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A Rapid Filtration Method for Receptor Binding: Characterization With Mu and Delta Opiate Receptors

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ZADINA, J. E. AND A. J. KASTIN. *A rapid filtration method for receptor binding: Characterization with mu and delta opiate receptors.* PHARMACOL BIOCHEM BEHAV 21(6) 947–952, 1984.—A commercially available (Skatron) cell harvester was adapted for use in mu (^{3}H-naloxone-labeled) and delta (^{3}H-DADLE-labeled) opiate receptor assays and compared with a widely used conventional manifold for a number of binding characteristics. Whatman GF/B glass fiber filters and the less expensive filters available with the harvester were also compared and produced similar binding characteristics on the harvester and manifold if the harvester filters were used double-ply, and if the rinse time was less than 12.5 sec. Longer rinse times produced lower binding with 2-ply Skatron filters. Kd values, Hill coefficients, and Scatchard plot regression coefficients were very similar for the two filtration devices and filter types. A significantly reduced maximum number of sites (Bmax) was observed after filtration on the harvester, reflecting the smaller filter surface area relative to that of the manifold. The filter surface area on the harvester, nevertheless, is considerably larger than that of other manifolds with microplate spacing. This provides the advantages of rapid filtration with less restriction on tissue concentrations. Specific binding was linear with protein concentration up to at least 800 μg protein, which is well within the range of most neurotransmitter and peptide receptor binding studies. At about 1 mg protein the rinse buffer flow was slower due to the high tissue concentration. Although the results of filtration with the harvester and the conventional manifold were similar, the time requirements differed considerably. With the harvester, one experimenter could conduct the filtration process 2–3 times faster than 2 experimenters using the manifold. Thus, the harvester provides a fast, easy method to study receptor binding and is suitable for opiates and peptides as well as neurotransmitters.

Opiate receptors Mu and delta opiate receptors Filtration Manifold Cell harvester Peptides

THE most commonly used methods for separation of bound and free radiolabeled ligand in studies of receptor binding involve pouring the incubation material over glass fiber filters under vacuum. Except when centrifugation or dialysis are required for rapidly dissociating receptor-ligand complexes (e.g., the GABA receptor), filtration is the method of choice [1]. However, the conventional filtration manifolds developed for this purpose generally require laborious and time-consuming individual handling of both incubation tubes and filters. This creates an important limitation on the size of the assays and speed with which they are processed.

In order to reduce the time and effort involved in filtration and filter handling, we tested a device originally designed for the rapid harvesting of multiple-well, ^{3}H-thymidine-labeled lymphocyte microcultures [3] for use in the measurement of mu (^{3}H-naloxone-labeled) and delta (^{3}H-DADLE-labeled) opiate receptors in rat brain membranes. We compared the binding characteristics after use of the harvester with those observed after filtration on one of the most widely used conventional manifolds and found that the harvester yielded comparable binding values at a considerable savings in time, effort, and expense.

METHOD

Separation Systems (Filtration Devices) and Filters

Figure 1 illustrates the two filtration devices used to separate bound from free ligand: (a) The Millipore model 1225 12-place sampling manifold (Millipore Corp., Bedford, MA), and (b) The Skatron model 7000 12-well cell harvester (Skatron, Inc., Sterling, VA). Three types of filters were used: (a) GF/B circles or sheets from Whatman, Inc. (Clifton, NJ); (b) the filtermats supplied with the Skatron cell harvester, which are similar to the Whatman 934-AH glass fiber filters. Nominal retention size with this filter is 1.5 μm vs. 1.0 μm for the GF/B. However, since nominal retention is determined in large part by thickness, and doubling the 0.30 mm thick Skatron filters provides a reasonably close approximation to the 0.71 mm thick GF/B at a considerably reduced price, we also tested double-ply Skatron filters; (c) In one experiment, we also tested the Schleicher and Schuell (S&S) #32, a 0.65 mm filter advertised as having been designed to replace GF/B filters at a lower cost.

