with standard acidic dyes, such as Coomassie Blue, that would bind with similar affinity to both sample and background. Therefore, Millipore provides Immobilon CD together with a staining kit (Cat. No. ICDM QS060), whose application is expected to result in a negative stain of the protein zones.

In a comparative investigation on the binding properties of Hep G-2 [20] proteins to immobilized metal chelate affinity chromatographic (IMAC) matrices, we showed how the sequence of two runs, on Cu- and Zn-loaded columns, was purifying a group of proteins, tentatively identified as the histone fraction [21]. In order to demonstrate positively the nature of these components, sequencing was selected as the most direct approach, since with histones an accurate assessment of the main physico-chemical parameters, p*I* and M_r, may be questioned in several respects [22–24]. Except for the H_2B polypeptide, the $-NH_2$ termini of histones are blocked by acetylation [25,26]; internal sequencing was then essential.

However, when detection of histone proteins resolved by SDS polyacrylamide gel electrophoresis (PAGE) and blotted to an Immobilon CD membrane was attempted according to the protocols and with the Millipore Quick Stain reagents [27], no signal could be detected, even at high protein concentrations, on the uniformly purple membrane (not shown).

This failure prompted the present investigation, aimed at defining optimum transfer procedures and searching for new, effective staining protocols.

2. Experimental

2.1. Blotting membranes

Immobilon P (standard PVDF membrane) and Immobilon CD (a basic PVDF derivative) were purchased from Millipore (Bedford, MA, USA).

2.2. Protein samples

A whole histone fraction was obtained from Hep G-2 cell lysate [20] by the sequence of Zn- and of Cu-IMAC [21]. Bovine serum albumin (BSA) and lysozyme were obtained from Sigma (St. Louis, MO, USA).

2.3. Electrophoretic separations and blotting procedures

Analytical protocols

Histones and their peptide fragments were resolved by SDS-PAGE on a 10–23% T PAA gradient (% T = total monomer concentration in a PAA medium), according to Schaegger and Von Jagow [28], in a Protean II vertical chamber (Bio-Rad, Richmond, CA, USA). Marker proteins were run on 5–20% T PAA gradients polymerized with the discontinuous buffer system of Laemmli [29] in a MiniProtean cell (Bio-Rad).

Blotting was either in 3-cyclohexylamino-1-propanesulphonic acid (CAPS) buffer (pH 11) [3] or in Tris–glycine (pH 8) [30]. The transfer, at 4°C, lasted 4 h at 300 mA in a 3-l cell or 2 h at 200 mA in a 1-l cell (Bio-Rad).

In order to assess the transfer efficiency under various experimental conditions, 25 μl of sample buffer, containing 2 μg of radiolabelled albumin or lysozyme (5000 cpm/μg) and 2 μl of prestained M_r markers (Bio-Rad), were loaded in triplicate on different lanes of four SDS-PAGE slabs. Blotting was towards Immobilon, either P or CM, at pH 8 and 11. Individual lanes were then cut and γ-counted, for 1 min, with background subtraction. The relative standard deviation among the replicas was similar to that with

repeated sample measurements with our Hamilton syringe.

Batch protocol

The sample was loaded on SDS-PAGE slabs across an 8-cm wide pocket. During the subsequent blotting two membranes, a 7-cm wide Immobilon CD foil in direct contact with the SDS slab and a 9-cm wide Immobilon P sheet anodal to it, were overlaid on the gel after stitching along one edge and marking with asymmetric cuts for easier alignment. At the end of the transfer, proteins on Immobilon P (two series of 0.5-cm-wide bands, one on each side) were detected according to standard Coomassie protocols [31]. After re-assembling this stained pattern with Immobilon CD, the position of the proteins of interest in the latter could easily be recognized.

2.4. Staining protocols

After transfer, the protein pattern was detected according to the following protocols:

(A) staining for 5 min with 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol; destaining for 5 + 5 min in 50% (v/v) methanol–10% (v/v) acetic acid [30];

(B) staining for 5 min with 0.2% (w/v) Ponceau Red in 3% (w/v) trichloroacetic acid; destaining for 2 + 2 min in 5% (v/v) acetic acid [31];

(C) according to Quick Stain (Millipore) instruction leaflet, 5 min staining, 5 min in developer solution, at 50°C [27];

(D) exposure of the dried membrane for 30 s to iodine vapours;

(E) staining for 5 min with 0.5% (w/v) Toluidine Blue in Tris–HCl (pH 6.8); destaining for ca. 1 min in 1% (v/v) acetic acid (modified from Ref. [32]);

(F) incubation for 10 min in biuret reagent, i.e., 0.02% (w/v) $CuSO_4$ [diluted from a 2.5% (w/v) stock solution, made up in 5% (w/v) sodium tartrate, with NaOH added to complete dissolution] in 2% (w/v) Na_2CO_3–4% (w/v) NaOH [33].

