METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

Cancer Biology Research Laboratory
Stanford University Medical Center
Stanford, California

VOLUME 26

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical

evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

CONTENTS

The Use of the Avidin-Biotin Complex as a Tool in Molecular Biology

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I. INTRODUCTION

The high affinity constant between the glycoprotein avidin and the vitamin biotin prompted early attention to the nature of this complex. To obtain further insight into the properties of the avidin-biotin complex, in the early 1950s Fraenkel-Conrat and co-workers (1952) purified avidin and studied the effect of chemical modification on its activity. No further interest was taken in the complex until the end of the decade when Wakil et al. (1958) and Lynen et al. (1959) discovered the coenzyme function of covalently bound biotin. It became clear that avidin could be used as a tool for characterizing biotin-requiring enzymes. In fact, the spatial position of the avidin-bound biotin-containing subunit of transcarboxylase was ultimately localized by high resolution electron microscopy (Green et al., 1972; Green, 1972).

Since 1963 Green has been the leading figure in the efforts to understand this unique interaction by various biophysical and biochemical methods (Green, 1975). However the innate reason for the strong interaction between biotin and avidin is not yet known. Judging from the structure of biotin (Figure 1), it is difficult to understand why such a simple molecule should possess such an unprecedented affinity for a given protein. Even more surprising, only the intact ureido ring is

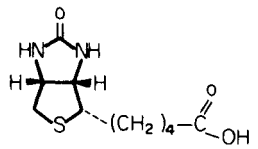

Figure 1. The structure of biotin.

required for this strong interaction.

Regarding the other partner of this complex (Table I), it is surprising that the four tryptophan residues of each avidin subunit vie for the biotin molecule. There is no perceivable reason for tryptophan, which generally participates in charge-transfer complexes or hydrophobic interactions, to have affinity for the ureido group instead of other more hydrophobic components of the biotin molecule. Nevertheless, even though we do not yet fully understand this interaction, it provides a powerful tool for study in the following areas: (1) the isolation of biotin-derivatized materials by affinity chromatography, (2) affinity labeling and identification studies, (3) affinity cytochemical labeling for localization studies by fluorescence and electron microscopy, (4) the inhibition of bacteriophages, and (5) the study of cell surface molecular interactions.

In this respect the avidin-biotin complex represents a complementary approach and/or a potential replacement for lectins and antibodies in biological interactions that exploit the specific binding between a protein and a ligand. This chapter describes in more detail previous contributions to the application of the avidin-biotin complex and provides some suggestions about the direction of its prospective use. Naturally, we will be unable to cover all possible applications; it seems that the potential of the

TABLE I

Some Important Characteristics of Avidin[a]

Molecular weight	67,000
Subunit molecular weight	15,600
K_D (avidin-biotin complex)	$\sim 10^{-15}$
E_{282}(1 mg/ml)	1.54
ϵ_{282}	96,000
Oligosaccharide/subunit	1
Mannose/subunit	4-5
Glucosamine/subunit	3
Tryptophan/subunit	4

[a]Modified from Green (1965).

avidin-biotin complex as a tool in molecular biology is unlimited, and that its successful implementation is directly dependent on the needs and imagination of the user.

II. PRINCIPLE

The rationale behind our approach is as follows: biotin, bound to a macromolecule, is still available for the high affinity interaction with avidin (Becker and Wilchek, 1972). Thus (in addition to biotin-requiring enzymes) biotin-derivatized hormones, phages, lectins, antibodies, and other binding proteins can interact with avidin; and if the avidin is immobilized or covalently bound to a potentially perceptible probe, the avidin-biotin complex can be used for the localization or isolation of the compounds above and/or their receptors (Figure 2). The major

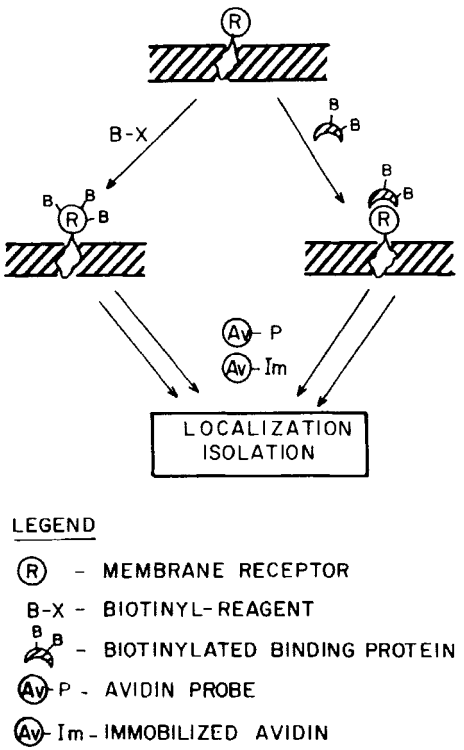

Figure 2. Schematic representation of the rationale behind the use of the avidin-biotin complex as a probe in molecular biology.

restriction concerns methods for the introduction (attachment) of biotin to a given component of the experimental system.

Only in one case—that of biotin-requiring enzymes—has nature provided us with a native, covalently bound, biotinylated protein. Various laboratories have demonstrated through the years that the biotin moiety of these proteins is capable of interacting with avidin (Knappe, 1970; Moss and Lane, 1971). Accordingly, avidin has been used for the isolation and structural determination of the biotin-containing subunits. Therefore, in other cases it is necessary to devise methods for the artificial emplacement or covalent attachment of the biotin molecule to a specified component of the experimental system. The latter is subsequently evaluated by an appropriate avidin-containing conjugate.

III. PREPARATION OF REACTIVE BIOTINYL DERIVATIVES

Proteins contain a variety of functional groups, some of which are important for their activity and some not. In any given protein, chemical modification of an essential functional group may destroy directly or indirectly, its biological activity and/or specificity. Since we are interested in preserving these properties of the protein, a selection of group-specific reagents must be available. Therefore it would be advantageous to have biotinyl derivatives that can be bound to different classes of functional groups. If a given biotinyl derivative interferes with the biological activity or specificity of a modified protein, an alternative derivative can be used in its place. Accordingly, we have prepared a selection of biotinyl derivatives that can be covalently bound to a variety of functional groups, including amines, thiols, imidazoles, and phenols, as well as carboxyls. Since many of the important cell receptors are glycoproteins, biotin derivatives that can interact with sugar residues have also been prepared. Some of the biotin derivatives that we have found useful are listed in the scheme in Figure 3, and the methods of preparation of a selected few are summarized in Sections III.1 to III.4. It should be noted that these reagents are not only applicable for direct coupling to a protein, but also can be used after prior enrichment of a given functional group and subsequent attachment with an appropriate biotinyl derivative to the extraneous functional group. For example, thiolylation of a protein with homocysteine lactone generates free sulfhydryl groups, which subsequently can be reacted with a bromoacetyl analog of biotin. A second example is the biotinylation of a glycoprotein by way of the oligosaccharide moiety, since the first step in such a procedure consists of periodate-oxidation of vicinal hydroxyls to aldehydes. The latter can be

