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Use of the Avidin–Biotin Complex for Specific Staining of Biological Membranes in Electron Microscopy*

(ferritin/*Acholeplasma laidlawii*/erythrocytes)

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ABSTRACT To expand the electron microscopist's options in localization and visualization, a new and general staining technique has been tested. The avidin–biotin complex serves as a coupling between the electron-dense marker, ferritin, and points of interest in biological samples. When specific cellular components are tagged with biotin, those components may be visualized with ferritin-linked avidin. Because of the remarkably strong affinity of avidin and biotin (characterized by an association constant of 10^{15} M^{-1}), the staining is rapid and stable.

The preparation of ferritin–avidin conjugate is described, and examples are presented of the application of this complex to biotin-tagged membranes. The ghosts of *Acholeplasma laidlawii* have been treated with biotinyl-*N*-hydroxysuccinimide ester to label protein amino groups. Erythrocyte membrane oligosaccharides have been oxidized by periodate or by galactose oxidase, and the resulting aldehydes labeled with biotin hydrazide.

The avidin–biotin complex in electron microscopy seems especially appropriate for seqential staining procedures, as well as for visualization of reaction sites of biotin-labeled, low-molecular-weight reagents.

The power of electron microscopy to define biological structures is greatly enhanced by labeling specific cellular components with electron-dense stains. Often the stain of choice is the iron-storage protein, ferritin. Ferritin has been coupled to specific biological components through intermediary protein–ligand complexes. Ferritin-linked antibodies stain appropriate antigens (1), such as trypsinogen, in bovine exocrine pancreatic cells (2). Ferritin–lectin conjugates tag specific saccharides (3), such as concanavalin-A binding sites on 3T3 fibroblasts (4).

We have applied another protein–ligand interaction for electron microscopic staining, the avidin–biotin complex (ABC). The affinity of the egg white protein avidin for biotin (vitamin H) is characterized by an association constant of 10^{15} M^{-1} (5, 6). This value is orders of magnitude larger than the association constants for antibody–antigen or agglutinin–sugar complexes; the association of avidin and biotin is remarkably rapid and stable. The binding is minimally affected by modification of the vitamin's carboxyl group (6). Thus, if biotin is attached through its carboxyl group to a molecule of interest, that molecule may be visualized in the electron microscope with ferritin-linked avidin. Avidin, alone, has already played a role as an electron microscopic stain for determining the subunit structure of the biotin enzyme transcarboxylase (7).

This paper describes the preparation of ferritin–avidin conjugate, and presents as examples of ABC staining, the labeling of proteins in *Acholeplasma laidlawii* membranes, and the tagging of saccharides in erythrocyte ghosts. The independent study by Broker and Davidson (T. Broker and N. Davidson, personal communication) has shown another application of avidin and biotin, the visualization of tRNA hybridized to DNA.

MATERIALS AND METHODS

Biotin Reagents. Biotinyl-*N*-hydroxysuccinimide ester (BOSu) was prepared from biotin (Sigma Chemical Co.) and *N*-hydroxysuccinimide by coupling with dicyclohexylcarbodiimide (8). The reactants were dissolved at 0.07 M concentration in dimethylformamide, and incubated at 50° for 16 hr. The mixture was cooled to room temperature, the dicyclohexylurea was filtered off, and the filtrate was dried on a rotary evaporator. The residue was crystallized from isopropanol; m.p., 208–210°.

Biotin hydrazide (9) was produced via biotin acid chloride (10). Thionyl chloride (15 ml) was added dropwise to solid biotin (0.50 g) and stirred until solubilization was complete. Excess thionyl chloride was removed by evaporation. To the biotin acid chloride, 40 ml of absolute methanol was added slowly, the mixture stirred until homogeneous, and the methanol removed on a rotary evaporator. The residue was redissolved in 5 ml of methanol, and added dropwise at 23° to a well-stirred solution of 1 ml of hydrazine in 4 ml of methanol. The reaction was continued overnight, and the solvent was then removed under reduced pressure. The dry residue was extracted with 10 ml of distilled water at 23° for 30 min. The solid remaining was collected on a filter, and crystallized from dimethylformamide; m.p., 227–229°. Calculated for $C_{10}H_{18}O_2N_4S$: C, 46.50; H, 7.02; N, 21.68. Found: C, 47.00; H, 6.89; N, 21.59.

