METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 26

METHODS OF BIOCHEMICAL ANALYSIS

Edited by **DAVID GLICK**

Cancer Biology Research Laboratory
Stanford University Medical Center
Stanford, California

VOLUME 26

An Interscience® Publication
JOHN WILEY & SONS, New York · Chichester · Brisbane · Toronto

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. *By Edward A. Bayer and Meir Wilchek, Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel* 1

Polarographic Measurement of Steady State Kinetics of Oxygen Uptake by Biochemical Samples. *By H. Degn, J. S. Lundsgaard, and L. C. Peterson, Institute of Biochemistry, Odense University, Odense, Denmark; A. Ormicki, Department of Computer Sciences, Institute of Mathematics, Odense University, Odense, Denmark* 47

Analysis of Biological Macromolecules and Particles by Field-Flow Fractionation. *By J. Calvin Giddings, Marcus N. Myers, Karin D. Caldwell, and Susan R. Fisher, Department of Chemistry, University of Utah, Salt Lake City, Utah* 79

Recent Developments in the Stopped-Flow Method for the Study of Fast Reactions. *By Keitaro Hiromi, Laboratory of Enzyme Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan* 137

Peptide Mapping of Proteins. *By Gordon T. James, Department of Neurology, University of Colorado Medical Center, Denver, Colorado* 165

Solid-Phase Methods in Protein Sequence Analysis. *By Richard A. Laursen, Department of Chemistry, Boston University, Boston, Massachusetts; Werner Machleidt, Institut für Physiologische Chemie, Physikalishe Biochemie und Zellbiologie der Universitat München, Munich* 201

Analysis of Cellular Electron Transport Systems in Liver and Other Organs by Absorbance and Fluorescence Techniques. *By Helmut Sies and Bolko Brauser, Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität Münchenm, Munich* 285

High Temperature Gas-Liquid Chromatography in Lipid Analysis. *By V. A. Vaver and A. N. Ushakov, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow* 327

Author Index 407

Subject Index 417

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

EDWARD A. BAYER and MEIR WILCHEK, *Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel*

I. Introduction 2
II. Principle 4
III. Preparation of Reactive Biotinyl Derivatives 5
1. Amino Reagents 7
2. Carboxyl and Sugar Reagents 7
3. Thiol Reagents 8
4. Phenol and Imidazole Reagents 8
IV. Assays for Avidin and Biotin 9
V. Purification Studies: Affinity Chromatography 9
1. Natural Biotin-Containing Systems 9
2. "Tailor-Made" Biotin-Containing Systems 11
3. Experimental Procedures 14
A. Preparation of Affinity Columns 14
a. Activation of Sepharose 14
b. Biotin Affinity Columns 14
Biocytin 14
Biocytin Sepharose 14
Polymeric Biotin-Containing Columns 14
c. Avidin Affinity Columns 15
Cyanogen Bromide Induced Immobilization 15
Periodate-Induced Immobilization 15
Other Coupling Methods 15
B. Isolation Procedures 15
VI. Localization Studies: Affinity Cytochemistry 16
1. General Considerations 16
2. Localization of Functional Groups 17
A. Sugars 17
B. Amino Acid Residues 20
3. Localization of Receptors 22
4. Other Systems 23
5. Restrictions of the Method 25
6. Experimental Procedures 26
A. Preparation of Avidin-Conjugated Markers 26
a. Ferritin-Avidin Conjugates via Reductive Alkylation 26

b. Glutaraldehyde Method 26
c. Other Coupling Methods 27
d. Other Markers 27
e. Analysis of Conjugates 27
B. Direct Biotinylation of Functional Groups 27
a. Sialic Acid Residues 27
b. Galactose and *N*-Acetylgalactosamine Residues 28
C. Biotinylation of Biologically Active Proteins 28
D. Interaction Between Biotinylated Binding Protein and Receptor ... 29
E. Localization of Biotinylated Sites 29
VII. Miscellaneous Systems 29
1. Phage Inactivation Studies 29
A. General Considerations 29
B. Experimental Procedures 29
a. Preparation of Biotin-Modified Bacteriophage 30
b. Inactivation of Bacteriophage by Avidin 31
2. Lymphocyte Stimulation 31
3. Hormone-Receptor Interactions 33
VIII. The Biotin Transport System: An Affinity Labeling Study 35
IX. Antibiotin Antibodies 40
X. Conclusions 41
Acknowledgments 42
References 42

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

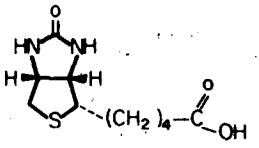

Figure 1. The structure of biotin.