Binding Assays

Tissue preparation: Male albino rats obtained from Zivic-Miller (Allison Park, PA.) weighing 200–300 g were decapitated and their brains were rapidly removed and placed on ice for dissection. After discard of the cerebellum, crude membrane fractions were obtained by homogenization of tissues in 50–100 volumes of 50 mM TRIS (pH 7.5 at 4°C) and centrifugation at 10,000 g for 10 min. Pellets were reconstituted in 20 or 40 volumes of TRIS, and, for some of the

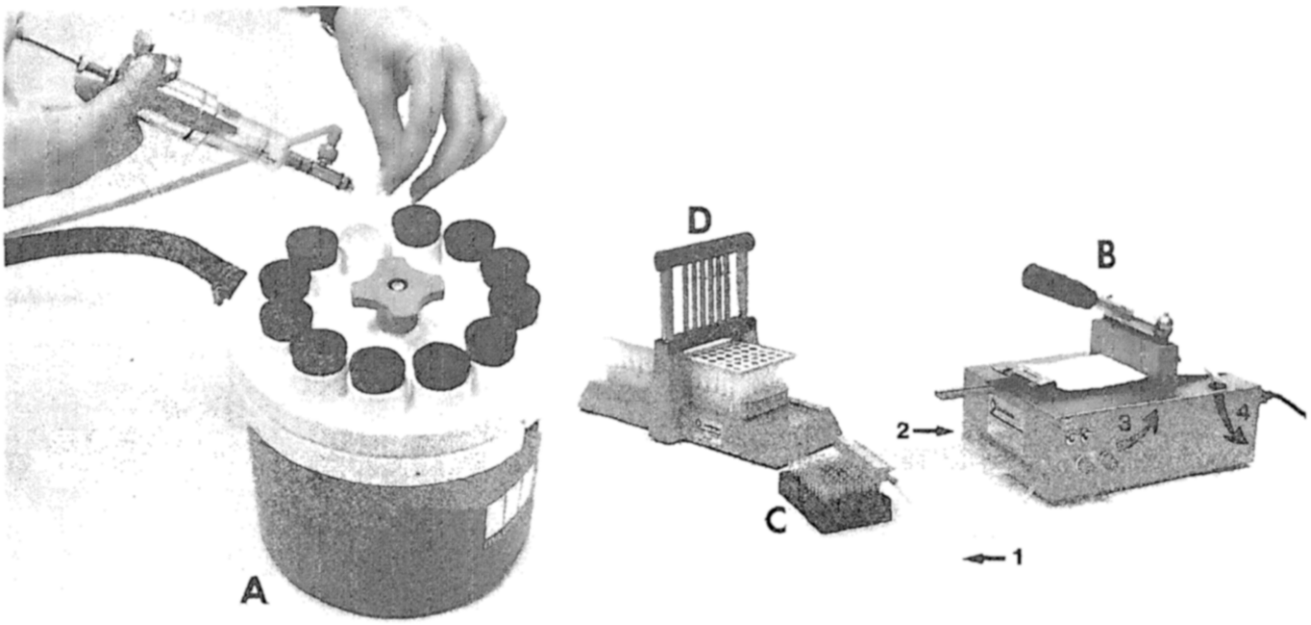

FIG. 1. Separation systems. Left: Millipore model 1225 12-place sampling manifold (A). Right: Skatron cell harvester (B) with microplate-spaced 1 ml tubes (C) and filterpunch device (D). Arrows indicate direction of flow: (1) Rinse buffer flows through single hose into 12 incubation tubes at same rate and volume; (2) incubation mixture and rinse buffer are drawn from tubes through 12 hoses and up (3) through filters; (4) unbound ligand is drawn out to waste trap.

assays, were quickly frozen with acetone and dry ice and stored at −70°C. Preliminary experiments indicated that negligible loss of binding occurred with this freezing procedure.