Protocols A and B were applied to Immobilon P foils and C–F to Immobilon CD.

2.5. In situ digestion and peptide analysis

In the batch experiment described above, after excision of the band of interest from Immobilon CD and cutting of the 2×70 mm^2 strip into ca. 1-mm^2 pieces, the latter were suspended in 70 μl of 0.1 *M* Tris–HCl buffer (pH 8.5) containing 1 *M* NaCl, 10% acetonitrile and 0.2 *M* urea [19]. A 1-μl volume of Lys-C (*Achromobacter* protease 1 [34], from Sigma, Cat. No. P-3428) was added, and the mixture was incubated for 15 h at 37°. After a second addition of proteolytic enzyme and a further incubation for 3 h, the sample was diluted to 1 ml with water and lyophilized, subjected to SDS-PAGE and blotted.

2.6. Protein sequencing

A 50-μg amount of β-lactoglobulin (from Sigma, Cat. No. L-4520) was subjected to SDS-PAGE and blotted either to Immobilon P (to be stained with Coomassie Blue) or to Immobilon CD (to be revealed with iodine, Toluidine Blue, Quick Stain and biuret). The –NH_2-terminus sequence was analysed on the five samples with an automated protein sequencer (Model 6625 Prosequencer).

3. Results and discussion

3.1. Batch application on Immobilon CD

Owing to the ample supply of purified proteins whose identity with a whole histone fraction we intended to establish, the batch protocol detailed under Experimental could be tried first. Fig. 1A shows the electrophoretic pattern (bands 1–4) of the starting material, as stained on the Immobilon P back-up. The strip corresponding to band 3 was excised for further treatment from the CD transfer membrane in direct contact with the SDS-PAGE slab. In Fig. 1B, the higher M_r

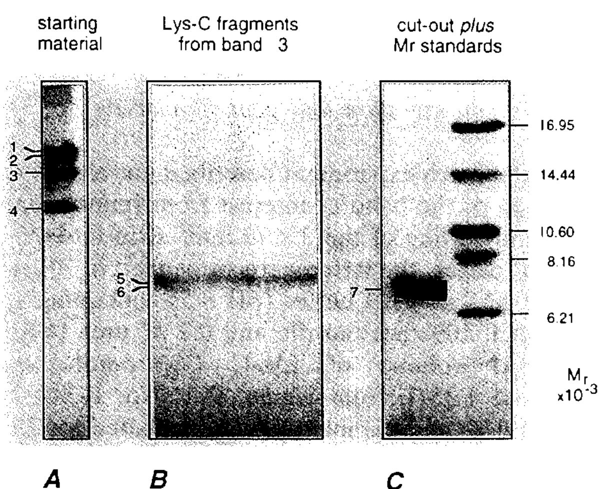

Fig. 1. Preparative fractionation of proteins on Immobilon CD with detection by Coomassie Blue staining on an Immobilon P back-up, prior to in situ proteolytic digestion and sequencing. A whole histone fraction [21] was subjected to SDS-PAGE on a 10–23% T PAA gradient [28] and blotted for 4 h at 300 mA in CAPS buffer [3]. (A) Protein pattern stained with Coomassie Blue on an Immobilon P foil, overlaid on an Immobilon CD membrane in direct contact with the SDS-PAGE slab. After excision of band 3 and cutting, the 1 mm^2 pieces were suspended in the digestion buffer and added with two aliquots of Lys-C endoprotease. (B) SDS pattern of the resulting higher M_r fragments (bands 5 and 6), on electrophoretic migration and blotting as for the sample in (A). (C) Cut-out (band 7) of the bands of interest along with CNBr–myoglobin fragments (from Merck) as M_r standards. All pictures are cropped to the area of interest.

proteolytic fragments (bands 5 and 6) from its Lys-C digestion [34] were stained on an Immobilon P blot, whereas from Fig. 1C the components to be analysed further by sequencing have been cut out. This protocol, resembling old proposals for preparative electrophoresis on slab gels, may only apply to readily available samples, with a simple banding pattern. For microtechniques, different approaches to protein detection had to be explored.

