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Post-separation detection of nucleic acids and proteins by neutron activation

(thermal neutrons/indirect labeling/autoradiography/nucleotides/chromatography)

ROBERT M. SNAPKA*†, KWAN KWOK‡, JOHN A. BERNARD‡, OTTO K. HARLING‡, AND ALEXANDER VARSHAVSKY*§

*Department of Biology and ‡Nuclear Reactor Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **We describe approaches to neutron activation analysis and their application to post-separation autoradiographic detection of biological compounds. Specifically, we have extended the use of a "direct-labeling" method to the post-separation detection of DNA after gel electrophoresis and to the detection of nucleotides separated by TLC. In addition, we describe a more generally applicable "indirect-labeling" method in which separated compounds of interest are selectively bound to ligands containing highly neutron-activatable elements, such as manganese (^{55}Mn), europium (^{151}Eu), or dysprosium (^{164}Dy), and then irradiated with thermal neutrons. This method is illustrated with nucleotides separated by TLC and with proteins separated by polyacrylamide gel electrophoresis. In contrast to the direct-labeling approach, the indirect-labeling method can be adapted to detect any class of substances for which a highly neutron-activatable, selectively binding ligand is available. The theoretically achievable sensitivity of the indirect-labeling method is in the attomole (10^{-18} mol) range.**

Radioactive labeling is one of the most sensitive analytical strategies for the detection of chemical compounds. However, applications of this approach in biochemistry are often subject to significant constraints. *In vivo* radiolabeling may be complicated by radiation damage to cells and by competition from intracellular pools of precursors (1–5). *In vitro* radiolabeling, though producing much higher specific radioactivities, often results in undesired chemical modification of the original compounds, which may greatly complicate their separation and identification unless radiolabeling is carried out *after* separation of the original compounds. As suggested more than 30 years ago (6), one general method for such post-separation labeling is that of neutron activation. This technique is based upon selective conversion (by neutron capture) of some of the atoms of the sample molecules to unstable isotopes whose radioactive decay is readily detected.

In the "direct-labeling" method of post-separation detection by means of neutron activation, one or more of the elements present in the separated compounds of interest are converted directly to their radioactive isotopes by irradiation with thermal neutrons *in situ*. In an early application of this approach, phosphorus (^{31}P)-containing compounds, such as phospholipids, were detected after their separation by paper chromatography by using neutron activation to convert ^{31}P into radioactive ^{32}P (7–12). In the present work we have extended this direct-labeling method to the detection of nucleotides separated by TLC and to the detection of DNA separated by agarose gel electrophoresis. Neutron activation of ^{31}P in the above compounds yields detection sensitivity comparable to that obtained by *in vivo* labeling and has the advantage that molecular species to be detected are labeled only after their separation.

In addition, we describe a more generally applicable "indirect-labeling" method of post-separation analysis in which separated compounds of interest are selectively bound to highly neutron-activatable elements and then irradiated with neutrons. This general method, illustrated below with nucleotides separated by TLC and with proteins separated by polyacrylamide gel electrophoresis, can be adapted to detect any class of substances for which a highly activatable, selectively binding ligand is available.

MATERIALS AND METHODS

Materials. Unlabeled nucleotides (Pharmacia P-L Biochemicals) were dissolved in H_2O and stored at −70°C. TLC plates were polyethyleneimine (PEI)-impregnated cellulose layers bound to mylar sheets (Polygram cell 300 PEI from Brinkmann or Bakerflex PEI-cellulose from Baker).