"Mu" receptor assays. Membranes prepared as described above were incubated in duplicate or triplicate with ^{3}H-naloxone (S.A. 37–60 Ci/mmole, New England Nuclear) and with either levallorphan (1 μM), a close congener of naloxone, to determine non-specific binding, or with the inactive enantiomer dextrallorphan (1 μM) to determine total binding. Stereospecific binding was defined by subtraction of mean nonspecific counts from mean total counts. All tubes contained 100 mM NaCl and 0.1% bovine serum albumin (BSA) and were incubated on ice for 60 min. "Single point" assays were conducted with a 1.5 nM concentration of ^{3}H-naloxone. For most experiments, however, in order to insure that the methodological comparisons reflected sampling over a binding isotherm useful for estimation of dissociation constants (Kd) and maximum number of binding sites (Bmax), 6 concentrations (0.25–8 nM) of tracer were employed and Scatchard analyses were performed.

"Delta" receptor assays. Membranes were incubated with ^{3}H-D-Ala^2-D-Leu-enkephalin (DADLE) (S.A. 40–54 Ci/mmole, Amersham) with or without Leu-enkephalin (1 μM) for 30 min at 23°C. Bacitracin (80 μg/ml) was included to inhibit degradation of peptides, and all tubes contained 0.1% BSA. Specific binding was determined by subtraction of counts in the presence of Leu-enkephalin from those observed in its absence. The concentrations of labeled peptide for Scatchard and "single point" assays were the same as those described above for ^{3}H-naloxone.

The use of these procedures for measurement of mu and delta opiate receptors neither assumes complete separation of receptor subtypes nor precludes the possibility of a single receptor with varying forms or conformations.

Filtration Procedure

Filtration with the Millipore manifold was performed as follows: filters were presoaked in buffer, positioned on the manifold, and the port plate (with stoppers in all ports except for the first) was fastened on top of the filters. To insure that the vacuum remained constant for different sets of tubes, different assays, and for both filtration systems, vacuum generated by a Welch Duo-Seal vacuum pump was adjusted with a bleeder valve to 50 cm H_2O (37 mm Hg) as measured by a Sears vacuum gauge. The assay incubation, which was conducted in 12×75 mm borosilicate tubes, was stopped with 4 ml of cold (4°C) buffer and the mixture was immediately poured over the filter followed by 3 rinses with 4 ml of cold buffer. The stopper from the second port was then transferred to the first port and the second incubation tube was processed in the same manner as the first. The procedure was repeated for the remaining 10 ports. Then the filters were removed, dried at 60°C for 2 hr, and placed in scintillation vials with NCS:OCS (Amersham) that was mixed according to the procedure recommended by the vendor for use with dried membranes bound to glass fiber filters: 55.5 ml of distilled water were added to a 500 ml bottle of NCS, that was then diluted 1 part NCS with 10 parts OCS, and 7.0 ml of the mixture was pipetted into each vial. The vials were covered with aluminum foil and kept at 4°C overnight to allow solubilization of membranes before scintillation counting.

For the cell harvester, one of 2 available suction heads could be used, allowing a choice of several different sizes of incubation tubes. The small suction head was used for 250 μl microplate wells and for 1 ml tubes with microplate spacing. The larger suction head was used for 10×75 mm or 12×75 mm tubes arranged in a standard test tube rack (1.75 cm spacing between tubes). Filtration on the cell harvester was con-

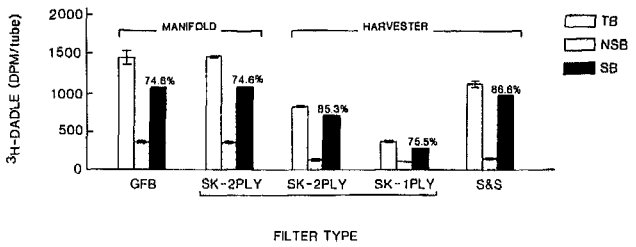

FIG. 2. ^{3}H-DADLE (delta opiate receptor) binding: effect of filter type and separation system. Rat brain membranes were incubated with 1.5 nM ^{3}H-DADLE and total (open bars), nonspecific (hatched bars) and specific (solid bars) binding were determined after filtration on the Millipore manifold (left 2 sets of bars) or the Skatron harvester (right 3 sets of bars). GF/B filters were compared to double-ply Skatron filters (SK-2ply) on the manifold and the SK-2ply filters were compared to a single-layer filter of the same type (SK-1ply) and to the Schleicher and Schuell (S & S) filter on the harvester. Percentage of total binding that was specific is given above the SB bars. Error bars are S.E.M.