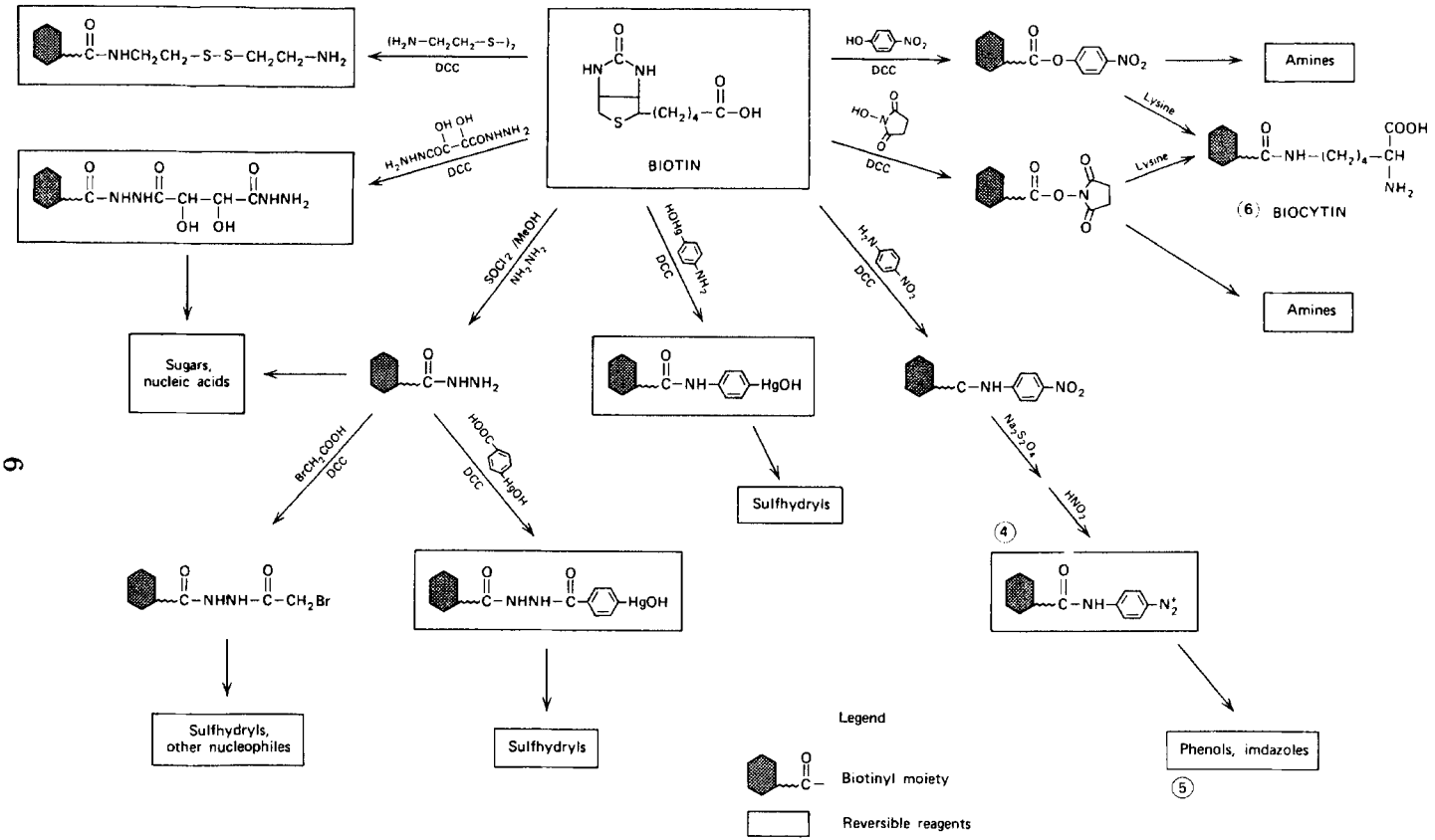

Figure 3. Reactive biotinyl derivatives useful for coupling to different functional groups.

used to introduce different functional groups to which a suitable biotin derivative subsequently can be bound.

1. Amino Reagents

1. *Biotinyl-N-hydroxysuccinimide ester (BNHS).* The method described by Becker et al. (1971) and Bayer and Wilchek (1974) is as follows. Dicyclohexylcarbodiimide (0.8 g) was added to a solution of dimethylformamide (12 ml) containing biotin (1 g) and *N*-hydroxysuccinimide (0.6 g). The suspension was stirred overnight at room temperature. The dicyclohexylurea precipitate was filtered and the filtrate evaporated under reduced pressure. The residue was washed well with ether, and the product was recrystallized from isopropanol.

An alternative method for preparation of this compound was described by Jasiewicz et al. (1976) by use of *N,N'*-carbonyl-diimidazole as a coupling reagent.

2. *Biotinyl-*p*-nitrophenyl ester (*p*BNP).* Bayer and Wilchek (1977) synthesized *p*-BNP by a slight modification of the earlier procedure described by Becker et al. (1971). To biotin (244 mg 1.0 mmole) suspended in 3 ml of methylene chloride was added *p*-nitrophenol (175 mg, 1.3 mmole) and dicyclohexylcarbodiimide (DCC) (206 mg, 1 mmole). After stirring this mixture for 24 hr at 25°C, it was filtered and the filtrate taken to dryness under reduced pressure. The yellow gummy residue was washed several times with absolute ether, and taken up in isopropanol. Following filtration, the solution was reduced to minimum volume and allowed to crystallize overnight. The crystals of *p*BNP were collected by filtration and washed with anhydrous ether.

Another preparative procedure for this compound was described recently by Bodanszky and Fagan (1977). The *o*- and *m*-nitrophenyl esters of biotin can be prepared in a similar manner.

2. Carboxyl and Sugar Reagents

Biotin hydrazide (BHZ) was synthesized by a modification of the procedure used by Heitzmann and Richards (1974). Thionyl chloride (1 ml) was added slowly to a chilled solution (10 ml) of methanol (in an ice-saline bath). To this solution biotin (1 g) was added, and it was left overnight at room temperature. The solvent was evaporated to dryness. Methanol (10 ml) was added, and the solvent was again evaporated to dryness. The residue was redissolved in 5 ml of methanol; hydrazine hydrate (1 ml) was added, and the reaction was allowed to proceed overnight at room temperature. The precipitate (biotin hydrazide) was filtered and washed with ether. A second crop may be obtained by con-

centration of the filtrate. The samples were recrystallized from dimethylformamide.

Biotin hydrazide can be used directly with the aldehyde derivatives of periodate-oxidized sugars. For reaction with carboxyl groups, carbodiimides (water soluble or otherwise) must be added as a coupling reagent. Other biotin derivatives for carboxyl groups can be prepared by monosubstitution of biotin to diamines and coupling of the latter derivatives to carboxyls via carbodiimide.

3. Thiol Reagents

Thiol reagents were prepared either by substitution of biotin hydrazide or monosubstituted biotinyl-amines with bromoacetyl-*N*-hydroxysuccinimide ester, or with bromoacetic anhydride (Wilchek and Givol, 1977). Biotinyl derivatives containing mercury were prepared by coupling biotin-hydrazide with the *N*-hydroxysuccinimide ester of *p*-hydroxymercuribenzoate.

Biotinyl-bromoacetyl hydrazide. Biotin hydrazide (260 mg) was dissolved in 0.5*M* sodium bicarbonate (10 ml) and treated with bromoacetic anhydride (520 mg) in 4 ml of dioxane at 0°C. After 15 min the precipitate was filtered off, dissolved in isopropanol and precipitated with ether.

4. Phenol and Imidazole Reagents

Biotinyl reagents for the phenol and imidazole reagent functional groups were prepared by reduction of biotinyl-*p*-nitroanilide with sodium dithionite, followed by diazotization with sodium nitrite. This reaction should be performed immediately before use.

1. *Biotinyl-p-nitroanilide.* Biotin (244 mg) was dissolved in dimethylformamide (3 ml), and triethylamine (0.14 ml) was added. The solution was cooled, and isobutylchloroformate (0.16 ml) was added. After 5 min, *p*-nitroaniline (150 mg) was added. The reaction mixture was left at room temperature overnight. Upon adidtion of ethyl acetate, the product precipitated and was collected. The product was recrystallized from isopropanol.

2. *Biotinyl-diazoanilide.* Biotinyl-*p*-nitroanilide was dissolved in dimethylformamide and water was added until the suspension became slightly turbid. To this suspension, an excess of crystalline dithionite was added. After 10 min the solution was acidified with 1*N* hydrochloric acid to pH 2.0 and treated with sodium nitrate at 0°C. After 5 min a sample was brought to pH 8.0 and reacted with phenol or imidazole. The appearance of a deep yellow or reddish color is a sign of reaction.

IV. ASSAYS FOR AVIDIN AND BIOTIN

A variety of method for the assay of avidin and/or biotin are presently available. Biotin may be analyzed by a selection of biological procedures based on the use of appropriate microorganisms (see McCormick and Wright, 1970). Biological assays, although by far the most sensitive (representative lower limit: 10 pg of biotin), are cumbersome to perform and typically take several days to obtain results. Biotin content may also be analyzed chemically, using *p*-dimethylaminocinnamaldehyde (McCormic and Roth, 1970).

Avidin may be assayed using [^{14}C]-biotin to a lower limit of 20 ng of avidin (Wei, 1970). Since avidin is highly antigenic (Korenman and O'Malley, 1970), the avidin content of a given solution can be assessed by radioimmunoassay.

Reciprocal methods for the detection of either avidin or biotin are also available, although the sensitivity is generally reduced. One method is based on the increased absorbance of avidin at 233 nm ($\Delta\epsilon_{223} = 2.4 \times 10^4 M^{-1}$/mole biotin) upon complex formation with biotin (Green, 1970). The quantitative displacement by biotin of the avidin-dye (4′-hydroxy-azobenzene-2-carboxylic acid) complex forms the basis for another assay of both biotin and avidin (Green, 1970). The quenching of tryptophan fluorescene in avidin upon complex formation with biotin provides yet another rapid and sensitive assay for both biotin and avidin in solutions free of fluoroescent contaminants (Lin and Kirsch, 1977). The latter procedure affords improved sensitivity, and free biotin may be determined in amounts as small as 20 ng. Biotin (5-10 ng/ml) or avidin (100-300 ng/ml) also can be assayed by the phage technique (Becker and Wilchek, 1972).