Direct Labeling: Neutron Irradiation and Processing of TLC Plates and Agarose Gels. After TLC, the PEI-cellulose plates were immersed in glass-distilled CH_3OH (Omnisolve) for 10 min and then air-dried. The backs of supporting mylar sheets were taped with Scotch repair tape (3M) to strengthen the sheets, which become brittle during irradiation. The plates were placed in polyethylene bags and irradiated with thermal neutrons within a vertical thimble in the graphite reflector of the Massachusetts Institute of Technology nuclear reactor for 96 hr at a flux of 4×10^{12} neutrons·cm^{-2}·sec^{-1}, unless stated otherwise. The samples were then "cooled down" for 2 days (longer in some experiments) to allow ^{24}Na and other short-lived isotopes to decay to sufficiently low levels. Thereafter, irradiated TLC plates were pressed, cellulose side down, onto a layer of Scotch photomount spray adhesive (3M) on cardboard and the mylar backing, together with the intermediate binding layer, was peeled off. Removal of the backing is necessary because it contains trace amounts of metals such as ^{75}As and ^{133}Cs, whose neutron activation interferes with detection of the phosphorus-containing compounds separated by TLC.

Agarose gels after DNA electrophoresis were washed in H_2O and then placed onto several layers of Whatman 3MM paper and dried under vacuum at ≈60°C. Dried agarose was peeled off the backing, placed between two stiff polyethylene sheets, and irradiated as described above.

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Abbreviations: BPS, bathophenantroline disulfonate; $LiDodSO_4$, lithium dodecyl sulfate.

†Present address: Department of Radiology, The Ohio State University, 103 Wiseman Hall, 400 West 12th Avenue, Columbus, OH 43210-1214.

§To whom reprint requests should be addressed at: Department of Biology, 16-520, Massachusetts Institute of Technology, Cambridge, MA 02139.

Indirect Labeling. *Nucleotides.* After TLC the PEI plate (Brinkmann) was immersed in 0.3 mM manganese acetate in glass-distilled CH_3OH for 10 min, unless stated otherwise, then immersed for an additional 10 min in CH_3OH alone, and thereafter irradiated for 6 hr at 10^{10} neutrons·cm^{-2}·sec^{-1}.

Proteins. After electrophoresis of a standard mixture of proteins in lithium dodecyl sulfate ($LiDodSO_4$)-containing 12% polyacrylamide minigels (0.75 mm thick, 8 × 10 cm, Hoefer, San Francisco), the gels were washed for 1 hr in 50% CH_3OH in H_2O, followed by washing in 10% CH_3COOH/25% CH_3OH for 1 hr and incubation for 20 min in the same solvent with a mixture of 3 mM bathophenantroline disulfonate (BPS, Sigma) and either 1 mM $EuCl_3$ or 1 mM $DyCl_3$ (Aldrich). The gels were then washed for 30 min in several changes of 10% CH_3COOH/25% CH_3OH. Dried gels were irradiated for 15 min at 1×10^{13} neutrons·cm^{-2}·sec^{-1} and exposed for autoradiography.

Autoradiography. Irradiated and cooled down samples were wrapped in SaranWrap and exposed at −70°C with Kodak X-Omat AR film, either with or without intensifying screens (DuPont Lightning Plus). Filters (either a 0.5-mm-thick aluminum foil or 0.2-mm-thick mylar sheets) were placed between the sample and both the film and intensifying screens to reduce interference from radiation due to traces of neutron-activated isotopes within the separation support materials.

RESULTS

Post-Separation Detection of Nucleotides and DNA by Direct-Labeling Neutron Activation. The sensitivity of the direct-labeling method for ^{31}P was determined by spotting a 1:10 dilution series of ATP onto a TLC plate and then irradiating the plate with thermal neutrons (13) to convert ^{31}P of ATP into ^{32}P. The lowest amount of ATP detected after 12 hr of autoradiographic exposure was ≈5 pmol in a spot ≈2 mm in diameter (Fig. 1). Considering that each ATP molecule contains three phosphorus atoms, the detection limit under these conditions is ≈5 pmol of ^{31}P per mm^2. The specific radioactivity of ATP obtained in this direct-labeling procedure is ≈13 × 10^3 cpm/μg or 2.7 mCi/mmol (1 Ci = 37 GBq), which is comparable to specific radioactivities of ATP produced by metabolic labeling of mammalian cells in culture with ^{32}P. Fig. 2 shows the results of post-separation detection of nucleotides separated by TLC. Under the conditions used, 0.1 nmol of ADP and ATP are readily detectable after 6 hr of autoradiographic exposure (Fig. 2). The relative intensities of spots are directly proportional to the number of phosphorus atoms present per nucleotide (Fig. 2 and data not shown). Total intracellular nucleotides resolved either in a short unidimensional TLC run (Fig. 2*A*) or in two-dimensional fractionations (data not shown) are also readily visualized by the direct-labeling neutron activation method.