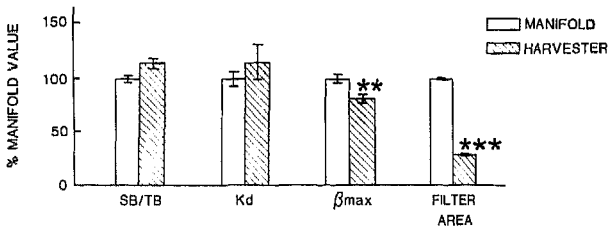

FIG. 3. ^{3}H-naloxone (mu opiate receptor) binding characteristics: comparison of manifold to harvester. Six concentrations (0.25-8 nM) of ^{3}H-naloxone were used to compare binding characteristics on the manifold with those on the harvester (100% value). SB/TB: specific relative to total binding (mean from all 6 concentrations). Kd: estimated dissociation constant (mean of 5 determinations in 1 assay); Bmax: estimated maximum number of binding sites.

ducted as follows: a filter mat was aligned, rough side up, in the first alignment notch and clamped, forming a 10 mm filter disk at each of the 12 pairs of O-rings in the filter clamp. Vacuum was then adjusted as described above to 50 cm. Two switches on the machine controlled the filtration operation. The "rinse" button allowed the gravity-fed buffer from a reservoir 1.3 m above the device to flow through the 18 ga. inflow cannulae in the suction head into 12 incubation tubes. The "air" button allowed the vacuum to draw the contents of the incubation tubes through 15 ga. outflow cannulae (positioned adjacent to the inflow cannulae on the suction head), up through the filters, and out to the waste trap. To presoak the filters, the suction head was placed in a row of empty tubes and both buttons pressed simultaneously for 5 sec. The "air" button alone was held for an additional 5 sec to insure clearance of the buffer. The suction head was then transferred to the first set of incubation tubes and when the vacuum was re-established, the "rinse" and "air" buttons were simultaneously pressed for the appropriate rinse time (usually 10 sec). The "air" button was held for an additional 10 sec to clear the buffer and to prevent backflow onto the filters. The filter mat was then released, advanced to the next notch, and the procedure was repeated. The distance between the filters on the harvester was sufficient to prevent cross-contamination within a set of tubes, and the 10 sec (5 ml) rinse eliminated residual radioactivity between sets. After 8 sets of 12 tubes, the filtermat was removed, dried at 60°C for 2 hr, and placed on the filter punch template (see Fig. 1). The template was placed on a rack of scintillation vials arranged in 16 rows of 6 tubes. The 6-place filter punch device was used to rapidly transfer the filters to the vials. The device was equipped with 4 prongs for punching the 10 mm circles out of the filter mat and a sleeve for ejecting the filters from the prongs into the bottom of the vials. The NCS:OCS mixture (3.5 ml) was easily added to the vials while still in the rack. The solubilization and counting procedure was the same as described above.

Statistical Analyses

Results were analyzed by analysis of variance followed, where appropriate, by the Newman-Keuls Multiple Range Test.