V. PURIFICATION STUDIES: AFFINITY CHROMATOGRAPHY

1. Natural Biotin-Containing Systems

It is interesting to note that in the early attempts at specific isolation of biologically active compounds (affinity chromatography), the avidin-biotin complex was used as a model system for demonstrating the applicability of such an approach. Again, the reason for this is the high affinity constant, that applies even under the most unfavorable conditions. Biotin was first coupled to cellulose by way of an ester bond, and some retardation of avidin was obtained on such columns (McCormick, 1965). In the first study using Sepharose as a carrier for affinity chromatography (Cuatrecasas and Wilchek, 1968), this complex was again used to show the superiority of this matrix over previously used carriers. Thus when

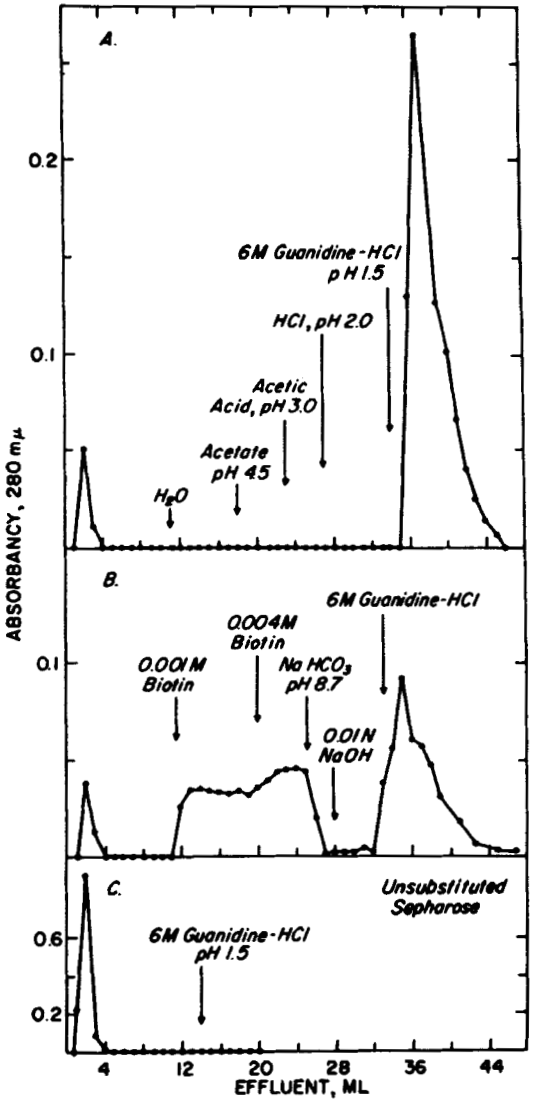

Figure 4. Affinity chromatography of commercial avidin on biocytin-Sepharose (*A*,*B*) and unsubstituted Sepharose (*C*) columns. The columns (0.5 × 5 cm) were equilibrated with 0.2 *M* sodium bicarbonate, pH 8.7, and 0.75 mg of avidin (in 0.5 ml of the same buffer) was attempted by varying the conditions as indicated (arrows). The small protein peak that emerges early in *A* and *B* represents an impurity that does not bind [^{14}C]biotin.

biocytin was coupled to cyanogen bromide activated Sepharose and a solution of avidin was passed over the column, the avidin was so strongly adsorbed that no single agent (e.g., salts, acids, bases, or concentrated solutions of biotin) was effective in eluting the avidin from the biotin column. Only the combination of 6 *M* guanidine-hydrochloride at pH 1.5 was capable of eluting the avidin (Figure 4). Even under these drastic conditions, however, avidin could be purified 4000-fold directly from egg white without appreciable loss of biological activity.

The reverse approach was also taken to purify biotin-containing compounds (Bodanszky and Bodanszky, 1970). Biotinyl peptides from transcarboxylase were adsorbed to an avidin-Sepharose affinity column and could be eluted only under the above-mentioned drastic conditions. In this case the yields were quite low due to the extraordinary interaction between immobilized avidin and the biotin-containing peptides. In a more recent study (Rylatt et al., 1977) the biotin-containing tryptic peptides of pyruvate carboxylases from liver mitocondria in various mammalian and avian species were isolated by complexing with soluble avidin. Subsequent separation of the protein-peptide complex from free peptides was achieved by precipitation of the former with zinc chloride. The avidin-biotinyl-peptide complex was irreversibly dissociated by 70% formic acid.

A more extensive investigation in which the avidin-biotin complex was used, involved the separation of biotin-containing subunits from the apoenzyme of biotin-requiring enzymes. Two different approaches have been employed. One study used an avidin column for the adsorption of transcarboxylase (Berger and Wood, 1974). The enzyme was dissociated into subunits at pH 9.0, and only the subunits containing the biotin remained bound to the column. In this manner the nonbiotinylated subunits were isolated in a nonactive state. Upon addition of purified biotin-containing subunits, the enzyme was reconstituted in a highly active form. A similar approach was used to isolate apo(acetyl-CoA-carboxylase) completely free of the holoenzyme (Landman and Dakshinamurti, 1973, 1975). In another approach, biotin was coupled to Sepharose followed by an excess of avidin. To this immobilized complex, pyruvate-carboxylase was added. Again, in this way the biotin-containing subunit could be separated from the remaining subunits (Lane et al., 1970).

2. "Tailor-Made" Biotin-Containing Systems

The avidin-biotin complex can be used as a tool for purification, even for systems in which biotin is not a native component (see Figure 2). Such systems usually employ a sandwich-type approach (Wilchek and Gorecki,

1973). The standard protocol involves the coupling of biotin or a hapten to one of the interacting species, followed by controlled incubation with the counterpart. The mixture is then applied to an avidin or antibody column. Alternatively, the biotin-containing partner may be bound initially to the immobilized avidin and a solution comprising the second species is then applied to the column. Theoretically, after extensive washing the interacting system can be dissociated and the underivatized member can be isolated. On the other hand, the interacting system can be separated theoretically as a complex by introduction of huge excesses of biotin. Because of the inefficiency of the latter procedure, considering the strong interaction between avidin and biotin, however, it is preferable to dissociate the interacting complex by other means (e.g., as described above for the isolation of transcarboxylase subunits).

As an example, we have tried both approaches on the purification of the receptor for insulin (Ginsberg and Wilchek, unpublished work). [^{125}I]Insulin was reacted with biotinyl-*N*-hydroxysuccinimide ester and mixed with a membrane extract from turkey erythrocytes. The complex was not adsorbed to the avidin affinity column. On the other hand, when the biotinylated insulin was bound to the avidin column and subsequently the extract was applied, the receptor was adsorbed to the column. It seems that prior formation of the biotin-derivatized insulin-receptor complex shields the biotin moiety from further interaction with the avidin column. Using the second approach, however, afforded no advantage over direct coupling of insulin to Sepharose because similar dissociation conditions (3*M* guanidine-hydrochloride) were required in both cases to remove the receptor from the column.

Our lack of luck with the insulin receptor, however, seems not to be a general phenomenon among other interacting systems. For example, a method for gene enrichment from the total DNA of an organism has recently been described (Pellegrini et al., 1977; Manning et al., 1977). Purified RNA, from the corresponding gene, was covalently attached to biotin by means of a cytochrome *c* bridge. The modified RNA was hybridized to the total DNA preparation. Only the DNA, which recognized RNA, underwent hybridization; thus the specific population could be separated from the other DNA through the action of the avidin-biotin interactions. This was accomplished either by affinity chromatography on an avidin column or by gradient separation on avidin-containing microspheres. The gene was obtained in high yields, 42-80% pure.

The avidin-biotin complex was also used for the retrieval of thymocytes artificially labeled with BNHS (Jasiewicz et al., 1976). In this study the cells were adsorbed to avidin immobilized on nylon mesh. No attempt was

made to release the cells from the solid support nor to reconstitute native cells. This study would have been more valid if the biotin had been bound to the cells in a reversible manner, either through the use of reagents that can be split chemically, or by enzymatic cleavage using biotinidase, which removes biotin from proteins in natural systems (Koivusalo et al. 1963).

We have used this technique to separate successive generations of proliferating yeast cells (Bayer, Niedermeyer, and Skutelsky, unpublished work). Yeasts were harvested in mid-log phase and subjected to biotinylation. The cells—still viable—were allowed to double in number, and after one generation two different populations of cells were observed in the electron microscope: one exhibiting prominent surface label following ferritin-avidin treatment, and a second that was completely void of surface label after the same treatment. In some cases unlabeled buds on labeled cells were seen. The biotin-derivatized parent generation thus could be separated from the daughter cells by sequestration of the former on an appropriate solid support containing either avidin or antibiotin antibodies (Berger, 1975). In the latter case the cells could be released after immobilization by concentrated solutions of biotin. We have also found that preferential agglutination on 10% fetal calf serum, bovine serum albumin, or sucrose provides a suitable method for separating larger numbers of cells.