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

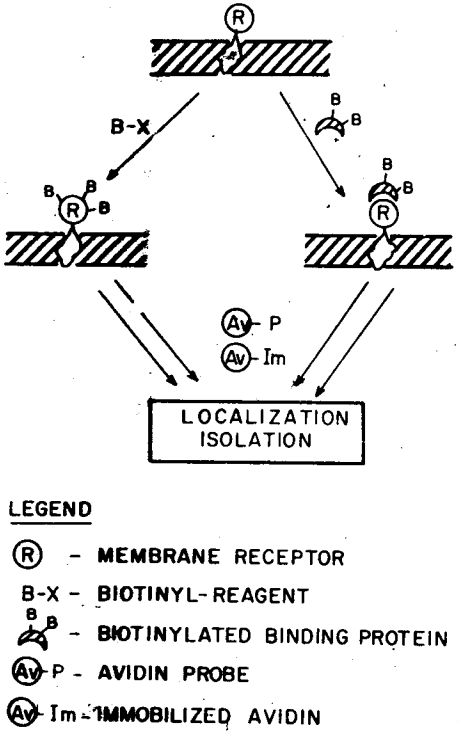

Figure 2. Schematic representation of the rationale behind the use of the avidin-biotin complex as a probe in molecular biology.

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

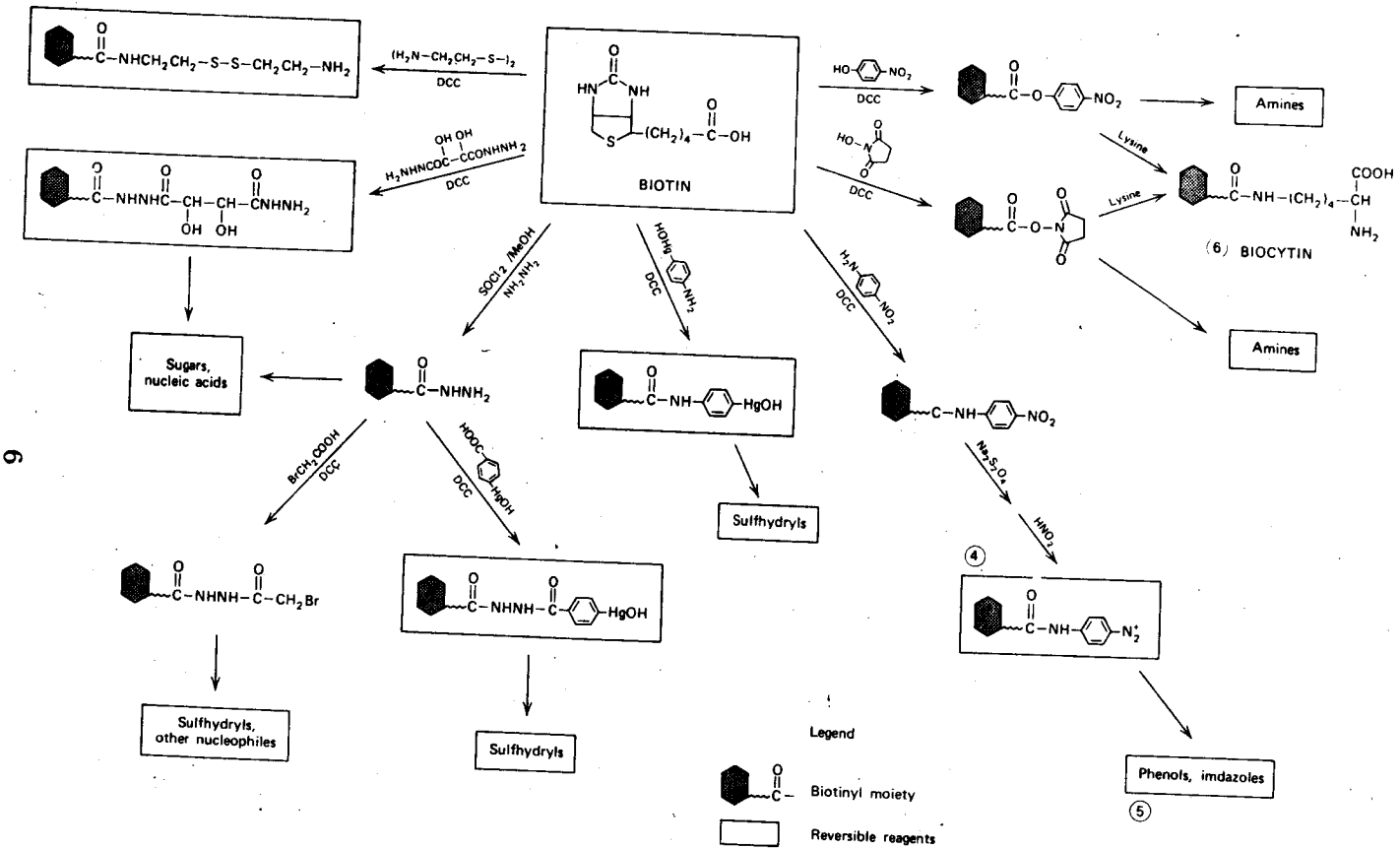

Figure 3. Reactive biotinyl derivatives useful for coupling to different functional groups.

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 ($\Delta\epsilon_{233} = 2.4 \times 10^4 M^{-1}$/mole biotin) upon complex formation with