Ferritin–Avidin Conjugate (FAv). Six times-recrystallized horse ferritin (Miles Laboratories) was crystallized once again from cadmium sulfate (11). The crystalline ferritin was redissolved at a concentration of 5–10 mg/ml in 2% ammonium sulfate. Ferritin concentration was estimated by assuming

Abbreviations: ABC, avidin–biotin complex; BOSu, biotinyl-*N*-hydroxysuccinimide ester; FAv, ferritin–avidin conjugate; PBS, phosphate-buffered saline.

* Preliminary results of FAv labeling of biotin-modified membranes from retinal rod outer segments were presented at the Seventeenth Annual Meeting of the Biophysical Society, February 1973; Heitzmann, H. and F. M. Richards (1973) *Biophys. Soc. Abstracts*, 232a.

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A_{440} to be 1.5 for a 1 mg/ml of solution (12). The solution was centrifuged at 40,000 × *g* for 20 min, and the supernatant was passed through a Sephadex G-25 column and eluted with 0.9% NaCl to free the protein from cadmium. Ferritin-containing fractions were pooled and concentrated to about 5 mg/ml. The ferritin was passed through a second Sephadex G-25 column and eluted with 0.10 M Na phosphate, pH 7.0, to change the buffer composition. The ferritin fractions were pooled and concentrated to 20 mg of ferritin/ml of 0.10 M Na phosphate, pH 7.0.

Avidin (Sigma Chemical Co.) was dissolved at 10 mg of protein per ml in phosphate buffer. Avidin solution (0.2 ml), mixed with 1.0 ml of ferritin solution, gave similar molar concentrations of the two protein species. It was observed that coprecipitation of ferritin and avidin at pH 7 was favored by low ionic strength and high protein concentration. To the ferritin–avidin solution was added 0.1 ml of 0.5% glutaraldehyde (Electron Microscopy Sciences); after 1-hr incubation at 23°, the reaction was quenched by adding 2 ml of 0.1 M NH_4HCO_3. The solution was dialyzed against 0.9% NaCl and stored in a sterile vial at 4°.

Binding and Precipitation Assays. Biotin-binding activity of FAv was measured using [^{14}C]biotin (Amersham-Searle). FAv (to give 10^{-5} M avidin) and [^{14}C]biotin (10^{-4} M at 0.50 mCi/mmol) were dissolved in 0.2 ml of 0.9% NaCl. The solution was incubated at 23° for 30 min, and then run through a Sephadex G-25 column. Fractions were dissolved in Bray's solution (13) in nylon vials for scintillation counting at 88% efficiency. The void volume peak of bound biotin was completely separated from the free biotin peak. The amount of biotin bound was compared with that calculated for the initial activity of the avidin present (1 mmol of biotin associated/20,000 mg of protein).

The yield of avidin–ferritin conjugates in the FAv preparation was measured by (*1*) labeling all active avidin present with [^{14}C]biotin, (*2*) precipitating all ferritin-linked avidin with ferritin-specific antibodies. A portion of FAv solution (0.2 ml, 8 mg of protein per ml) was incubated with excess [^{14}C]biotin (0.5 mCi/mmol), and passed through a Sephadex G-25 column to remove unbound biotin. The void volume fractions were pooled, and aliquots (0.5 ml, 1 mg of protein per ml) were withdrawn. To some aliquots were added amounts of antiserum against horse ferritin (Miles Laboratories) appropriate to precipitate all the ferritin present. To other aliquots were added equivalent volumes of 0.9% NaCl. After incubation at 23° for 1 hr, 0.8 ml of 0.9% NaCl was added to each sample, and antibody–antigen complexes were sedimented by centrifugation. Fractions of each supernatant were mixed in Bray's solution for scintillation counting.

Biotin Labeling of Proteins. For testing the association of avidin with biotin-labeled macromolecules, proteins were tagged with biotin. A solution of ovalbumin (Worthington), 10 mg in 1.0 ml of 0.1 M $NaHCO_3$, was mixed with 0.1 ml of BOSu solution, 12 mg in 1 ml of dimethylformamide. The mixture was kept at 23° for 1 hr, loaded on a Sephadex G-25 column, and eluted with 0.9% NaCl.