3.2. Comparing staining protocols on Immobilon CD

Although disappointing, it is not surprising that the staining contrast between a basic protein and a basic membrane was lost on Immobilon CD blots. Alternative detection procedures were then devised, with the proviso that detection of the bands of interest should interfere minimally with the following steps (digestion, fractionation, sequencing). At the same time, it should have a fair sensitivity and a broad specificity towards the revealed proteins; in addition, it should be as simple and inexpensive as possible.

Three new staining protocols for Immobilon CD were tried on blots to which increasing amounts (from 0.1 to 100 μg per lane) of two proteins, bovine serum albumin and lysozyme, had been transferred from SDS-PAGE slabs. The resulting patterns are shown in Fig. 2, together with control staining of Immobilon CD with Quick Stain (C: a negative band against a purple background) [27] and the standard results on Immobilon P with (A) Coomassie Blue and (B) Ponceau Red [31].

In the experiment in Fig. 2D, the dried membrane was briefly exposed (for ca. 30 s) to iodine vapour. The protein bands stain positively, with a typical brownish hue. Much of adsorbed I_2 may subsequently be removed by evaporation, most extensively if under reduced pressure. Resorting to this protocol was suggested not only by a long tradition in TLC but also by our own experience with oligopeptide detection following carrier ampholyte isoelectric focusing [35], although, in the latter instance, a negative instead of a positive signal could be obtained. In native PAGE, albumins may be specifically detected by iodine staining as transparent spots against a brown background, whilst other proteins are not detected [36]. Being reversible, iodine staining may be followed, if required, by any other detection protocol.

Fig. 2E shows the results obtained on staining with 0.5% Toluidine Blue at pH 6.8 and destaining in 1% acetic acid after extensive rinsing with distilled water. Treatment with acetic acid must be kept as short as possible and requires continuous and vigorous shaking. The bands appear clear against a blue background; the staining contrast increases as the membrane dries. The rationale for this trial was our confidence that the behaviour of a basic reagent would be just the opposite to that of typical acidic dyes, with

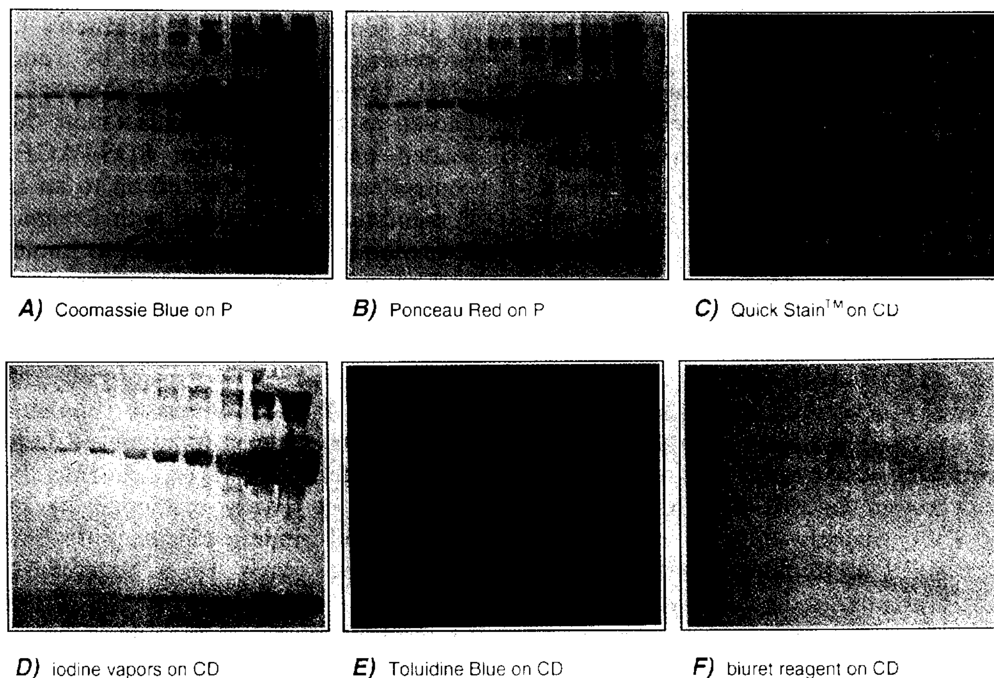

Fig. 2. Comparison of standard and new staining protocols for proteins blotted on to Immobilon CD. Various amounts of two proteins, BSA and lysozyme (from left to right 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μg per lane) were run on 5–20% T PAA gradients [29]. After blotting on to (A and B) Immobilon P and (C–F) Immobilon CD, the protein pattern was detected as follows: (A) Coomassie Blue stain; (B) Ponceau Red; (C) Quick Stain; (D) iodine vapour; (E) Toluidine Blue; (F) biuret reagent. All pictures were taken on the dried membranes.