Other biotinylated proteins have also been prepared for receptor perturbation and subsequent purification studies. Thus adrenocorticotropic hormone (ACTH) (Hofmann and Kiso, 1976) and insulin (Hofmann et al., 1977; May et al., 1978) have been modified with biotin for the purpose of isolating the respective receptors. However no such isolation has yet been described.

Biotinylated-lectins and antibodies have also been retrieved on avidin columns for their separation from the underivatized protein (Bayer et al., 1976b; Skutelsky and Bayer, 1976b). This procedure, however, was instituted for analytical purposes rather than for purification. The use of this class of biotinylated binding proteins is described below Section VI, on affinity cytochemistry.

The avidin-biotin complex has also been used for purification studies in a relatively new approach termed "affinity partitioning" (Flanagan and Barondes, 1975). This technique involves the action of an aqueous, polymer two-phase system. The addition of a polymer-ligand that partitions predominantly into one phase has been shown to cause a shift of the ligand-receptor into the same phase. Thus biotinylated-binding proteins can be complexed with poly(ethylene-oxide) avidin, and this complex is capable of partitioning the respective receptor from the dextran phase.

The avidin-biotin complex was used to isolate an affinity-labeled oligonucleotide fragment from *E. coli* 23-*S* ribosomal RNA. The avidin was used to "fish out" biotinylated affinity-labeled nucleotides. The basicity of avidin then permitted the isolation of the complex on a phosphocellulose column (Eckermann and Symons, 1978).

3. Experimental Procedures

A. PREPARATION OF AFFINITY COLUMNS

a. Activiation of Sepharose (Axen et al., 1967). Sepharose 4B (10 g) was washed well with distilled water, resuspended in 10 ml of distilled water, and the suspension was stirred constantly with a magnetic stirrer. Cyanogen bromide (1.25 g) was added and the pH maintained between 10.0 and 11.0 by the dropwise addition of 2*N* sodium hydroxide. After 10 min the activated gel was filtered and washed extensively with cold 0.1*M* sodium bicarbonate. The activation procedure can be performed in the presence of water-miscible organic solvents. The typical procedure in our laboratory includes dissolving cyanogen bromide (100 g) in dimethylformamide (50 ml), and the activation is performed in 2*M* Na_2Co_3 for 2 min. Dimethylformamide is more advantageous than acetonitrile, because a yellow product is often obtained with the latter solvent.

Coupling of ligands to activated Sepharose is usually performed in 0.1*M* bicarbonate solutions for 16 hrs at 4°C.

b. Biotin Affinity Columns

Biocytin (Bayer and Wilchek 1974). Biocytin is synthesized as follows: BNHS (340 mg, 1 mmole) was suspended in 3 ml of dimethylformamide, hot α-*t*Boc-lysine (400 mg) dissolved in 4 ml of sodium bicarbonate was added, and the suspension was adjusted to pH 8.5. The reaction was carried out for 4 hr. The solvent was evaporated under pressure and *t*Boc-biocytin was precipitated by the addition of 5 ml of 10% citric acid. The crystals were filtered and washed with water. A second crop of crystals can be isolated from the mother liquor.

The *t*Boc-group was removed by treatment with 4*N* hydrochloric acid in dioxane (5ml). After 20 min ether was added. The crystals formed are filtered, washed well with ether, and dried *in vacuo*.

Biocytin Sepharose. Biocytin-hydrochloride was coupled to activated Sepharose as described above. About 25 mg of biocytin was used to prepare 20 ml of substituted Sepharose.

Polymeric Biotin-Containing Columns. The synthesis of poly-L-lysine or polyacrylic hydrazide and its subsequent coupling to Sepharose was

described earlier in this series (Wilchek and Hexter, 1976). Poly-L-lysyl-Sepharose (3 g, containing about 60 μmole of lysine) was suspended in absolute dioxane (3 ml). An excess of biotin (65 mg) was added followed by an equimolar amount of DCC (50 mg). The reaction was allowed to proceed overnight. The biotin-conjugated poly-lysyl-Sepharose was washed several times with large volumes of dioxane, methanol, and distilled water, respectively, and stored in about 5 ml of distilled water.

Alternatively, the column above can be prepared using BNHS at pH 8.5. Other biotin-containing columns were prepared by coupling biotin to diamine- or dihydrazide-coated Sepharose.

c. Avidin Affinity Columns

Cyanogen Bromide Induced Immobilization (Bodanszky and Bodanszky, 1970). Avidin (20 mg in 20 ml of 0.1*M* sodium bicarbonate) was added to 10 g of activated Sepharose (above), and the suspension was stirred overnight at 4°C. The gel was washed (the supernatant and washings checked by absorption at 282 nm to determine the amount of protein coupled) and stored suspended in water with the addition of a few crystals of sodium azide.

Periodate-Induced Immobilization. Avidin (20 mg) was dissolved in 20 ml of 0.1*M* sodium acetate buffer, pH 4.5, and sodium *m*-periodate (22 mg) was added. The reaction was carried out for 3 hr at 4°C, after which ethylene glycol (2 ml) was added. The solution was dialyzed overnight at 4°C. The contents of the dialysis bag were then allowed to interact for 3 hr at room temperature with either adipic-hydrazido Sepharose or poly acryl-hydrazido Sepharose. The extent of immobilization (>95%) was checked by measuring the absorbance (282 nm) of supernatant fractions. Using this method, the affinity constant for biotin was somewhat reduced.

Other Coupling Methods. In addition to the above-mentioned methods, avidin has been coupled to other columns, notably glass beads via the *N*-hydroxysuccinimide active ester (Manning et al., 1977). Avidin has also been immobilized on nylon mesh by partial hydrolysis of the nylon and subsequent coupling using a water soluble carbodiimide (Jasiewicz et al., 1976). Polymethacrylate spheres have also been used for the same purpose (Manning et al., 1975). We have also coupled avidin to fixed, intact, erythrocyte membranes as a mode of immobilization.

B. ISOLATION PROCEDURES

The lack of chromophore in biotin renders the isolation of biotin-containing peptides difficult to follow. Two approaches were taken by Bodanszky and Bodanszky (1970) to overcome this obstacle. One

approach employed radioactive biotin-containing peptides. In another more elegant method 4-hydroxyazobenzene-2′-carboxylic acid was applied to the avidin column, giving a pink color. The color was displaced upon addition of biotin-containing peptides. In a typical experiment using this approach, a 1-ml column of avidin (2 mg/ml) was able to bind 0.06 μmole of biotin. The same amount of biotin-containing peptides was adsorbed. However elution was extremely difficult; using 6*M* guanidine hydrocholoride, pH 1.5, only 25% of the biotin-containing peptide was eluted from the avidin column.

A different approach to the isolation of biotin-containing peptides was taken by Rylatt et al. (1977). Soluble avidin was used to bind the biotin-containing peptides in solution. It was found that zinc sulfate is capable of precipitating the avidin-biotin complex without concomitant dissociation. After washing out non-biotin-containing peptides, the avidin-biotin complex was dissociated by incubating with 70% formic acid for 1 hr at room temperature. Under these conditions avidin was irreversibly denatured and could no longer interact with biotin.

VI. LOCALIZATION STUDIES: AFFINITY CYTOCHEMISTRY

1. General Considerations

One of the most cogent areas of recent interest concerns the specific localization of functional groups, biologically active components and receptors on cell surfaces. The use of affinity methods for the localization, visualization, and subsequent evaluation of specific cellular components by light, fluorescent, or electron microscopy has been termed "affinity cytochemistry" (Bayer et al., 1976b). In general, the technique is based on the preparation of a mixed conjugate, comprising a biologically active molecule (e.g., antibodies, lectins, hormones) attached chemically to a potentially demonstrable probe (e.g., fluorescein, ferritin, peroxidases, hemocyanin), whereby the resultant product retains both detectibility and biological activity. For use in light, fluorescent, and electron microscopical studies, these probes have been coupled to a wide spectrum of biologically active molecules, including antibodies (Singer and Schick, 1961; Avrameas, 1969; Raff, 1976), lectins (Nicholson and Singer, 1971; Ash and Singer, 1976), hormones (Jarrett and Smith, 1975), lipoproteins (Anderson et al., 1976), vitamins (Bayer et al., 1978b), sugars (Monsigny et al., 1976), cations (Danon et al., 1972), and anions (Bayer and Skutelsky, unpublished work). Because the defined electron-opacity of the ferritin iron core affords superior resolution qualities, ferritin is the electron microscopic marker of choice.