In another application of the direct-labeling approach, as little as 0.5 ng of polyoma DNA separated by agarose gel electrophoresis could be readily detected by direct-labeling neutron activation after 12 hr of autoradiographic exposure (Fig. 3).

Indirect-Labeling Neutron Activation: Post-Separation Detection of Nucleotides. The major limitations of the direct-

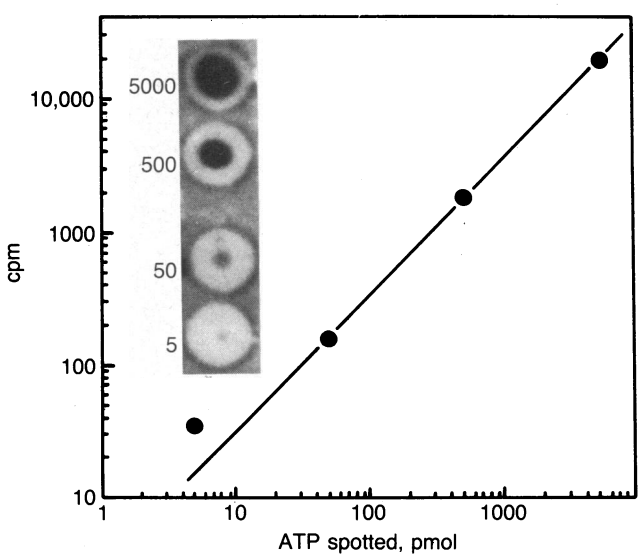

FIG. 1. Detection of phosphorus-containing compounds by direct labeling (neutron activation of ^{31}P). Identical aliquots (10 μl) of ATP solution in H_2O were spotted onto a TLC strip of PEI-cellulose (Polygram cell 300 PEI, Brinkmann) in a 1:10 dilution series. The strip was processed, irradiated with neutrons, and autoradiographed. The ATP spots and an equivalent background area were cut out and assayed with Ultrafluor (National Diagnostics, Somerville, NJ), with energy windows set at 0.157 to 1.71 MeV (1 eV = 1.602 × 10^{-19} J). (*Inset*) Autoradiogram after 12 hr of exposure, with the number of pmol spotted indicated on the left. The "halo" around each spot is due to removal of neutron-activatable impurities from the PEI cellulose by diffusion of water in which the ATP was dissolved.