The glycoprotein apotransferrin (human, gift of Dr. J. Shaper) was treated mildly with $NaIO_4$ (14). The oxidized protein (5 mg/ml in 0.1 M sodium acetate, pH 5.6, 0.15 M NaCl) was mixed with an equal volume of 0.02 M biotin hydrazide in the same buffer, allowed to stand for 3 hr at 23°, and then dialyzed against 0.9% NaCl.

Biotin Labeling of Membranes. Acholeplasma laidlawii ghosts (the gift of Prof. D. M. Engelman) were modified by BOSu. The membranes were prepared from *A. laidlawii* B which had been grown in stearic acid-supplemented medium (15). The ghosts were washed twice by suspension and centrifugation in 0.1 M $NaHCO_3$; membranes (4 mg of protein) were resuspended in 2 ml of 0.1 M $NaHCO_3$. Half of the suspension was treated with 0.1 ml of BOSu solution (20 mg/ml in dimethylformamide); no addition was made to the other half. The samples were incubated at 23° for 1 hr, and washed twice with bicarbonate buffer.

Erythrocyte membranes were labeled with biotin hydrazide after membrane saccharides were modified by galactose oxidase (16). The cells from whole human blood (5 ml, heparinized, less than 1 week old) were washed three times by suspension and centrifugation in phosphate-buffered saline (PBS, 0.15 M NaCl, 0.01 M Na phosphate, pH 7.3). The packed cells were incubated at 23° for 30 min after addition of 0.5 ml of 0.05 M $NaBH_4$ in PBS. The borohydride was added to quench endogenous reducible components of erythrocytes (16). The cells were washed three times in buffer, and divided in half. To each aliquot was added 1.0 ml of 0.01 M biotin hydrazide in PBS. One aliquot was treated with 0.3 mg of galactose oxidase of *Polyporus circinatus* (Sigma Chemical Co., Type I, 130 units/mg). Both portions were incubated at 37° for 2 hr, and then diluted with 10 ml of PBS. The suspensions were centrifuged and then washed twice in buffer. Each portion of cells was lysed by osmotic shock, and then washed in PBS.

Erythrocyte membranes were also labeled with biotin hydrazide after periodate oxidation of membrane sugars. Human erythrocytes from 2 ml of whole blood were washed and resuspended in 2.5 ml of buffer. To the supension was added 0.1 ml of 0.02 M $NaIO_4$ in PBS. After incubation at 23° for 15 min, the mixture was diluted with 10 ml of buffer and the cells pelleted. The cells were washed twice more in buffer; hemolysis was slight. The cells were resuspended in 1 ml of PBS, and treated with 0.4 ml of 0.01 M biotin hydrazide in PBS. After a 2-hr incubation at 23°, the mixture was diluted with 10 ml of PBS, centrifuged, and the cells washed twice in buffer. The erythrocytes were then lysed by osmotic shock.

Electron Microscopy. A copper grid, supporting a carbon-coated formvar film, was floated face down for 5 min on a drop of 5% bovine serum albumin in 0.2 M NH_4HCO_3. The grid was touched to a drop of buffer and then treated with a drop of buffer in which were suspended the membranes (*A. laidlawii* or erythrocyte ghosts). The drop was retained on the grid for 5 min, and then the grid was washed in 10 drops of buffer. Each drop was withdrawn by touching the grid to filter paper, but the grid was not allowed to dry completely. The washed grid was treated with a fresh drop of 5% serum albumin for 2 min, followed by a drop of buffer. Then the grid received a drop of FAv (1–2 mg of protein per ml) for 5 min. After 10 buffer washes, the grid was dried, viewed in a Hitachi 7-S electron microscope, and photographed at ×20,000 magnification.

RESULTS

Ferritin–avidin conjugate

Binding of [^{14}C]biotin to FAv showed that an average of 60% of initial biotin-binding activity was retained in three different preparations. Experiments with anti-ferritin serum indicated that an average of 80% of total biotin-binding activity in two

different preparations was precipitated by the antiserum. Therefore, the overall yield of active avidin coupled to ferritin was about 50%. The conjugate was retested after several weeks' storage at 4°, and found to have retained about 90% of its capacity for biotin uptake.

We did not find it necessary at this stage to purify one-to-one complexes of ferritin and avidin. An appropriate gel filtration column could be useful for removing oligomers from the FAv reagent if accurate quantitation of single binding sites in the membrane preparations were required.