The preparation of ferritin conjugates represents the major problem inherent in the above-described method. Procedures currently available for covalent coupling of ferritin to the biologically active counterpart are, for the most part, cumbersome and inefficient. The resultant complex is of high molecular weight—often a multimer—thus affecting both the physical and chemical binding characteristics as well as the biological activity of the conjugate.

It has been shown, however, that the use of the high affinity avidin-biotin complex can circumvent some of the problems relating to ferritin-protein conjugation (Bayer et al., 1976a). In addition, this method may be employed to unify and facilitate certain aspects of affinity cytochemical techniques (Heitzmann and Richards, 1974; Bayer et al., 1976b, 1978c; Skutelsky and Bayer, 1976a).

The following steps are involved in this approach. (*a*) Biotin is attached via an appropriate reactive derivative, either directly to cell surface functional groups (sugars, amino acids, etc.) or to a biologically active molecule (antibody, lectin, etc). (*b*) In the latter case, the biotinylated conjugate is incubated with an appropriate target (intact cells or tissue, enzymatically or chemically treated cells, membrane preparations, subcellular fractions, defined macro molecules). (*c*) Subsequent incubation with ferritin-avidin conjugates permits ultrastructural visualization of the given cell surface receptor. (*d*) Proper controls using nonbiotinylated preparations and/or unconjugated ferritin should always be implemented.

An alternative method, which constitutes a permutation of the foregoing method, has also been attempted in our laboratory. Biotinylated membrane sites are saturated with free avidin. Since the latter is a tetramer, subsequent interaction with biotin-conjugated ferritin also results in specific labeling of cell surfaces. This method is somewhat less tedious than that involving ferritin-avidin conjugates because preparation and analysis of protein-protein conjugates are precluded. However the resultant cell surface label is much less uniform, and consequently the method is less reliable. Figure 5 summarizes both approaches.

2. Localization of Functional Groups

A. SUGARS

The avidin-biotin complex was first used as an affinity cytochemical probe by Heitzmann and Richards (1974) for the localization of biotin-tagged sites on membrane preparations of *Acholeplasma laidlawii* and on erythrocyte ghosts.

In our laboratory, in collaboration with Dr. Ehud Skutelsky, ferritin-

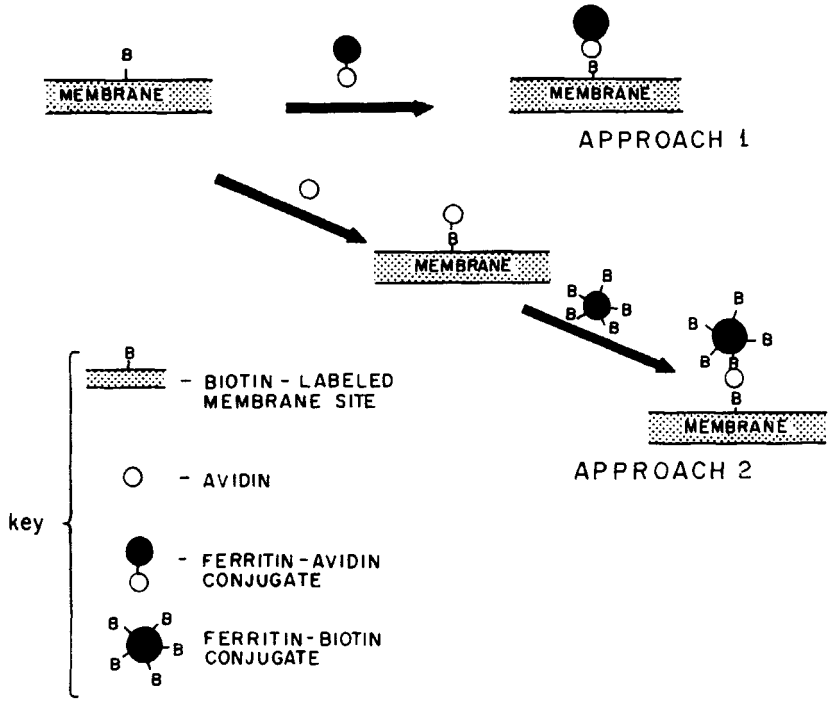

Figure 5. Schematic representation of two possible approaches for ultrastructural cyto chemistry using the avidin-biotin system.

avidin conjugates (FAv) were used in a variety of studies on intact erythrocytes and lymphocytes. Thus erythrocytes from various mammalian species (e.g., mouse, rat, rabbit, and human) were treated with periodate under conditions causing the *cis*-hydroxyl groups of sialic acid (SA) to be selectively oxidized to the corresponding aldehydes (Skutelsky et al., 1977). The latter were reacted with biotin hydrazide, resulting in biotinylated erythrocytes. The reaction is schematically shown in Figure 6. Treatment of these cells with FAv revealed the SA in the cell surface (Figure 7). This method was shown to be superior to such other electron microscopic methods previously used for the localization of SA as cationized ferritin (Danon et al., 1972) and colloidal iron (Gasic et al., 1968), since only surface SA and not anionic groups were detected. As Figure 7 indicates, the ferritin particles were somewhat removed from the membrane surface. Therefore we were able to calculate the average distance of the SA from the erythrocyte surface. Values of 50 to 70 Å were

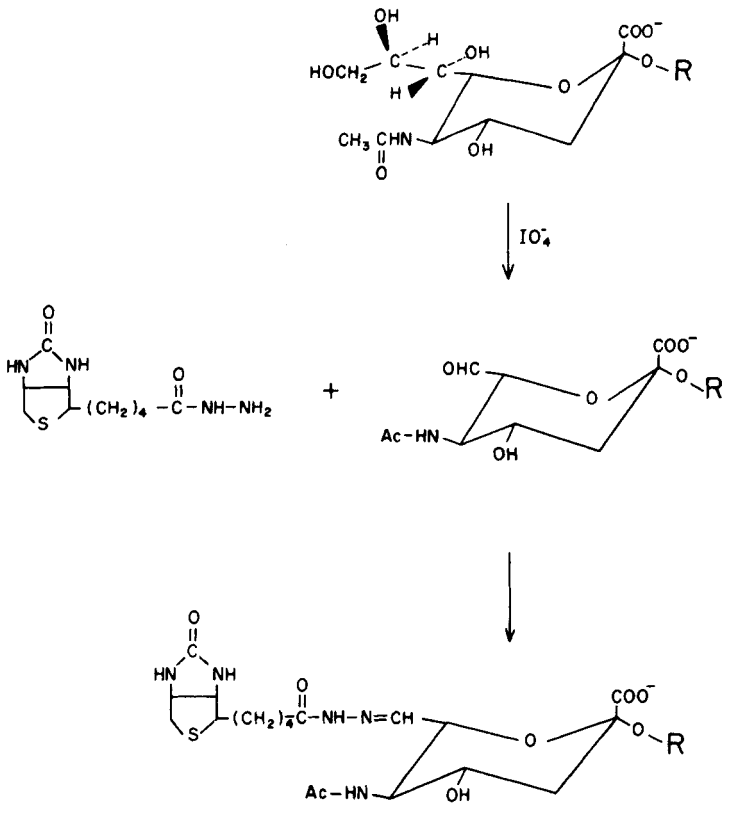

Figure 6. Schematic description of selective biotinylation of membrane-bound sialic acid residues.

obtained for rat and human, whereas in rabbits the SA was juxtaposed to the lipid bilayer (Bayer et al., 1977). We were also able to follow the fate of SA during the development of the erythrocyte from its precursors (Skutelsky et al., 1977).

Very recent studies have shown that the same procedures can be used to study alterations in the surface in various examples of diseased blood cells. Thus striking alterations in the topography of SA were observed on erythrocytes derived from thalassemic patients (Kahane et al., 1978). These observations were in accordance with the decreased life span of these cells in the circulation. In another study with lymphatic leukemia cells from both human and bovine sources, we have observed dramatic increases in the surface labeling of SA, compared with that of normal

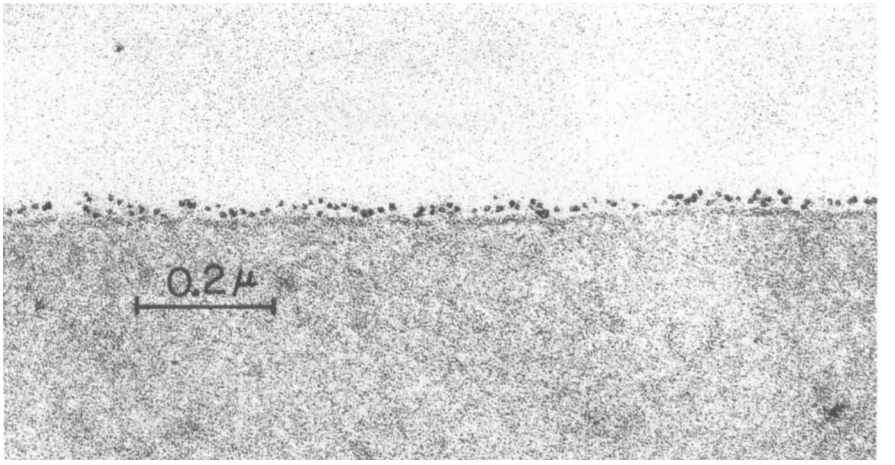

Figure 7. Labeling of membrane-bound sialic acid sites on the human erythrocyte by periodate-induced biotinylation followed by treatment with ferritin-avidin conjugates. Note the measurable distance separating the ferritin particles from the plane of the membrane.

lymphocytes (Skutelsky et al., 1978). In the bovine leukemic cell, a dense, multilayered deposition of FAv was obtained, extending to more than 500 Å from the lipid bilayer. Likewise, the density of SA on lymphoid cells, derived from chronic lymphatic leukemia patients, was nearly twice that of lymphocytes derived from healthy donors.