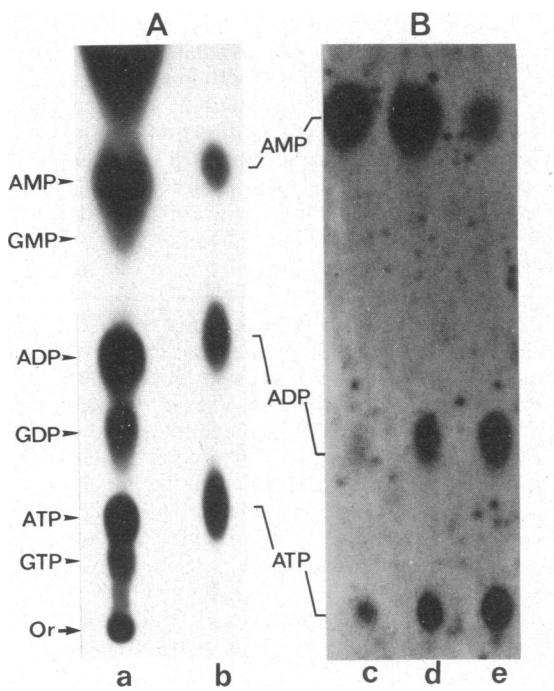

FIG. 2. Post-separation detection of total cellular and standard nucleotides by direct labeling (neutron activation of ^{31}P). (*A*) Total intracellular nucleotides (lane a) from mouse NIH 3T3 cells (see below) and a standard nucleotide mixture (5 nmol each of ATP, ADP, and AMP) (lane b) were separated by unidimensional TLC in 0.9 M guanidinium chloride (15) on a Brinkmann PEI TLC plate. Or, origin. (*B*) Standard nucleotides separated on a Bakerflex PEI TLC plate (Baker). Lane c, 0.1 nmol of ATP and ADP, 5 nmol of AMP; lane d, 0.5 nmol of ATP and ADP, 5 nmol of AMP; lane e, 1 nmol each of ATP, ADP, and AMP. See text for irradiation and autoradiography (for 12 hr). The background noise due to the presence of traces of highly activatable elements in PEI-cellulose plates was approximately constant for different lots of a given brand of PEI-cellulose but varied greatly between brands, with the plates from Brinkmann consistently yielding the lowest background (compare *A* and *B*). Preparation of intracellular nucleotides: a confluent monolayer of mouse NIH 3T3 cells in a 10-cm plate was rinsed with ice-cold 0.2 M NH_4HCO_3 (pH 8.0). The cells were scraped into the same buffer, pelleted by centrifugation, and extracted with an equal volume of 20% formic acid at 4°C for 30 min. The sample was centrifuged at 12,000 × *g* for 10 min, the supernatant was dried in a SpeedVac (Savant), and the residue was dissolved in 0.2 M NH_4HCO_3 (pH 8.0).

FIG. 3. Post-separation detection of DNA by direct labeling (neutron activation of ^{31}P). Purified polyoma viral DNA (5 ng) was electrophoresed in a 1.3% agarose gel in 1 mM Tris/EDTA/40 mM Tris acetate (pH 8.0). The gel was processed, irradiated, and autoradiographed for 12 hr (after 2 weeks of "cool-down" incubation). I and II, circular supercoiled and nicked forms of polyoma DNA, respectively.

labeling method are the range of its applicability (only compounds that contain highly activatable elements can be detected) and its relatively low sensitivity. For instance, post-separation labeling of nucleotides by direct neutron activation as described above required irradiation at high neutron fluxes (4×10^{12} neutrons·cm^{-2}·sec^{-1}) for relatively long intervals (96 hr for the experiments in Figs. 1–3). This requirement results from the relatively low neutron capture cross section of ^{31}P (0.19 barn; 1 barn = 1×10^{-24} cm^2) and the relatively long half-life of the product nucleus, ^{32}P (≈14 days). The problem could be circumvented if it were possible to detect nucleotides indirectly through neutron activation of elements other than ^{31}P. For example, manganese (^{55}Mn) has a 74-fold higher neutron capture cross section, 13.4 barns, and ^{56}Mn, the product of neutron irradiation of ^{55}Mn, decays with a half-life of only 2.6 hr. Thus, if Mn^{2+} ions bind to separated nucleotides more efficiently than they bind to a chromatographic support matrix, it should be possible to detect these compounds with shorter, lower-flux irradiations than those required in the direct-labeling method. Indeed, when one-dimensional chromatograms of adenosine nucleotides were treated with manganese acetate solutions and thereafter irradiated for 6 hr at the relatively low flux of 1×10^{10} neutrons·cm^{-2}·sec^{-1}, the spots of ATP (3 nmol), ADP (10 nmol), and AMP (2 nmol) were detectable after 12 hr of autoradiographic exposure (Fig. 4 *A* and *B*). Upon reexposure of this chromatogram 24 hr later, no nucleotide spots were detectable (Fig. 4*B*; compare with Fig. 4*C*), demonstrating that the autoradiographic signal was due to ^{56}Mn rather than ^{32}P.

This *indirect-labeling* approach is readily generalized for the detection of any class of substances for which a highly activatable, selectively binding ligand is available, as shown below with proteins separated by gel electrophoresis.