Double diffusion test

The Ouchterlony plate was a simple and rapid test for biotin modification of diffusible molecules. Avidin formed insoluble aggregates with other macromolecules substituted with biotin at more than two loci. This aggregation, analogous to the antibody-antigen precipitin reaction, was assayed by double diffusion (Fig. 1). Avidin precipitated biotinyl-ovalbumin, but not unmodified ovalbumin. Avidin did not precipitate biotinyl-apotransferrin (probably because the glycoprotein was labeled at only one or two loci). Nevertheless, biotin-tagging of the apotransferrin could be detected by the interference with the precipitation by avidin of biotinyl-ovalbumin. Unmodified apotransferrin did not block this precipitation.

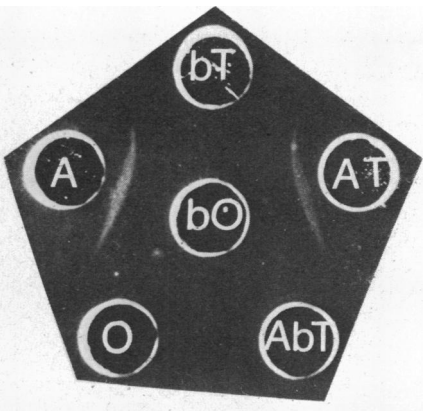


FIG. 1. Double-diffusion test for biotin modification of macromolecules. The wells in the Ouchterlony plate were filled as follows: (A) avidin, (bO), biotinyl-ovalbumin, (AT) avidin plus apotransferrin, (AbT) avidin plus biotinyl-apotransferrin). The lines between (A) and (bO), and between (AT) and (bO), show the expected precipitation of avidin with labeled ovalbumin and the lack of interference by apotransferrin. The *absence* of a line between (A) and (bT) shows that the biotinyl-apotransferrin does not form a precipitate with avidin. However, the fact that biotinyl-apotransferrin binds to avidin and inhibits its precipitation of biotinyl-ovalbumin is shown by the *absence* of a line between (AbT) and (bO).

A. laidlawii membrane proteins

The reaction of BOSu with *A. laidlawii* ghosts was expected to label proteins almost exclusively, because *A. laidlawii* membrane lipids and carbohydrates bear few primary amino groups (17, 18). The distribution of biotinyl-proteins was revealed by specific staining with FAv (Fig. 2a). Nonspecific binding of FAv to the membranes was negligible (Fig. 2b); bare membranes appeared as dark areas against the light background of the carbon-coated film.

Erythrocyte membrane saccharides

Biotin hydrazide serves as a general reagent for the detection of aldehydes through hydrazone formation. Aldehyde groups on erythrocyte membranes were produced from galactose and *N*-acetylgalactosamine by galactose oxidase (19), coupled to biotin with the hydrazide reagent, and visualized by FAv staining (Fig. 3a). After initial borohydride reduction, membranes which were treated with biotin hydrazide without enzymatic oxidation were not appreciably labeled by FAv (Fig. 3b). Furthermore, biotin-substituted membranes were stained only at background level (Fig. 3c) by FAv which had been saturated with biotin (the FAv solution was preincubated with 0.5 mM biotin for 30 min at 23°). Thus FAv was not binding by Schiff base formation to free aldehydes (if any remained after treatment with biotin hydrazide).