the extra bonus of selectivity between proteins, which release promptly any bound colour, and membrane, which destains at a slower rate.

Finally, when the blotting membrane is immersed in an alkaline copper tartrate solution (biuret reagent [33]), proteins pick up within 10 min an obvious, if pale, pink shade, most evident when wet Immobilon CD is overlaid to a matt white background, and largely lost on membrane drying (Fig. 2F). Neither increasing the concentration of copper in the staining solution nor adding Folin–Ciocalteau reagent [37] seems to increase the staining intensity (not shown). From visual inspection, copper appears to be completely removed from the membrane by further incubation with 5 m*M* EDTA.

Across the concentration range used in our experiments, which is typical of the (micro)-preparative techniques for which Immobilon CD was devised, the staining intensity of protein zones is almost constant, and only their width varies. This is most evident for albumin, for which the 100-μg band is distorted into a 7×7 mm^2 circle versus a 7×4 mm^2 oval for the corresponding lysozyme sample. From this perspective, the detection limit is alike for the six staining protocols, since all of them can reveal even the lowest protein amount (i.e., 0.1 μg per lane). However, the band-to-background contrast each reagent is able to produce depends heavily on the colour shade it develops, and on whether the staining is positive or negative. By this criterion, we may rank the newly proposed staining protocols as follows: iodine vapours >toluidine solution >> Quick Stain >>> biuret reagent.

Quick Stain is much more expensive than the chemicals included in the other staining protocols and, contrary to them, requires both long-term storage of the stock solutions at −20°C and

blot incubation at 50°C. Thus, for cost and ease, the sequence is: biuret ⩾ iodine ⩾ toluidine >>> Quick Stain.

A further staining protocol was also tried: it has been reported that poly(vinyl alcohol) (PVA) can saturate "instantaneously" both nitrocellulose and PVDF blotting membranes [38], and it was hoped that its coating would quench the ionic interaction between the basic groups on derivatized Immobilon CD and acidic dyes. Among the latter, Coomassie Blue was compared with copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CPTS), reported to give a reversible stain [39]. It was hoped that its lower staining affinity yet high molar absorptivity would result in a satisfactory signal-to-background contrast. Different concentrations of PVA, namely 0 or 1 mg/ml for Immobilon P and 1 or 10 mg/ml for Immobilon CD, and one protein load, i.e., 35 μg of each of albumin and lysozime per lane, were used. Immobilon P and CM membranes were processed in parallel. On the former, the staining intensity after Coomassie Blue treatment is the same with and without PVA treatment. With CPTS and no PVA, a fair band-to-background contrast is observed as long as the membrane is stored wet, whereas the staining fades on drying. After PVA treatment, lysozyme stains negatively against a darker background, while the albumin band can no longer be detected. Unfortunately, no protein zone can be revealed on Immobilon CD under either set of conditions, except for a hint of a BSA band position on Coomassie Blue staining. With CPTS, the background was pale blue after incubation with 1 mg/ml PVA and clear on saturation with 10 mg/ml PVA (not shown). No further attention was devoted to the alternative staining protocols for PVDF already assessed previously [40].

3.3. *Structural analysis on blotted and stained proteins*

Efficient detection is a prerequisite for an effective analytical procedure, and it is of the utmost importance to ascertain that staining procedures do not bring about structural alterations in the sample components. The adequacy of the blotted and stained proteins for further processing was then tested by running their $-NH_2$ terminus sequence. β-Lactoglobulin was chosen as the reference substance; 50 μg were loaded per lane on an SDS-PAGE slab. A control sample was blotted on to an Immobilon P membrane and stained with Coomassie Blue; four samples were transferred to Immobilon CD and processed as the strips in Fig. 2C–F. Fig. 3 compares the maximum initial yields from the various membranes (average for cycles 3–5), expressed as percentage recovery by reference to Immobilon P and Coomassie Blue staining. All samples processed on Immobilon CD provide an approximately 50% lower yield than the control. This may be partly explained by differential transfer and binding efficiency on blotting (see below), and partly by a progressive if slow desorption of bound proteins from derivatized PVDF during Edman degradation. Among the test runs, the maximum initial yield (52%) was obtained on band detection with iodine vapour. Some spurious peaks were observed,