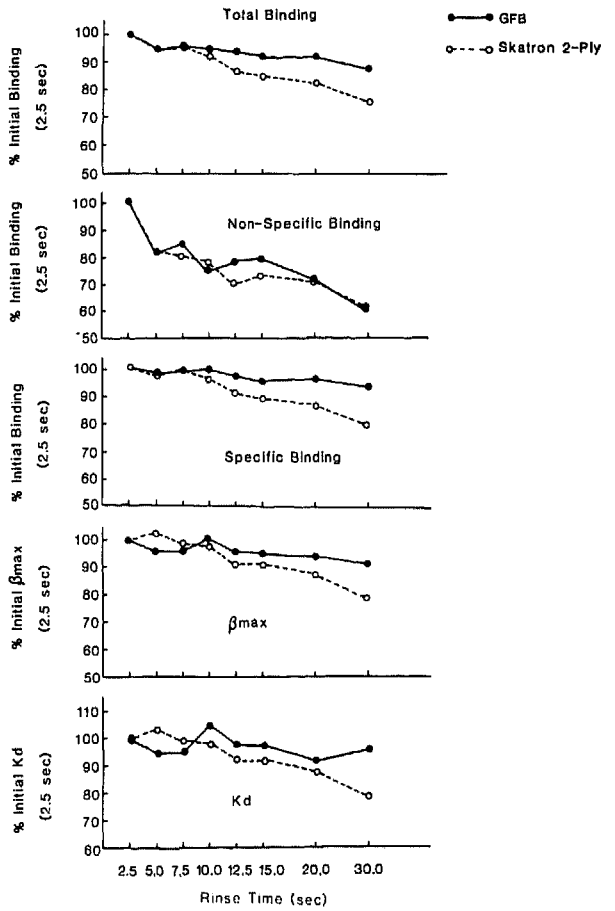

FIG. 4. Effect of rinse time and filter type on binding parameters. Binding levels at each time point for each of 6 concentrations of ^{3}H-naloxone were standardized relative to binding of the same concentration at 2.5 sec (100%). Filled circles and solid lines: GF/B filters. Open circles and broken lines: double-ply Skatron filters.

RESULTS

1. Comparison of Skatron Harvester to Millipore Manifold and GF/B to Skatron Filter Mats

The results of Experiment 1, shown in Fig. 2, demonstrate that, for ^{3}H-DADLE binding, the double-ply Skatron

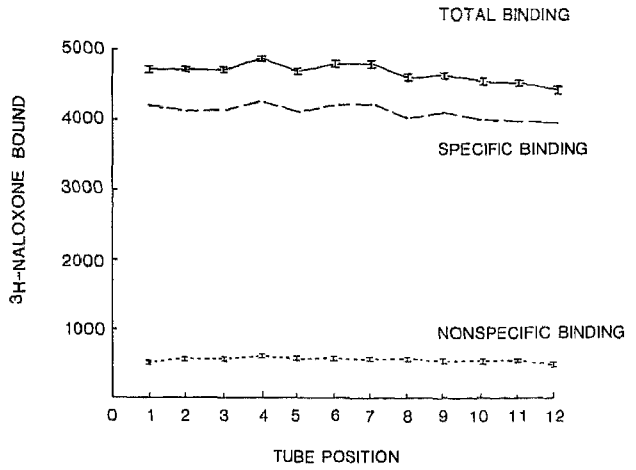

FIG. 5. Effect of tube position on binding. Membranes were incubated with 1.5 nM ^{3}H-naloxone and total, nonspecific, and specific binding determined at each position.

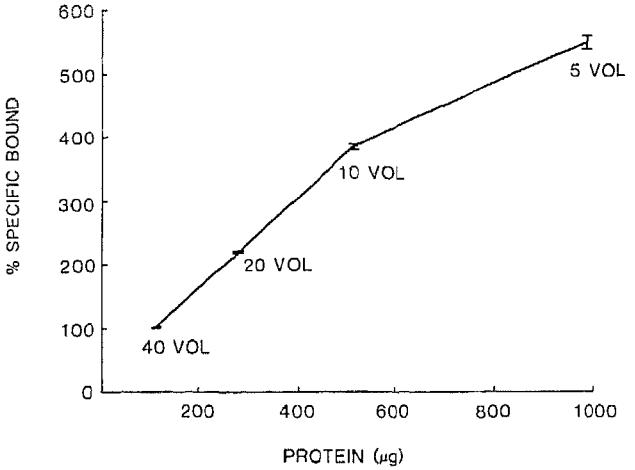

FIG. 6. Effect of protein load (tissue concentration) on specific binding. The lowest concentration (110 μg) was used as the 100% binding level for each of the 6 concentrations of ^{3}H-naloxone.