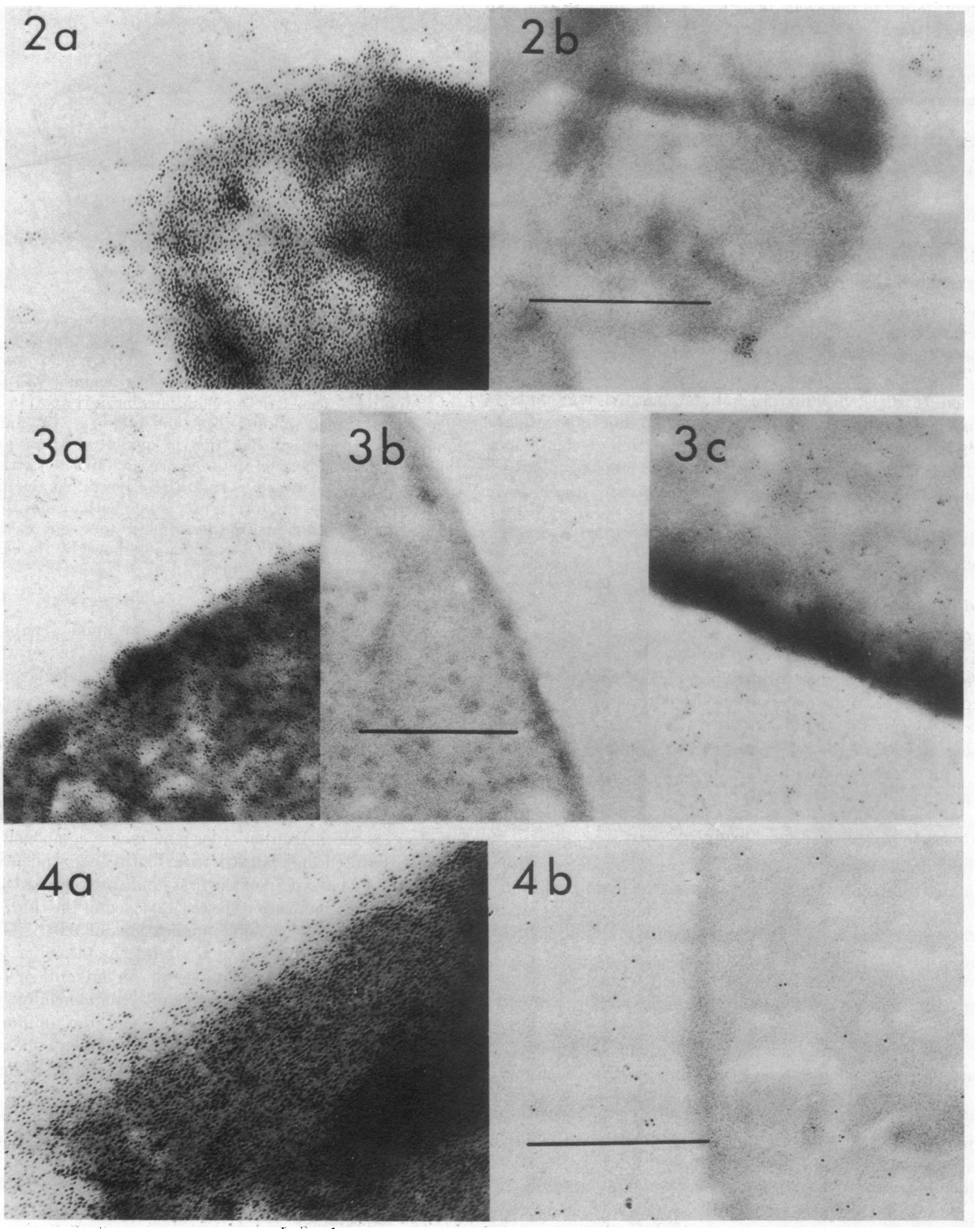
Aldehydes were also generated in erythrocytes by reaction with periodate, by means of a procedure which had been found to oxidize primarily sialyl residues among membrane sugars (20). Membranes which were modified by biotin hydrazide after periodate oxidation appeared densely stained with FAv (Fig. 4a). Control experiments with biotin-blocked FAv showed that the staining was specific (Fig. 4b).

DISCUSSION

Useful ABC staining will depend on the successful attachment of biotinyl groups to the sites of interest. We have provided a few examples of general class staining: the proteins in *A. laidlawii* ghosts, galactose and galactosamine groups in the sialoglycoproteins and glycosphingolipids of erythrocyte membranes (21), and sialyl residues in the sialoglycoproteins which are also in erythrocytes (20).

In general, ABC can be useful in staining biological specimens when biotin is introduced by (*1*) chemical reaction specific for a class of components, (*2*) affinity labeling of enzyme or receptor sites (for such ligands as inhibitors, hormones, neurotransmitters, growth factors) by a ligand analog that contains biotin, (*3*) reconstitution of systems whose components have been separated and labeled with biotin independently. Our experiments have been of the first kind. Broker and Davidson (personal communication) have succeeded in the third kind of labeling. Thus, the possible applications of ABC staining in electron microscopy appear as useful complements to those using antibody–antigen complexes. An advantage of ABC is that crystalline avidin is available commercially; the immunological method requires time for the immunization process and for the appropriate immunoglobulin to be purified. Further, some molecules whose visualization is desired—especially low-molecular-weight reagents—may elicit a low titer of immunoglobulin. In many cases the synthesis of a biotin-containing reagent may be quite simple and fast. On the other hand, antibodies, once obtained, have an important advantage in that they may be applied without modification of the sample.