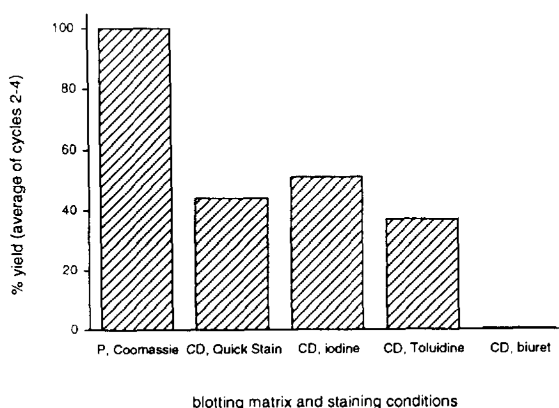

Fig. 3. Initial yield on automatic sequencing by Edman degradation of β-lactoglobulin blotted to different immobilizing membranes and stained according to various protocols. A 50-μg amount of β-lactoglobulin was blotted either to Immobilon P or to Immobilon CD. The protein sequence was analysed on the stained patterns with an automated protein sequencer (Model 6625 Prosequencer). Average values for the percentage yield over cycles 3–5 (by comparison with a Coomassie Blue-stained sample, taken as reference) are reported. On the abscissa, P represents Immobilon P and CD represents Immobilon CD.

notably an interfering signal with a retention time similar to that of Trp-phenylthiohydantoin (PTH) derivative. A lower recovery was obtained from the Toluidine Blue-stained blot (37% in comparison with the Coomassie Blue-stained sample). The sequence was clearly readable throughout, except during the first cycle, the chromatogram for which was obscured by the presence of a large interfering band with a retention time intermediate between those of Asp- and Tyr-PTH derivatives. In contrast, no amino acid residue could be removed from the protein terminus after copper–EDTA treatment; the yield was zero. Finally, Quick Stain tracing was obscured by many interfering peaks, and in this respect was much worse than for all other samples, whereas, in a quantitative sense, its initial yield (44%) ranked it between the former two. For this test, the scores among staining protocols may thus be summarised as: toluidine solution ⩾ iodine vapour >> Quick Stain.

3.4. Assessing transfer conditions

All blotting experiments reported so far were performed in CAPS–NaOH (pH 11) [3] instead of in standard Tris–glycine (pH 8) buffer [30]. The former medium was selected in order to lower to zero the glycine background on amino acid analysis and to avoid Tris buffers with their HCHO contaminants that may artefactually react with free $-NH_2$ [41]. Moreover, Patterson et al. [17] reported a similar binding affinity of BSA at either pH, as evaluated by the staining intensity on a nitrocellulose foil backing up the Immobilon CD membrane. We wondered, however, whether the same would hold not only for a mildly acidic protein (the pI of apo-albumin is about 6) but also for alkaline components. As the pK of the basic groups on Immobilon CD has not been disclosed, it is merely speculative to evaluate ratios between their dissociated and undissociated forms on membranes under different conditions. On the other hand, if the interacting species was a protein–SDS micelle, there should be no difference in its surface charge between pH 8 and 11, or in the electrostatic attraction between the membrane and acidic proteins, once stripped free of SDS. For basic proteins, the electrostatic repulsion towards positive charges should decrease as their pI is approached (and would change to attraction, were the pH higher than the pI). To clarify this point, we reacted with ^{125}I [42] both BSA and lysozyme. We then loaded either albumin or lysozyme on SDS-PAGE slabs. Each sample contained ca. 10 000 cpm of labelled and 2 μg of "cold" protein, together with pre-stained M_r standards. Blotting was towards Immobilon P and CD membranes, either at pH 8 (Tris–glycine) or at pH 11 (CAPS–NaOH). Individual lanes, detected after the stained bands, were cut and their radioactivity was measured in a γ-counter. The results, expressed for each protein with reference to counts on Immobilon P after blotting at pH 11, are plotted in Fig. 4. In all instances, more radioactivity binds to the filter at pH 8 than at pH 11. The difference is low for albumin (Fig. 4A) on Immobilon P, negligible for lysozyme on Immobilon CD (B) and substantial in the remaining two cases (ca. 50% increase for lysozyme on Immobilon P, >100% increase for albumin on Immobilon CD). Overall, the binding to Immobilon CD is ca. 25 (at pH 11) to 50% (at pH 8) lower than to Immobilon P for lysozyme, ca. 20% higher for albumin at pH 8 and 50% lower for albumin at pH 11.