filter mats produced binding characteristics [total (TB), nonspecific (NSB), and specific binding (SB)] very similar to those observed with the GF/B filters. As described below, the two filter types also produced similar binding characteristics when ^{3}H-naloxone was the labeled ligand. The proportion of specific binding relative to the total binding was at least as high on the harvester as it was on the manifold. This was true with both single and double-ply Skatron filters and with S&S filters. As expected, filter surface area and thickness were important factors in the amount of ligand retained. The diameters of the portion of the filtermat through which the membranes were filtered (diameter within the sealing ring) is considerably lower on the harvester (10 mm) than the manifold (19 mm), resulting in a surface area that is 27% that of the harvester. This difference is likely to have contributed to the different levels of binding on the two devices. Thinner (1-ply) and slightly thicker (S&S) filters produced binding levels that were lower and higher, respectively.

These findings were confirmed in saturation assays with ^{3}H-naloxone. As shown in Fig. 3, the amount of specifically bound ligand relative to total bound was again slightly higher on the harvester than on the manifold. While not statistically significant, this pattern was observed in all assays comparing the two filtration devices and reflected slightly lower nonspecific binding on the harvester.

The Kd values estimated by the manifold (0.50 nM) and the harvester (0.57 nM) were not significantly different. Hill coefficients (1.095, 1.078) and regression coefficients of Scatchard plots (0.982, 0.995) were also not significantly different. The estimated maximum number of sites (Bmax) on the harvester, however, was 82% of that estimated by the manifold (153 vs. 188 fmol/mg protein, $F(1,6)=19.67$, $p<0.005$). This difference is probably due, at least in part, to the considerably smaller, $F(1,10)=4209$, $p<0.001$, surface area of the filters on the harvester.

2. *Effect of Rinse Time on Binding*

Figure 4 shows 5 binding parameters (total, nonspecific, and specific binding, Bmax and Kd) measured on the Skatron harvester as a function of rinse time and filter type (GF/B vs. 2-ply Skatron filters). For the first 3 parameters, the values at each time point represent the mean of the values from 6 concentrations of ^{3}H-naloxone (0.25–8.0 nM). The differences in binding levels at these varying concentrations were standardized by expressing the binding value at a given concentration and time point relative to the binding for that concentration at the 2.5 sec time point. Thus, the 2.5 sec value was 100% at each concentration of naloxone. Both filter types showed a relatively rapid decrease in both total and nonspecific binding between 2.5 and 5 sec, producing little change in the remaining 3 parameters. As expected from the results described above, very little difference between 2-ply Skatron and GF/B filters was observed in any of the parameters before the 12.5 sec rinse time. While the 5 parameters were fairly stable over the entire set of rinse times tested for the GF/B filters, however, a noticeable decline in specific binding, and therefore total binding and Bmax values, was observed beginning at about the 12.5 sec rinse time for the Skatron filters.

It is apparent from Fig. 4 that the optimal rinse time is between 5 and 10 sec, when the binding parameters are stable and similar for both filter types. Although the values at each time were expressed relative to the same filter type at the 2.5 sec rinse time, the Bmax values and mean specific binding at 2.5 sec for the 2 filter types were within 2% of each other. For rinse times longer than 12.5 sec, however, the GF/B filters are preferable.

Four replications of the 10 sec rinse time with 6 concentrations of tracer were used to determine intra-assay variability with the two filter types. Duplicate (2TB, 2NSB) tubes were used. For the Skatron 2-Ply filters, coefficients of variation (CV: standard deviation divided by the mean) were 3.05% for TB, 7.9% for NSB, 9.16% for Bmax, and 9.88% for Kd. Corresponding values for GF/B filters were 3.96%, 5.26%, 4.7%, and 9.43%. Inter-assay variability (CV) for 3 separate comparable assays was 8.4% for Bmax and 9.8% for Kd.