The same biotinyl derivative can also be used for the localization of galactose residues on the cell surface by prior treatment with galactose oxidase (Heitzmann and Richards, 1974). In a similar manner this procedure, combined with enzymatic techniques, can be used as a tool for the localization of sialyl-blocked, membrane-bound, galactose-containing sites (Figure 8). Treatment of aldehydes generated by galactose oxidase with an unrelated hydrazide, followed by reduction with borohydride, blocks free galactosyl groups. Subsequent enzymatic digestion of SA residues with neuraminidase exposes penultimate galactose (or *N*-acetylgalactosamine), which may now be subjected to successive treatment with galactose oxidase and biotin hydrazide.

B. AMINO ACID RESIDUES

The avidin-biotin complex is not only useful for the localization of cell surface sugars, it can also be used for the study of amino acid functional groups of cell surface proteins or polypeptides. Thus the *N*-hydroxy-

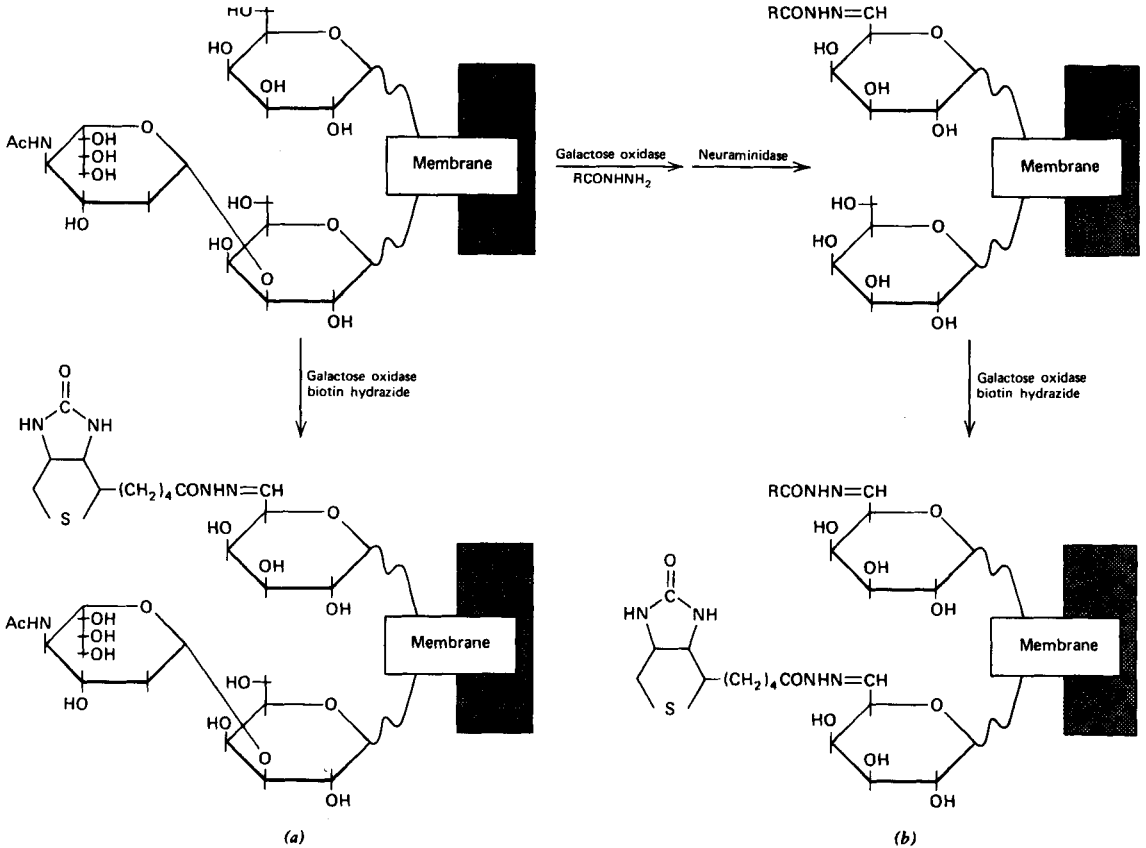

Figure 8. Schematic illlustration representing differential biotinylation of membrane-bound galactosyl (or *N*-acetylgalactosamine) residues in the free (*a*) or sialyl-blocked (*b*) form. The hydrazide derivatives of R is structurally unrelated to biotin and does not form a complex with avidin.

succinimide ester of biotin reacts fairly specifically with lysine residues. Use of this reagent at pH 5.0 limits its interaction to a α-amino groups. The diazonium salt of biotin can be used in the study of tyrosine and histidine residues, and is reversible upon reduction with dithionite.

The bromoacetyl derivative can be used for cysteines, or, following reduction with thiols, it is also suitable for localization of cell surface cystine bridges. Depending on pH, the bromoacetyl reagent may also be used to localize methionines, histidines, and lysines. Thus a variety of amino acid group-specific reagents are available, and comparative studies of the localization and analytical isolation of labeled surface proteins from various cell types and species are currently in progress in our laboratory.

3. Localization of Receptors

Of greater significance is the use of the avidin-biotin complex for the localization and evaluation of receptors on cell surfaces. Cell surfaces are known to possess a variety of receptors for hormones, antibodies, lectins, drugs, toxins, effectors, and a variety of other biologically active compounds. The amount of these receptors is usually very low, and very highly radioactive labeled compounds must be used to quantify the number of receptors per cell.

The use of electron microscopic techniques obviates some of the difficulties by permitting specific labeling with non-penetrating markers for quantitative analysis. The direct use of ferritin-conjugated binding proteins introduces a factor of uncertainty, since the binding proteins are usually of much lower molecular weight than the marker. The conjugated binding protein may exhibit reduced activity and/or altered specificity, thus restricting the reliability of the method. Coupling a small molecule, such as biotin, to the binding protein, under defined conditions, will only slightly affect the binding characteristics. Following interaction of the biotinylated binding protein with the cell surface enables more specific labeling of the receptor. The cell may be fixed either before or after the labeling procedure; consequently, these conditions are amenable to kinetics studies. Following fixation, the biotin-tagged sites are available for further interaction with FAv. Figure 9 presents the procedure for specific labeling of receptor sites.

We have exploited this approach (Bayer et al., 1976b) and have coupled biotin to various lectins, including concanavalin A (Con A) peanut agglutinin (PNA), soybean agglutinin, phytohemagglutinin, and wheat germ agglutinin. The biotinylated Con A (B-Con A) had properties similar to those of the native protein—namely, it bound to Sephadex and agglutinated erythrocytes. When we applied the B-Con A to erythrocytes

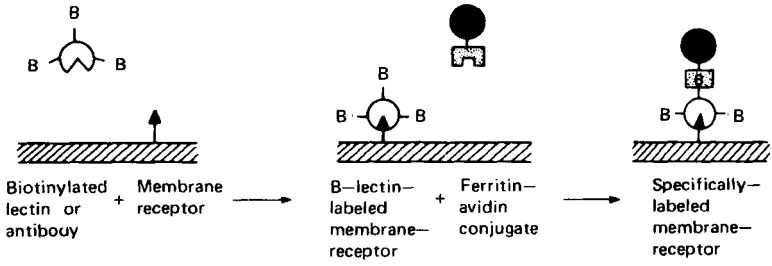

Figure 9. Schematic procedure for specific labeling of receptor sites by a biotinylated binding protein (antibody, lectin, hormone, effector, etc.), followed by interaction with an appropriate avidin-conjugated marker (ferritin, hemocyanin, peroxidase, fluorescein, etc.).

followed by FAv, the cell surface was heavily labeled with ferritin. α-Methyl-mannoside was effective in preventing interaction between the surface receptor and B-Con A but did not interfere with that between FAv and B-Con A. In a cognate study, when B-PNA, which has no exposed receptors on human erythrocytes, was incubated with the latter cells, no ferritin label was detected. When sialyl residues were removed with neuraminidase, however, the newly exposed B-PNA-receptor complex could be localized by the treatment above.