For the pH 11 experiment, the more or less conspicuous traces of prestained M_r standards left behind by electroblotting prompted a further evaluation of transfer efficiency by γ-counting the corresponding lanes in PAA gels. In comparison with the loaded amounts, the recovery in the Immobilon P experiment was 46% on the blot, 16% in the gel for albumin and 16% and 13%, respectively, for lysozyme. On Immobilon CD the figures changed as follows: albumin, 23% on the blot, 47% in the gel; and lysozyme, 11% on the blot, 9% in the gel. These data indicate once again how difficult it may be to adjust experimental parameters for an efficient transfer of all components in a highly heterogeneous sample. Moreover, the choice of a different reference parameter to that of Patterson et al. [17] allows a re-evaluation of the optimum blotting conditions for Immobilon CD: a pH 8

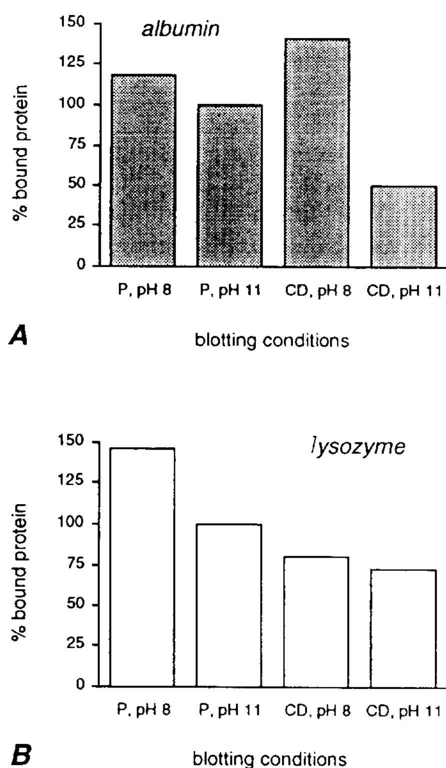

Fig. 4. Relative binding efficiency to Immobilon P and CD membranes under various blotting conditions. Radiolabelled albumin or lysozyme, together with prestained M_r markers, were run and blotted towards either Immobilon P or CM at pH 8 and 11. Individual lanes were cut and γ-counted. Average values from triplicate replicas were calculated as percentage binding efficiency, by comparison with the yield on Immobilon P at pH 11 taken as reference. (A) albumin; (B) lysozyme. On the abscissa, P represents Immobilon P and CD represents Immobilon CD.

buffer is always to be preferred to more alkaline media for this membrane.

4. Conclusions

From the evidence above, Tris–glycine buffer, according to Towbin et al. [30], is to be used when electroblotting from SDS-PAGE slabs to Immobilon CD; even under optimum transfer conditions, however, the binding to this basic membrane was found to be less efficient for a model basic protein (lysozyme) than for a neutral molecule (albumin).

On Immobilon CD, Toluidine Blue staining has at least equal sensitivity in protein detection to the commercial Quick Stain kit. Similarly to the latter, Toluidine Blue provides a negative stain, which allows for a negligible background, limited in fact to a single spurious peak during the first cycle of protein sequencing. Overall, the sequence data describe the blotted protein (β-lactoglobulin) as structurally integer, in provision for further analysis. Staining according to the proposed protocol is indeed performed at neutral pH with just a short exposure to dilute acetic acid.

Slightly higher contamination, with the constant presence of a peak co-eluting with the Trp derivative, is observed on Edman degradation after membrane exposure to iodine vapour. Although this may be a minor nuisance for most applications, and nothwithstanding an initial yield one third higher than with Toluidine Blue, we rank iodine treatment as a second-choice procedure, and Toluidine Blue staining is the preferred method for detecting both acidic and basic proteins blotted to Immobilon CD. In contrary, heavy chromatogram contamination and practical and economic considerations disfavour, in our opinion, the use of the commercial Quick Stain kit.

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