biotin (Green, 1970). The quantitative displacement by biotin of the avidin-dye (4′-hydroxyazobenzene-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

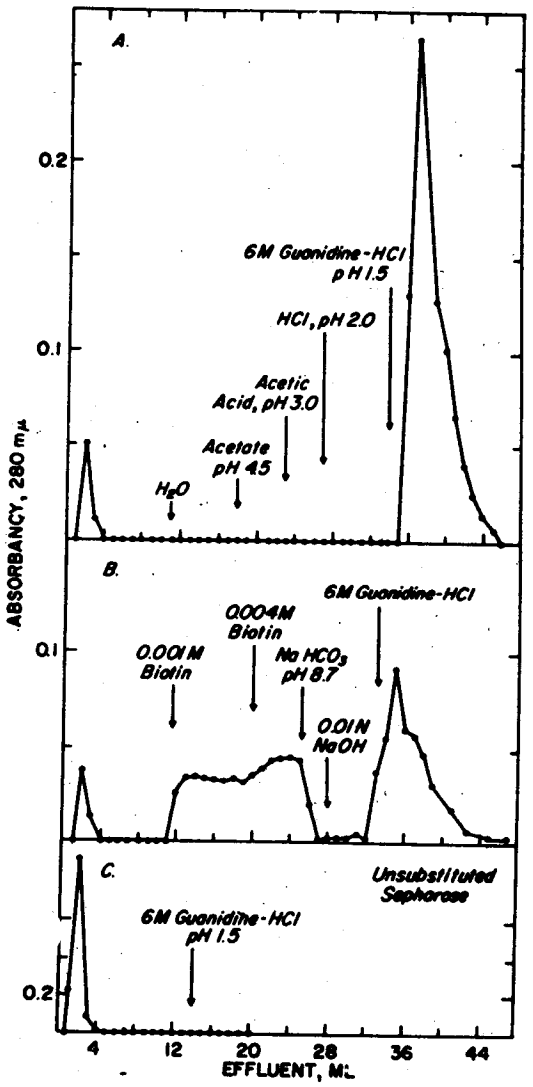

Figure 4. Affinity chromatography of commercial avidin on biocytin-Sepharose (*A*,*B*) and unsubstituted Sepharose (*C*) columns. The columns (0.5 × 5 cm) were equilibrated with 0.2 *M* sodium bicarbonate, pH 8.7, and 0.75 mg of avidin (in 0.5 ml of the same buffer) was attempted by varying the conditions 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,