3. *Effect of Tube Position on Binding Characteristics*

Hung *et al.* [4] showed that with a manifold such as the Millipore unit used in this study, a binding artifact can arise simply as a consequence of the sequence of processing the incubation tubes if the vacuum is not carefully controlled at

each port by the use of stoppers in the other ports. We used the stopper method recommended by Hung *et al.* [4] in all assays on the manifold and also tested for the possibility of any similar artifacts on the harvester. Figure 5 shows the mean TB, NSB, and SB produced by 6 replications of incubation tubes processed through each of the 12 positions on the harvester. No statistically significant differences were obtained among any of the positions for TB or SB. In one experiment, however, the nonspecific binding in the first and the last positions were significantly lower than in other tubes. This was not confirmed in 2 other experiments, and the SB was not significantly changed. However, one precaution that we now take, whenever possible, to ameliorate the possibility of an artifactual change in SB arising from position is to place corresponding TB and NSB tubes in the same positions in adjacent rows.

4. Effect of Protein Load (Tissue Concentration) on Binding

Figure 6 shows the effect of increasing the concentration of the tissue in the incubation (0.5 ml) on the specific binding observed after filtration on GF/B filters on the harvester. A dilute concentration (100 μl of membrane homogenate at 40 vol or 40 ml/g original wet weight, corresponding to 110 μg of protein) provided the baseline level (100%) of specific binding. Data from 6 concentrations of ^{3}H-naloxone were normalized by expressing each value as a percentage of that observed at the same tracer concentration at 110 μg protein. The increase in specific binding was a linear function of protein (tissue) concentration up to at least 515 μg of protein (10 vol) in this first experiment. One mg of protein (5 vol dilution), however, was too viscous a solution to filter rapidly. An increase in the time required for the tissue to pass through the filter resulted in accumulation of buffer in the tubes and a prolonged and incomplete wash since some of the 5 ml of buffer was not drawn out of the tubes in the 10 sec vacuum period after the 10 sec rinse. In this experiment, a deviation from linearity in specific binding was observed at the high concentration of tissue. In a second experiment, however, specific binding observed at 1000 and 1300 μg protein showed no deviation from linearity despite slight (about 0.5 ml) and moderate (about 2 ml) accumulation of buffer. At 800 μg, neither accumulation of buffer nor deviation from linearity occurred, indicating that the upper limit of protein concentration for which the harvester can be used is at or above that level. The Millipore manifold showed no deviation from linearity up to 1.5 mg protein and a slight deviation from linearity and increase in rinse time at 2 mg.

Since the standard protein concentration (about 300 μg) used by us in assays of opiate receptors was well within the linear portion of the curve for the harvester, it is clearly suitable for standard assays and even for studies using varying protein concentrations up to about 800 μg.

5. Comparison of Processing Times

The use of microplate-spaced tubes increased the speed of initiating incubations largely because of the use of pipets with multiple tips designed for these plates (e.g., the 4-tip pipet from Titre-Tek). With the harvester, we allowed 3 min intervals between sets of 24 tubes. With an extra interval added between every other 96-tube plate for waste disposal, we were able to process 448 tubes per hour. For the manifold, we allowed 4 min intervals between sets of 12 tubes, with an extra interval every 2 racks (144 tubes) and a second experimenter to transfer the filters. We therefore processed 196 tubes per hour with the manifold. Thus, processing for 1 experimenter on the harvester was 2.3 times faster than for 2 experimenters on the manifold. Although processing can be accomplished faster with both methods, we found that these intervals allowed reasonable time for vacuum adjustments, waste disposal, and minimization of errors. Further savings in time resulted from the faster addition of scintillation fluid that was possible with the small tubes in the rack used with the harvester, a procedure that was more than 2 times faster than addition to the larger, individual vials used with the manifold. Lower costs resulted from less expensive filters, smaller scintillation vials, and less scintillation fluid.