Post-Separation Detection of Proteins by Indirect-Labeling Neutron Activation. As a protein-binding, neutron-activatable ligand we initially chose complexes of an aromatic dye, BPS, with either europium (Eu^{3+}) or disprosium (Dy^{3+}) ions. ^{151}Eu and ^{164}Dy are among the most highly activatable elements, with thermal neutron activation cross sections of 3000 and 2600 barns, respectively (^{152m}Eu, $t_{1/2} \approx 9.3$ hr; ^{165}Dy, $t_{1/2} \approx 2.3$ hr). BPS (in a complex with multivalent metal ions) binds to proteins and has been proposed as a general protein stain (14).

A LiDodSO$_4$/12% polyacrylamide gel containing electrophoretically separated standard proteins was incubated with the Dy^{3+}–BPS complex, washed to remove the free complex, and irradiated with neutrons for 15 min (see *Materials and Methods* and legend to Fig. 5). Autoradiography of the gel 29 hr later showed that this procedure allows detection of

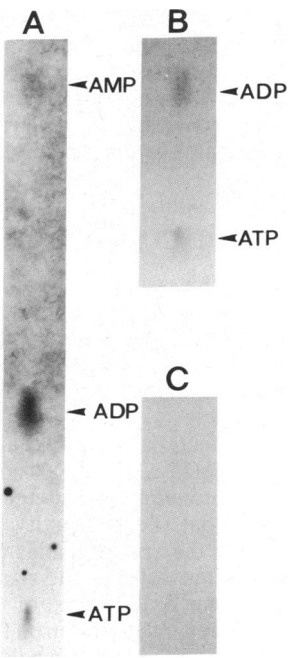

FIG. 4. Post-separation detection of nucleotides by indirect labeling (neutron activation of nucleotide-bound ^{55}Mn). (*A*) ATP (3 nmol), ADP (10 nmol), and AMP (3 nmol) were separated on a Brinkmann PEI TLC plate (see legend to Fig. 2), which was then processed and irradiated. Two hours after irradiation the chromatogram (with mylar backing still attached) was placed face down onto Kodak X-Omat AR film and exposed for 12 hr at −70°C. (*B*) Experiment identical to that in *A* but the TLC plate was immersed for 20 min in 0.03 mM manganese acetate in CH_3OH. (*C*) Same chromatogram as in *B* was reexposed under the same conditions 24 hr after the end of the first exposure.

separated proteins at <0.1 μg of protein per protein band (Fig. 5*A*). Analogous results were obtained using the Eu^{3+}–BPS complex (Fig. 5*B*). Since the background noise in the autoradiographic patterns of Fig. 5 decayed with virtually

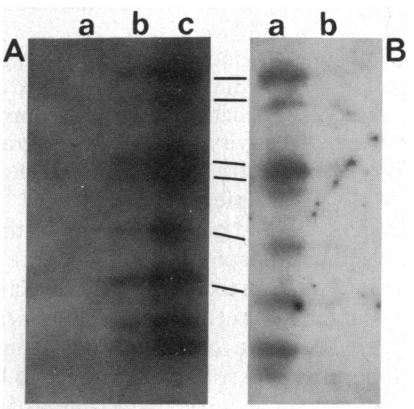

FIG. 5. Post-separation detection of proteins by indirect labeling (neutron activation of protein-bound metal–dye complexes). (*A*) A standard mixture of proteins (Sigma) containing phosphorylase B (92 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa) was electrophoresed in a LiDodSO$_4$-containing 12% polyacrylamide gel. After electrophoresis the gel was incubated with BPS–$EuCl_3$ and irradiated. Autoradiography of the gel was carried out 29 hr later for 2 hr. Lanes a–c contain approximately 0.01, 0.1, and 1 μg of protein in each of the major bands, respectively. (*B*) A gel processed in parallel to the one in *A* was incubated with 1 mM $DyCl_3$ (Aldrich)/3 mM BPS, followed by processing and neutron irradiation as in *A*. Seventeen hours later autoradiography of the gel was carried out for 30 min as in *A*. Horizontal bars indicate positions of the protein species listed above.

the same half-life as the corresponding signal (data not shown), most of the background noise in Fig. 5 is due to the incomplete removal of dysprosium and europium not bound to proteins (since BPS–metal complexes used in the present work bind proteins noncovalently, more stringent washing of the gels removes the complexes from the support matrix and the proteins).

