PHARMACOLOGICAL REVIEWS
Vol. 25, No. 2

Printed in U.S.A.

Pharmacological Applications

CHAIRMAN: SYDNEY SPECTOR, PH.D.

Principles and Methods for the Preparation of Drug Protein Conjugates for Immunological Studies

BERNARD F. ERLANGER

Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York

IN 1917, when Landsteiner began to prepare what he called "artificial conjugated antigens," it was "to investigate an almost dogmatic belief . . . that a special chemical constitution, peculiar to proteins, was required for the production of antibodies" (48). We know better now, of course, but it is to these studies by Landsteiner that we owe all of the papers presented at this Symposium.

The earliest of his conjugated proteins were prepared by the acylation of the amino groups of serum albumin with chlorides or anhydrides of butyric, isobutyric, mono-, di-, and trichloroacetic, anisic, and cinnamic acids. This was followed by his better known studies in which diazonium compounds were allowed to react with histidine, tyrosine, and tryptophan residues of a protein. With these conjugates, he established that the original specificity of the protein carrier was changed by the newly introduced groups which, by themselves, were not antigenic, and that cross reactions among sera depended now upon the structural relationships among the acyl or azo groups that were covalently linked to the protein. He also noted that, in most cases, antibody was produced to the protein carrier as well and that to be certain of antibodies to the new determinant group, one had to test the sera with conjugates made with an unrelated or homologous (to the immunized animal) protein. It was his practice to remove any of the anticarrier antibody by absorption of the serum with the free protein. This is still done, although it is not always necessary for radioimmunoassays. Landsteiner also sought to determine the optimal number of haptenic groups that gave the best antibody response, and he concluded that too much or too little hapten led to a poor response. With serum albumin as the carrier, 10 haptenic groups seemed to be optimal. The major finding by Landsteiner, however, related to the exquisite specificity of the antisera, as was so beautifully demonstrated by his classical studies with L-, D-, and *meso*-tartaric acids.

Thus, Landsteiner's work established many of the ground rules by which we operate today. Our contributions since his time have been mainly refinement of techniques and procedures and the expansion of his ideas. The major exception to this statement, and a crucial one, indeed, is the development by Berson and Yalow (12) of the technique of radioimmunoassay. It is this procedure that has led to the dramatic expansion of immunological techniques into the fields of biochemistry and pharmacology.

In 1956, our laboratory, in collaboration with Beiser and Lieberman, became inter-

ested in preparing steroid-protein conjugates which were to be used to elicit the formation of antisteroid antibodies. An examination of the literature at that time showed that the azo coupling techniques of Landsteiner were still dominant. Like him, we chose to use the serum albumins, in particular, bovine serum albumin (BSA) as the protein carrier, since they were inexpensive and likely to yield soluble conjugates. However, an examination of the amino acid content of BSA (fig. 1) convinced us that substitution by such relatively complex haptens as steroids should be attempted by reaction with the more plentiful *epsilon* amino groups of the lysine residues rather than by an azo coupling reaction with tyrosine, tryptophan and imidazole residues. This meant formation of peptide bonds, for which a number of convenient new methods had been developed recently (39). A systematic approach was developed in which carboxylic acid groups were introduced into the haptens in various ways so that reaction with the amino groups of the protein carrier could be effected.

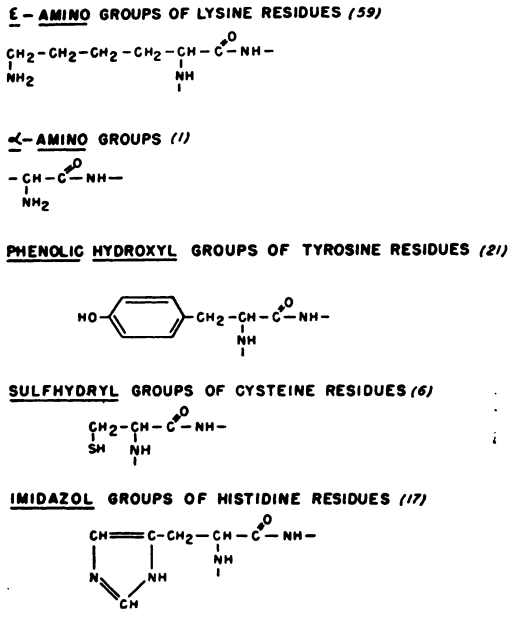

FIG. 1. Amino acids of bovine serum albumin (BSA) that can be linked to reactive haptens. In parentheses are their number per molecule of BSA.