DISCUSSION

Adaptation of the cell harvester for measurement of opiate receptors provided estimates of binding parameters comparable to those measured with the conventional filtration manifold, but with a considerably reduced expenditure of time and effort. These studies thus demonstrate the suitability of the harvester for opiate receptor studies and characterize its effects on a number of variables involved in the performance of the assays.

The filters delivered with the machine, which are similar in content and, when used double-ply, approximate the thickness of GF/B filters, produced binding characteristics comparable to those of the GF/B filters when the rinse time did not exceed 10–12 sec. In addition to the advantage of the filtermats being precut and notched to fit the harvester, the main advantage is cost. At the time of this writing, the cost of the Skatron (double layer) filters relative to the GF/B filters ranged from 29–59%, depending on volume and size specifications.

Comparison of the binding values of the harvester to those of the conventional manifold revealed no significant differences in Kd, Hill coefficients, or Scatchard plot correlation coefficients. Differences in the surface area of the filter outlined by the O-ring seal, however, may contribute to differences in the proportion of membrane retained, and hence the binding levels (including the estimate of Bmax). These were found to be lower on the harvester than on the manifold. The differences were not large but were statistically significant. This should have little effect on most receptor studies since the different groups in relevant comparisons will be similarly processed. The finding may need to be considered, however, if binding values are slightly lower than those previously found within a laboratory or in other laboratories. Where more precise estimates of binding differences between the systems are required, the amount of protein retained on the filters could be measured for each method after filtration of the relevant tissue concentration from the appropriate source, and the difference used to adjust binding levels. A similar decrease in binding may occur with recently available microplate manifolds with filter areas even smaller than those on the harvester.

An incubation concentration of 1 mg protein/0.5 ml appeared to overload the filters. However, this concentration was well above the range commonly used for standard opiate receptor and most established peptide and neurotransmitter receptor assays (c.f. [1]).

Specific binding consistently represented a slightly greater proportion of the total binding on the harvester than on the manifold due to lower non-specific binding. Inter- and intra-assay variability were low with the harvester even when only duplicate tubes were used. Two sizes of suction head permitted flexibility in the type of incubation tubes used. We

did notice that incubations in the range of 0.5 ml were best conducted with the 1 ml tubes, which produced less variable results than the 12×75 tubes in this range, but that 1 ml incubation did not mix well in the 1 ml tubes and that 10-or 12×75 mm tubes are preferable in that instance.

A number of variables that can change the "signal level" and "signal/noise" ratio (e.g., specific activity of the tracer, concentration and volume of tissue to be assayed, proportion of binding that is specific) need to be considered in choosing between smaller, faster, and more convenient manifolds and the larger, rather cumbersome, conventional manifold. The harvester provides an intermediate-size system with high speed of processing and reliability, as well as reasonably large sample capacity.

The findings reported here, therefore, should also be applicable to other neurotransmitter and peptide receptor systems. Preliminary experiments in our laboratory indicate that results similar to those reported above can be observed for binding of the tetrapeptide ^{125}I-Tyr-Pro-Leu-Gly-NH_2 (^{125}I-Tyr-MIF-1) [5] and for dopamine (^{3}H-apomorphine-labeled) receptors. Hall and Thor [2] showed that binding of the muscarinic cholinergic agent ^{3}H-quinoclidinyl benzilate (^{3}H-QNB) to rat brain membranes could be assayed with the harvester. Coefficients of variation and constants from Hill and Scatchard plots with the harvester were reported as similar to those with the manifold and with the literature, but only data for coefficients of variation were presented, and in contrast to our experiments, direct comparisons of the 2 systems with similar tissue concentrations were not conducted.

In conclusion, adaptation of the cell harvester for measurement of opiate receptors provided estimates of binding parameters comparable to those measured with the conventional filtration manifold, but with a considerably reduced expenditure of time, money and effort. The results of these experiments indicate that the harvester is suitable for many peptide and neurotransmitter receptor assays.

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