DISCUSSION

The major result of this work is the demonstration of feasibility of the indirect-labeling approach to post-separation detection of specific compounds by means of neutron activation. This method can be adapted to detect any class of substances for which a highly activatable, preferentially binding ligand is available.

The sensitivity of detection in the first applications of the indirect-labeling method (Figs. 4 and 5) is not higher than that available with more conventional detection techniques. However, it is clear that the sensitivity of, for example, protein detection using the above ligands (Fig. 5) is limited largely by the background noise as the signal itself could be readily increased by several orders of magnitude using >15-min irradiation times, higher neutron fluxes, and longer autoradiographic exposures. As shown below, the sensitivity of detection using the indirect-labeling method can in principle vastly exceed the sensitivity achievable by direct-labeling protocols. The sensitivity of detection in neutron activation analysis depends in particular on the cross section of the element to be activated and on the half-life of the corresponding unstable isotope. Specifically, for w grams of an isotopically pure element with atomic weight of A,

$$S = \frac{N\omega\sigma\phi(1 - e^{-\lambda t})}{A},$$

where S is the induced radioactivity (in disintegrations per second) after an irradiation time t (in seconds), N is Avogadro's number, σ is the neutron activation cross section of the element (in barns), ϕ is the neutron flux (in neutrons·cm^{-2}·sec^{-1}), and λ is the decay constant ($\lambda = 0.693/T$, where T is the half-life of the activated isotope in seconds) (see refs. 13 and 16).

Using natural Eu as the indirect-labeling ligand (Fig. 5*B*), and assuming a readily achievable neutron flux of 5×10^{13} neutrons·cm^{-2}·sec^{-1}, application of the above formula shows that the specific radioactivity obtained after irradiating the sample for one half-life of the metastable Eu isotope, ^{152m}Eu (9.3 hr), is $\approx 8.4 \times 10^9$ disintegrations·min^{-1}·μg^{-1} (dpm/μg). (Longer irradiations would produce not more than a 2-fold increase in specific radioactivity.) Furthermore, with conventional autoradiographic techniques, the detection limit for ^{152m}Eu is comparable to that for ^{32}P, ≈ 0.5 dpm/mm^2 for an autoradiographic exposure of 12 hr (Fig. 1 and data not shown). Assuming that a spot of a substance to be detected occupies 2 mm^2, that on average five Eu atoms are bound to a molecule to be detected (e.g., to a protein molecule), and that residual binding of an Eu-containing labeling ligand to the separation matrix is negligible, application of the above formula yields a detection limit of $\approx 3 \times 10^{-19}$ mol (0.3 amol).

Thus, it is clear that the sensitivity of detection achieved in the initial applications of the indirect-labeling method described in the present work is limited almost entirely by the background noise due to unbound ligands. One way to decrease background noise and to increase the relative amount of bound activatable ligand in future refinements of the indirect-labeling method is to employ reagents that can effect the selective and covalent attachment of neutron-activatable ligands to separated compounds of interest. Removal of the excess ligands by diffusion or other means could then be carried out much more efficiently and under more stringent conditions than are currently possible.

Achievement of no more than 10% of the theoretical sensitivity of detection by the indirect-labeling method should allow, among other things, post-separation detection and quantitation of low- to medium-abundance proteins from single mammalian cells. Since the indirect-labeling method can be adapted to detect any class of substances for which a highly neutron-activatable, selectively binding ligand is available, future applications of this method may be limited more by the chemical ingenuity of its users (construction of tightly binding activatable ligands) than by intrinsic limitations of the approach itself.

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