Biotinylated antibodies, elicited against erythrocytes, could be used to detect membrane-bound antigens in a similar manner. It is interesting to note that biotinylated whole antiserum can be used, since only the active cell-binding antibodies complex with the membrane, while other nonrelated biotinylated proteins are removed during subsequent centrifugation steps.

Because the number of receptor sites to Con A, PNA, and antibodies is relatively high, we are now trying to apply this approach to the localization of biologically important receptors present in very limited quantities on the cell surface, that is, hormone receptors (Riesel et al., 1977). Thus biotinylated insulin and human chorionic gonadotropin (hCG)) are presently being used for the localization of these receptors on lymphocytes and ovary cells, respectively.

4. Other Systems

The planar distribution of surface membrane proteins in *Acholeplasma laidlawii* has been investigated using the avidin-biotin complex (Wallace et al., 1976). The membranes were labeled with biotin via the *N*-hydroxysuccinimide ester. Under the conditions reported, only membrane protein constituents were derivatized. Biotinylated-membrane prepara-

tions were then treated with ferritin-avidin conjugates in a temperature-controlled chamber and fixed by drying in an atmosphere of dry nitrogen gas. It was found that membrane proteins were relatively dispersed either in the paracrystalline or smectic phases (below or above the phase transition, respectively). On the other hand, patches were observed at temperatures in the midphase transition range. These results indicate that the physical state of membrane lipids can influence the relative location of protein constituents within the plane of the membrane surface.

We have used the avidin-biotin complex for the direct visualization of the interaction between liposomes and the cell surface (Bayer et al., 1978a). Biotin was covalently attached to the head groups of appropriate lipids, again via biotinyl-*N*-hydroxysuccinimide ester. Liposomes, consisting of 5% biotinylated lipids, were interacted with various types of cells. Following fixation at an appropriate time interval, the distribution of biotinylated lipids on the cell surface was evaluated using ferritin-avidin conjugates. It was shown that the extent and mode of liposome-cell interaction was dependent upon both the lipid content of the liposome and the cell type.

Ferritin-avidin conjugates were also used to determine the relative position of the 4*S* and ribosomal RNA genes in HeLa cell mitochondrial DNA (Angerer et al., 1976). These authors found that at least 19 4*S* RNA genes are present in the HeLa mitochondrial-DNA genome.

Macromolecules have also been a subject for electron microscopic investigations using the avidin-biotin complex. In a pioneering work, Green and co-workers (Green et al., 1971) used bivalent biotin-containing compounds to study the orientation of avidin subunits. A modification of this approach (Green et al., 1972) permitted determination of the position of the biotin carboxyl carrier protein of native transcarboxylase. In an application of this technique to macromolecules that are not native biotin-associated systems, we have used the avidin-biotin complex to localize the positions of oligosaccharide residues on collagen (unpublished work).

The use of the avidin-biotin complex in ultrastructural or related labeling studies is not limited to FAv, and other markers can be used. Although ferritin exhibits superior resolution qualities for the analysis of labeled material in thin sections, freeze-etching replicas, shadow casting, or negatively stained sections by transmission electron microscopy; other markers—namely peroxidases, hemocyanin, and phages—are appropriate for evaluation by the latter techniques. Horseradish peroxidase may be used for labeling studies in both ultrastructural and light microscopic analysis. Hemocyanin or phages, conjugated to avidin, may eventually prove to be excellent markers for the detection of minute

amounts of receptors or as defined markers for adaptation of the technique to scanning electron microscopy.

A fluorescent form of avidin has already been applied to fluorescent microscopy. Heggeness and Ash (1977) used biotinylated heavy-meromyosin and biotinylated antiactin antibodies to visualize the distribution of nonmuscle contractile proteins in fibroblast cells. Extension of this technique to ultrastructural analysis may enable double-labeling studies for determination of the interrelationship between exocellular receptors and intracellular contractile components. The combination of two ultrastructural labels (e.g., peroxidase as the intracellular marker and ferritin conjugates as the extracellular label) may pave the way for such studies.

The use of the avidin-biotin complex as a general probe in affinity cytochemistry is appealing for a variety of reasons:

1. Only one conjugate (e.g., ferritin-avidin, fluorescent-avidin) need be prepared and characterized for all affinity systems.
2. Biotin can be attached to most small ligands and macromolecules efficiently and under very mild conditions.
3. In most cases, the size, the physical characteristics, and the biological activity of biotin-derivatized proteins are only nominally affected.
4. Crude preparations of binding proteins (e.g., whole antiserum instead of antibodies or plant extracts instead of lectins) can be biotinylated, and, following dialysis, may be used for localization studies without further purification.
5. The biotin-avidin complex is of exceptionally high affinity and stability.
6. The use of this system allows kinetics studies, since fixation and subsequent localization via the conjugated marker can be performed at any stage during the probe (binding protein) receptor interaction.
7. The avidin-biotin complex, in conjunction with standard affinity cytochemical systems (direct conjugation of binding protein to marker), may be used for double-labeling studies.
8. Both avidin and biotin are commercially available in large quantities.

5. Restrictions of the Method

Although the avidin-biotin complex affords a highly versatile method for specific ultrastructural labeling studies, the potential user should be aware of several limiting or interfering factors. Initially, when applying the biotin-avidin interaction to a given experimental system, it must be

determined whether the latter comprises a biotin-containing, biotin-recognizing, or biotin-free system. Avidin also possesses a biological role, albeit as yet undefined, and is produced in the oviducts of various avian and reptilian species as well as by selected strains of bacteria (Green, 1975). Avidin has been found to bind selectively to condensed chromatin (Heggeness, 1977) in a manner apparently unrelated to its biotin-binding properties. Our own observations confirm this phenomenon, since FAv binds "unspecifically" to subcellular fractions that are prepared from osmotically shocked intact cells and undoubtedly contain large amounts of adsorbed nucleic acids. It should be noted that avidin is a basic glycoprotein, and either ion-exchange properties or its oligosaccharide moiety might be responsible for a variety of side interactions. Consequently, it is emphasized that proper controls should be implemented in all applications of the avidin-biotin complex to affinity cytochemical studies.

6. Experimental Procedures

A. PREPARATION OF AVIDIN-CONJUGATED MARKERS

a. Ferritin-Avidin Conjugates via Reductive Alkylation. (Bayer et al., 1976a). Commercial avidin (15 mg) in 5 ml of acetate-buffered saline, (ABS) pH 4.5, was added to ferritin (100 mg, 1 ml). Sodium *m*-periodate (Merck, 0.66 ml, 0.1*M* solution) was added to a final concentration of 10m*M*. The mixture was stirred for 3 hr in ice, dialyzed for 6 hr against ABS at 4°C, and followed by a second dialysis overnight at 4°C against borate-buffered saline, pH 8.5. A fresh solution of sodium borohydride (10 mg/ml in 0.01*M* sodium hydroxide) was prepared, and 0.5 ml was added to the ferritin-avidin conjugates in an ice bath. After 1 hr the solution was dialyzed against phosphate-buffered saline (PBS), pH 7.0. The conjugates were washed twice by centrifugation (100,000 × *g*, 3 hr) and resuspension in PBS, and finally resuspended to 1 mg of ferritin per milliter.

This method of unidirectional conjugation has been shown to provide increased yields of active, unit-paired conjugates. Reductive alkylation is therefore recommended over the conventional glutaraldehyde techniques for the preparation of ferritin avidin conjugates.

b. Glutaraldehyde Method. (Heitzman and Richards, 1974). Commercial avidin (15 mg in 3 ml of PBS) was added to a solution of ferritin (1 ml, 100 mg) and stirred at room temperature. Glutaraldehyde (440 μl, 0.5% solution) was added slowly to a final concentration of 0.05%. The reaction was allowed to proceed for 1 hr at room temperature and

then stopped with 0.1*M* ammonium bicarbonate. The conjugates were dialyzed overnight against PBS. Large aggregates were removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was subsequently centrifuged at 100,000 × *g* for 3 hr. The pellet, consisting of free ferritin and ferritin-avidin conjugates, was resuspended in PBS, and the 100,000 × *g* centrifugation step was repeated. (The supernatant fractions containing free avidin were saved for future preparations of ferritin-avidin conjugates.) The washed conjugates were resuspended in PBS to a final concentration of 1 mg of ferritin per milliliter (A_{440} = 1.1).