In using biotin-containing small-molecule reagents, one may rely on the properties of the reagent for localization; for example, restriction to the external surface of a cell because of impermeability. Alternatively, for reagents which are permeable or whose permeability properties are unknown, one may use the impermeability of the large macromolecular component, FAv, to provide such specificity. Conversely, if all sites of reaction of the small-molecule biotin-containing reagent are to be visualized, one must ensure accessibility of all parts of the sample to FAv.



FIGS. 2–4. (2) FAv staining of *A. laidlawii* membranes (a) ghosts modified by BOSu, (b) untreated ghosts. Bar equals 0.4 μm. (3) Erythrocyte ghosts (a) labeled with FAv after treatment with galactose oxidase and biotin hydrazide, (b) labeled with FAv after incubation with biotin hydrazide alone, (c) treated with biotin-blocked FAv after reaction of the membranes with galactose oxidase and biotin hydrazide. Bar equals 0.4 μm. (4) Erythrocyte ghosts (a) stained with FAv after reaction with periodate and biotin hydrazide, (b) treated with biotin-blocked FAv after reaction of the membranes with periodate and biotin hydrazide. Bar equals 0.4 μm.

The clearest advantage of ABC staining is the powerful affinity of biotin and avidin. The high rate of association—10^8 M^{-1} sec^{-1} (5)—means that low concentrations of FAv will stain samples quickly, thus minimizing nonspecific background. Moreover, the half-time for dissociation requires months, which makes sequential staining possible without risk

of exchange. For example, a single sample could be tagged with one biotin reagent, labeled with FAv, then reacted with a different biotin reagent, and stained with hemocyanin–avidin complex. Both labels could then be seen and identified simultaneously in a single micrograph. This sequential labeling capability, plus the flexibility possible in chemical coupling of biotin to biological samples, indicate that ABC staining may be generally useful in cytochemistry.

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1. Singer, S. J. & Schick, A. F. (1961) *J. Biophys. Biochem. Cytol.* **9**, 519–537.
2. Kraehenbuhl, J. P. & Jamieson, J. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1771–1775.
3. Nicolson, G. L. & Singer, S. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 942–945.
4. Nicolson, G. L. (1972) *Nature New Biol.* **239**, 193–197.
5. Green, N. M. (1963) *Biochem. J.* **89**, 585–591.
6. Green, N. M. (1974) *Advan. in Protein Chem.*, in press.
7. Green, N. M., Valentine, R. C., Wrigley, N. G., Ahmad, F., Jackson, B. & Wood, H. G. (1972) *J. Biol. Chem.* **247**, 6284–6298.
8. Becker, J. M., Wilchek, M. & Katchalski, E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2604–2607.
9. Hofmann, K., Melville, D. B. & du Vigneaud, V. (1942) *J. Biol. Chem.* **144**, 513–518.
10. Wolf, D. E., Valiant, J. & Folkers, K. (1951) *J. Amer. Chem. Soc.* **73**, 4142–4144.
11. Wu, M. & Davidson, N. (1973) *J. Mol. Biol.* **78**, 1–21.
12. Schick, A. F. & Singer, S. J. (1961) *J. Biol. Chem.* **236**, 2477–2485.
13. Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285.
14. VanLenten, L. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1889–1894.
15. Engelman, D. M. (1971) *J. Mol. Biol.* **58**, 153–165.
16. Gahmberg, C. G. & Hakomori, S.-I. (1973) *J. Biol. Chem.* **248**, 4311–4317.
17. Gilliam, J. M. & Morowitz, H. J. (1972) *Biochim. Biophys. Acta* **274**, 353–363.
18. Shaw, N., Smith, P. F. & Koostra, W. L. (1968) *Biochem. J.* **107**, 329–333.
19. Avigad, G., Amaral, D., Asensio, C. & Horecker, B. L. (1962) *J. Biol. Chem.* **237**, 2736–2743.
20. Liao, T.-H., Gallop, P. M. & Blumenfeld, O. O. (1973) *J. Biol. Chem.* **248**, 8247–8253.
21. Steck, T. L. & Dawson, G. (1974) *J. Biol. Chem.* **249**, 2135–2142.