Rather than deal with the steroid work separately, we will incorporate it into a general survey of the methods of preparing immunogenic hapten-protein conjugates in which the hapten is a pharmacologically interesting compound. The arrangement of the review will be governed by the nature of the reactive functional groups of the hapten. In this way, it is hoped that the information can be applied most easily to new compounds being considered for use as determinant groups. No attempt will be made to present an exhaustive review of the literature. For this, the reader should refer elsewhere (15). Instead, the various procedures described will be illustrated by specific examples to which the reader can refer for practical aspects of the experimental methods.

Choice of Carrier

The protein carriers used in various laboratories include globulin fractions, the serum albumins of various species, hemocyanin, ovalbumin, and fibrinogen. Hapten-protein conjugates of serum albumin were, in general, more soluble than conjugates of γ-globulin or of ovalbumin. Thus, for example, steroid-protein conjugates of bovine, rabbit, and human serum albumin were soluble[1] above pH 5.5 (30, 31); similar conjugates made with γ-globulin and egg albumin precipitated out of solution during preparation and could not be redissolved. Insoluble conjugates can be used for immunization, but subsequent characterization of the antibody then becomes a more difficult problem.

Under certain circumstances, it may be advantageous to have both soluble and in-

[1] It has been our experience and judging from inquiries, the experience of others, that lyophilized steroid-BSA conjugates tend to become insoluble in water after standing on the shelf for several months. It is preferable to store them in solution, frozen or refrigerated. In the latter case, merthiolate or other suitable antimicrobial agent should be added. In many cases, lyophilized material can be made water-soluble again by stirring a suspension in 8 M urea for 1 or 2 days; most will dissolve. This is followed by removal of the urea by dialysis.

soluble conjugates containing the same determinant group. The latter can be used for the isolation and purification of hapten-specific antibody (66). For a review of insoluble hapten-carrier conjugates, in which the carrier is cellulose, we refer the reader to Weliky and Weetall (73).

Nature of the Hapten

Haptens with Carboxyl Groups

In this class of haptens may be included those which have a carboxyl group naturally, such as the small peptides angiotensin and bradykinin or acetylsalicylic acid (aspirin). In addition, many haptens, such as some steroids, may have reactive groups to which a carboxyl group may be attached. For conjugation to proteins, the same procedures may be used regardless of whether the carboxyl group is present as an inherent part of the naturally occurring hapten or as an added moiety.

Mixed Anhydride Procedure

The mixed anhydride procedure, developed for peptide synthesis (70), was introduced by us for the preparation of steroid-protein conjugates (30, 31). This is a simple procedure that does not require the isolation of an active derivative. The coupling procedure (fig. 2) is carried out directly with the hapten, and the resulting conjugate can be made to contain 15 to 30 haptenic groups per molecule of albumin. An example of this method is the coupling of cortisone-21-hemisuccinate to protein (30). Uridine 5′-carboxylic acid (47), testosterone-17-hemisuccinate, other steroid derivatives (30, 31), thyroxine (18), and prostaglandin (45) have been coupled to protein in this manner.

Carbodiimides

This is another direct method which has been used extensively in preparing conjugated antigens. Uridine 5′-carboxylic acid was coupled to a multichain polypeptide, poly DL-alanyl-poly L-lysine with dicyclohexylcarbodiimide in a 95% dimethylformamide medium as solvent (61). Coupling reactions can be carried out in aqueous solution by use of the water soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl or 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate (62, 63), both commercially available reagents. Angiotensin and bradykinin, both small polypeptides (m.w. *ca* 1000), were coupled to proteins in the first utilization of this procedure (38). The authors believed that the reaction was between the N-terminal amino group of the peptide and the protein, but provided no evidence for this. On the other hand, they later used similar techniques to couple angiotensin to polylysine with the water-soluble reagent, *N*-ethyl-benzisoxazole (37). Similar procedures were carried out by Dietrich (28) and by Haber *et al.* (41). Additional components of biological interest which were coupled to carriers with water soluble carbodiimides include gastrin (74), adenosine 3′,5′-cyclic phosphate (65), morphine (69), lysergic acid diethylamide (68), and prostaglandin (52). In the last three cases, the carrier used was polylysine and immunization was done with a complex of the conjugate and succinylated hemocyanin. This improved technique minimized the extent of the immunological response to the carrier portion of the immunogen.