Alternatively, to achieve unidirectionality, ferritin can be treated with an excess of glutaraldehyde (15%) and subsequently interacted with avidin following gel filtration on Sephadex G-25 to remove free glutaraldehyde (Otto et al., 1973).

c. Other Coupling Methods. Another unidirectional approach for the preparation of ferritin-avidin conjugates was described by Angerer et al. (1976). In this procedure, ferritin was bromoacetylated and reacted with thiolated avidin.

d. Other Markers. Other markers (e.g., peroxidases, hemocyanin) can be substituted for ferritin, and the respective avidin conjugates can be prepared as above, using equimolar amounts of the prospective marker. In the case of large markers (hemocyanin, phages, etc.), free avidin can be removed from the reaction mixutre by centrifugation as above. With smaller markers (peroxidases, etc.) the conjugates can be separated from the reactants by gel chromatography.

Fluorescein-derivatized avidin was prepared as follows. To a solution of avidin (14 mg) in 0.01*M* phosphate buffer, pH 7.4 (1 ml), was added fluorescein-isothiocyanate (300 ug in 0.1 ml of 0.5*M* sodium carbonate buffer, pH 9.5). The solution was stirred overnight in the cold. The conjugate was separated from the free ligand by passage of the reaction mixture over Sephadex G-25. The conjugate was visibly present in the void volume. About 6 fluorescein groups were attached per avidin molecule by this procedure. A somewhat different procedure was described by Heggeness and Ash (1977).

e. Analysis of Conjugates. The relative size of ferritin-avidin conjugates can be tested by gel filtration on a Sepharose 6B column. The extent of ferritin conjugation was assayed by affinity chromatography on a biotin-containing affinity column. The difference in absorbance (A_{440} applied effluent) represents the amount of active ferritin-avidin conjugate.

B. DIRECT BIOTINYLATION OF FUNCTIONAL GROUPS

a. Sialic Acid Residues (Skutelsky et al., 1977). Cells (10^8 ml) were washed and resuspended in PBS (1 ml). Sodium *m*-periodate was added to a final concentration of 1m*M*, and the reaction was allowed to proceed for 30 min in an ice bath. The cells were then washed twice with PBS and resuspended in a solution of biotin hydrazide (2.5 mg/ml). After 1 hr at room temperature, the cells were washed three times in PBS and fixed in 2% glutaraldehyde (1 ml in PBS).

b. Galactose and N-Acetylgalactosamine Residues (Heitzmann and Richards, 1974). The primary hydroxyl groups of these sugars were treated with galactose oxidase, and the resultant aldehydes were interacted with biotin hydrazide. Cells (approximately 10^8) were washed twice with PBS and mixed with sodium borohydride (2m*M* in PBS) to quench the effect of endogenous, oxidized membrane components. The cells were washed twice in buffer, and biotin hydrazide (2.5 mg/ml PBS) was added. The suspension was treated with galactose oxidase (10 units, Sigma Chemical) for 3 hr at 37°C, washed twice with buffer, and fixed in glutaraldehyde.

C. BIOTINYLATION OF BIOLOGICALLY ACTIVE PROTEINS

For relatively stable, biologically active proteins, such as antibodies, lectins, and polypeptide hormones, the following biotinylation procedure has proved to be effective.

Biotinyl-N-hydroxysuccinimide ester (BNHS) dissolved in dimethyl formamide (DMF), was added to a solution, pH 7.0 or higher, of the desired protein in a 1-10 to 1-100 v/v and 5-1 mole/mole ratio. For example, an aliquot (0.1 ml) containing 0.5 μmole of BNHS (1.7 mg/ml DMF) was added to a solution containing goat anti-rabbit IgG antibodies (16 mg of protein in 1 ml of PBS). The solution was kept at room temperature for 4 hr and dialyzed overnight at 4°C against PBS, with one buffer change. Biotinylated antibodies or lectins may be stored at −20°C. Whole antiserum or unpurified lectins can be biotinylated in the manner just described and used for affinity cytochemical studies in their unrefined state. The reaction with BNHS can be restricted mostly to α-amines by performing the reaction between pH 5.0 and 6.0.

Relatively unstable proteins (or those subject to loss of biological activity upon chemical modification) may require additional or alternative treatment—for example, modification of cysteines or tyrosines, or separation of biotinylated proteins from underivatized material after milder biotinylation procedures.

D. INTERACTION BETWEEN BIOTINYLATED BINDING PROTEIN AND RECEPTOR

Viable cells (10^8) or cells fixed with 2% glutaraldehyde for 30 min, were washed and incubated with an appropriate solution of biotinylated lectins or antibodies (0.5-1.0 mg of protein per milliliter of PBS for 30 min at room temperature. Normal or optimal conditions of interaction should be used with any other protein type (hormones, toxins, effectors, etc.). Controls comprise labeling with underivatized protein samples or use of appropriate inhibitors. After incubation, cells are washed, fixed with glutaraldehyde, and treated with 2% bovine serum albumin.

E. LOCALIZATION OF BIOTINYLATED SITES

Further treatment with ferritin-avidin conjugates (FAv) (1 mg of protein per milliliter) results in specific labeling of biotin-tagged sites. Cells whose functional groups have been directly biotinylated are labeled with FAv by similar treatment. Samples prepared in this manner are then processed for electron microscopy (Luft, 1961). Controls, comprising pretreatment of FAv with biotin, should be employed.

VII. MISCELLANEOUS SYSTEMS

1. Phage Inactivation Studies

A. GENERAL CONSIDERATIONS

One of the earliest studies that demonstrated the availability of biotin, artificially complexed with a living system, for subsequent interaction with avidin, involved phage inactivation (Becker and Wilchek, 1972). In this work phages, to which biotin was covalently attached by means of the *N*-hydroxysuccinimide ester, were found to be inactivated with avidin (Table II). It was known previously that bacteriophages, modified by chemical attachment of haptens, are still viable and can be inactivated by specific antibodies against the covalently linked modifier. This technique was first demonstrated by Makela (1966) and Haimovich and Sela (1966) and was used for the detection and quantification of antibodies and haptens. An analogous study was performed by Becker and Wilchek (1972) to show that not only antibody-antigen complexes are capable of phage inactivation, but other interacting systems can be used, provided one component can be bound to the phage and the counterpart is multivalent. The avidin-biotin system was an ideal subject for this study, once again because of the high affinity constant of interaction. It was assumed that the latter property would render this method the most sensitive for

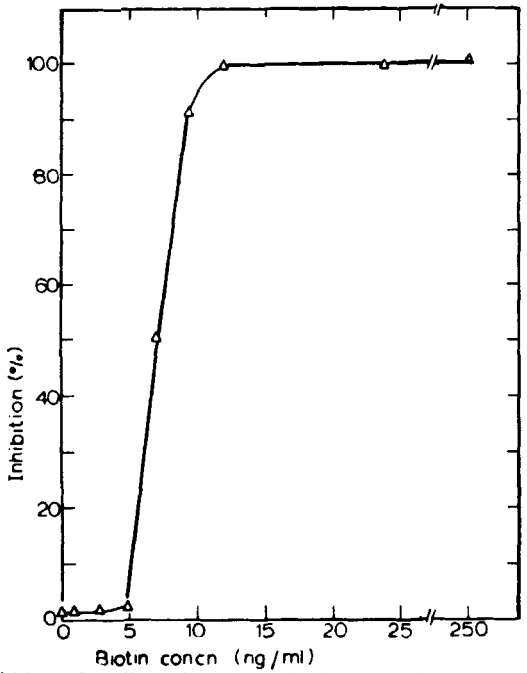

Figure 10. Inhibition of avidin-induced inactivation of biotin-derivatized phages by free biotin. Biotin (0.1 ml) at the concentration given was incubated with 0.1 ml of an avidin solution (20 μg/ml avidin in 50 m*M* sodium phosphate buffer, pH 6.8). A suspension (0.1 ml) of biotin-modified bacteriophage (see text) was added and the mixture incubated at 37°C for 1 hr. The surviving phage titer was determined, and the percentage inhibition of avidin-induced phage inactivation was calculated by comparing plaque-forming units per milliliter in the absence and in the presence of various concentrations of biotin.

quantitative estimates of biotin. It was found that avidin was indeed capable of inactivating the biotin-derivatized T_4-bacteriophage from forming plaques, and that free biotin in solution can prevent the observed inactivation. An interesting observation derived from this study was that only 2 moles of biotin per mole of avidin is required for the complete inhibition of phage inactivation. Figure 10 indicates that at least 2 biotin binding sites per avidin molecule must be unoccupied before avidin can inactivate the biotin-modified bacteriophage. The extremely narrow range in which inhibition by free biotin occurs may be a consequence of the relative homogeneity of avidin preparations versus the heterogeneous population of antibodies. Inhibition by free hapten of inactivation of chemically modified bacteriophages by antibodies usually occurs over several orders of magnitude of free hapten concentration.

B. EXPERIMENTAL PROCEDURES

a. Preparation of Biotin-Modified Bacteriophage. The bacterio-