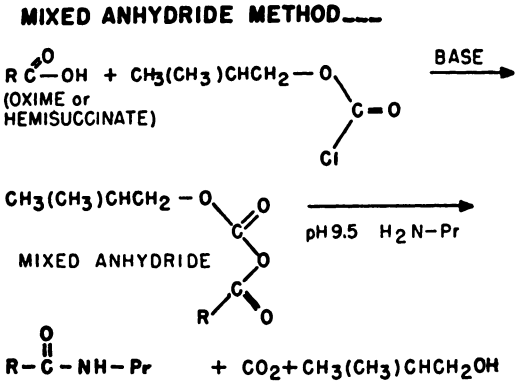

FIG. 2. Mixed anhydride procedure.

The conditions of the reaction are very simple. The carrier, an excess of hapten, and the reagent are simply stirred together in an aqueous solution for 30 min to several days,

depending upon the procedure. The reaction is followed by dialysis and the product is isolated by lyophilization. The reaction mechanism is as shown in figure 3. We see that there are two possible reactions, the desired one being catalyzed by H^+. The protein carrier, however, is most reactive at higher pH where dissociation of the lysine ammonium groups occurs. Thus, it is necessary to compromise, *i.e.*, to operate at a pH near neutrality to provide the most favorable conditions. In our experience, the use of water soluble carbodiimides has not always been successful. Extensive alteration of the carrier has occurred with little if any substitutions by haptenic groups. It is possible to be led astray since antibody is produced to the altered protein, and this antibody does not react with the protein in its original state.

Folic acid (58) and the hemisuccinate of digitoxigenin (54) have been conjugated to protein carriers with carbodiimides. It is also of interest that water-soluble carbodiimides have been used to couple nucleotides directly to proteins, presumably by formation of a P-N bond (42, 43).

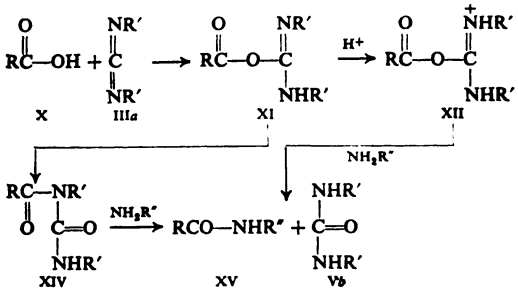

FIG. 3. The reactions of carbodiimides with carboxylic acids.

Miscellaneous Carboxyl Methods

An aspirin-protein conjugate was prepared by first converting aspirin (acetylsalicylic acid) to the acetylsalicylazide (14). The azide was coupled to rabbit serum globulin in a 1:1 dioxane-water solution maintained alkaline to phenolphthalein by the addition of base. About 25 to 35 haptenic groups were conjugated per molecule of globulin. A similar procedure was used for thyroxine (19). Antibodies specific for thyroxine have also been obtained by using, as antigen, tetra-iodothyropropionic acid coupled to protein by the mixed anhydride method (18).

The conversion of aspirin to an acid chloride which can react directly with protein was also reported (72).

Recently, the insect juvenile hormone DL-10,11-epoxyfarnesoic acid (fig. 4) was coupled to protein by a procedure that should find extensive use (49). We had been unable to effect the reaction with water-soluble carbodiimides, obtaining only unsubstituted, altered protein (see above). The N-hydroxysuccinimide ester was prepared by reaction of the juvenile hormone with N-hydroxysuccinimide (commercially available) in the presence of dicyclohexylcarbodiimide (fig. 5). N-Hydroxysuccinimide esters are used in peptide synthesis (6). They are quite stable if kept dry but react quickly and in good yield with amino groups to form amide or peptide bonds. Conjugates containing 20 juvenile hormone groups were used to raise specific antibodies in rabbits.

Haptens with Amino Groups

This class of hapten can be divided into two subclasses—the aromatic amines and the aliphatic amines.

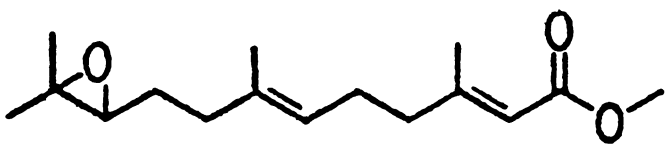

FIG. 4. DL-10,11-Epoxyfarnesoic acid, the insect juvenile hormone of *Manduca sexta* (tobacco hornworm).

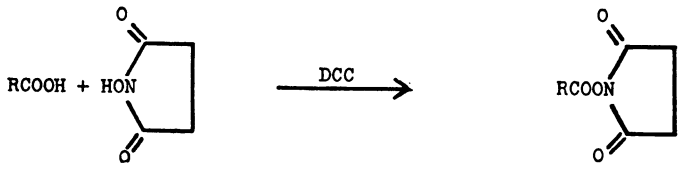

FIG. 5. Preparation of N-hydroxysuccinimide esters.

Aromatic Amines

Haptens with aromatic amino groups can be conjugated to proteins by the classical diazotization procedure of Landsteiner (48). A representative procedure can be found in Kabat (46) (p. 798 *et seq.*). An extremely interesting application of this procedure was the preparation of a chloramphenicol-protein conjugate which was used to elicit antibodies specific for chloramphenicol (44). In this case, a prior reduction of the nitro group of chloramphenicol to an amino group was required. As early as 1937, carcinogenic compounds were conjugated to protein carriers by means of their isocyanate derivatives which were prepared from amines (25). Immune sera were raised and their properties were studied (24, 25).

Aliphatic Amines

These can be reacted with proteins with carbodiimides as reagents (38). Or, they can be converted to *p*-nitrobenzoylamides by reaction with *p*-nitrobenzoylchloride. The amide derivatives can then be reduced by catalytic hydrogenation or other procedures to the *p*-aminobenzoyl derivative, which, upon diazotization, can be coupled to protein. In this way, Anderer (3) and Anderer and Schlumberger (4, 5) have prepared immunogenic conjugates of a series of oligopeptides that are part of the C-terminal peptide of tobacco mosaic virus proteins. A similar procedure was used by Deodhar (26, 27) to prepare a protein conjugate of angiotensin for immunochemical studies. Angiotensin has also been attached by its N-terminal amino group to the amino groups of a carrier by means of the bifunctional reagent *m*-xylylene diisocyanate (41). Toluene 2,4-diisocyanate has been used in a similar manner to prepare bradykinin conjugates (64). More recently, haptens containing amino groups were covalently linked to amino groups of protein carriers with glutaraldehyde. Among the haptenic groups conjugated in this manner are adrenocorticotrophic hormone (ACTH) (57), glucagon (33), and normetanephrine (55).

Haptens with Available Hydroxyl Groups

This class of haptens includes alcohols, phenols, sugars, polysaccharides, nucleosides, *etc.* In most cases, derivatives of this class of compounds must be made in order to introduce functional groups capable of reacting with proteins.

Hemisuccinates

A simple procedure, first introduced in our work with steroid-protein conjugates, is the conversion of the alcohol to the half ester of succinic acid (*i.e.*, the hemisuccinate). The hemisuccinate has an available carboxyl group that can be made to react by any of the procedures described above. Conversion to the hemisuccinate requires a reaction with succinic anhydride in pyridine. A representative procedure can be found in the papers on steroid-protein conjugates (30, 31). A more recent example is the preparation of the hemisuccinate of cyclic adenosine monophosphate (cAMP) (65).

Chlorocarbonates

Another alternative is the reaction of the determinant group with an equimolar quantity of phosgene to yield the highly reactive chlorocarbonate which reacts directly with the amino groups of the protein in the presence of bicarbonate. An example is the con-

version of testosterone to testosterone-17-chlorocarbonate (30).

Aminophenyl Derivatives

Phenols can be converted to active reagents by reaction with diazotized *p*-aminobenzoic acid. In this way, a carboxyl group is introduced into the molecule. This type of reaction was carried out successfully with 17-β-estradiol (73).

The classical procedure for the coupling of sugars involves the formation of *p*-nitrophenylglycosides, the conversion of the latter by hydrogenation to *p*-aminophenylglycoside and then attachment to the protein by diazotization. This method was used by Landsteiner (48) for a number of preparations. A representative procedure can be found in Kabat (46). A variant of this method, used by Goebel (34, 35) and Goebel and Hotchkiss (36), was conversion to the aminobenzyl ether followed by diazotization.

Oxidation to Dialdehydes

A more recent very simple procedure developed for the preparation of nucleoside- and nucleotide-protein conjugate (29, 32) makes use of the reaction of vicinal hydroxyl groups with periodate to yield dialdehydes (fig. 6). The dialdehydes, without isolation, are caused to react with the amino groups of protein at pH 9.5 in aqueous solution to yield aldimines which are stabilized by reduction with sodium borohydride. Only the final conjugate is isolated in this procedure, which is simple to run and yields conjugates with as many as 30 determinant groups per molecule of albumin. It should be applicable to all compounds with vicinal hydroxyl groups such as glycols, glycerol derivatives, glycosides, *etc.*, and has been used successfully for the preparation of digoxin-protein conjugates (17).

Oxidation to Carboxyl

Oxidation of the 5′-hydroxyl groups of uridine (47, 60), pseudo-uridine (47), and other nucleosides (61), has made it possible to conjugate these compounds to proteins by methods amenable for the reaction of carboxylic acid derivatives with proteins.

Miscellaneous Hydroxyl Methods

Another method of seemingly general applicability to carbohydrates was used by Coat *et al.* (20) to conjugate uridine to proteins. The isopropylidine derivative was allowed to react with *p*-nitrobenzoyl chloride to yield the 5′ ester. Removal of the isopro-

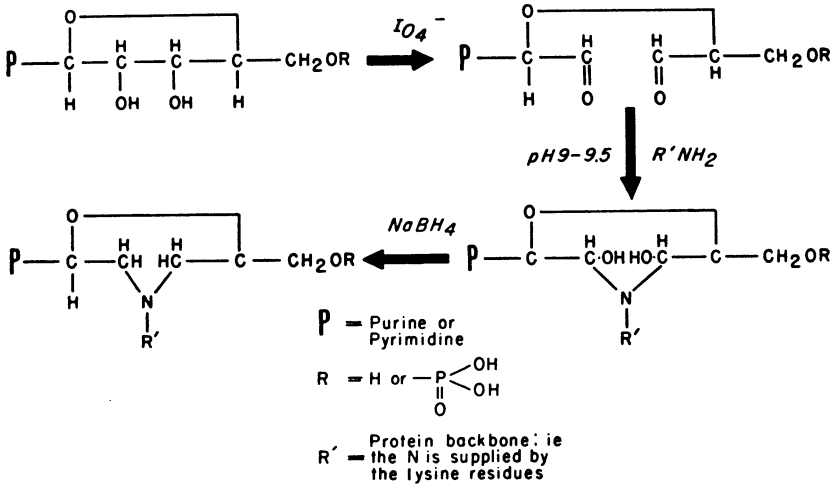

FIG. 6. The periodate method of preparing nucleoside or nucleotide-protein conjugates. (From B. F. Erlanger and S. M. Beiser: Antibodies specific for ribonucleosides and ribonucleotides and their reaction with deoxyribonucleic acid (DNA). Proc. Nat. Acad. Sci. U.S.A. 52: 68–74, 1964.)

pylidine protecting group and hydrogenation of the nitro group made it possible to link the uridine derivative to the protein by a diazotization reaction.

The bifunctional reagent, sebacoyl dichloride, has been used to convert alcohols to acid chlorides, which, at pH 8.5, react readily with proteins. This procedure was used by Bailey and Butler (7) to prepare a cholesterol-protein conjugate.

Haptens with Other Functional Groups

Ketones and aldehydes can be used as haptenic determinant groups by converting them to O-(carboxymethyl) oximes. This is done by reacting them with O-(carboxymethyl) hydroxylamine (NH_2OCH_2COOH, sold commercially as carboxymethoxylamine or aminooxyacetic acid). This serves to introduce a carboxyl group which is exploited as described above. Examples of this methodology can be found in the coupling of testosterone-3-(O-carboxymethyl) oxime, estrone-17-(O-carboxymethyl) oxime and progesterone-20-(carboxymethyl) oxime to bovine serum albumin with the mixed anhydride technique (30, 31). The ketone groups of aldosterone, corticosterone, and cortisol were derivatized with *p*-hydrazinobenzoic acid. The resulting carboxylic acid derivatives could be linked to BSA with water soluble carbodiimide (2). Aldehydes can be conjugated to proteins directly by Schiff base formation followed by stabilization of the bond by reduction with sodium borohydride. Pyridoxal and pyridoxal phosphate are examples of haptens conjugated in this manner (23, 67).

Penicillenic acid was conjugated to protein by an interesting procedure which included modification of the protein carrier (71). Penicillenic acid has a reactive sulfhydryl group which is capable of forming disulfide bonds with other sulfhydryl groups. The carrier proteins (*e.g.*, human γ-globulin or bovine γ-globulin) were artificially enriched by reaction with N-acetylhomocysteine thiolactone (11). The coupling reaction with an excess of penicillenic acid was then carried out in acetate buffer at pH 4 in the presence of H_2O_2.

bis-Diazotized benzidine can be used as a bridging reagent between proteins and haptens containing aromatic groups that react with diazonium compounds. A conjugate of thyrotropin releasing hormone (which contains a reactive histidine residue) was obtained in this way (8).

A novel approach has been to react serotonin with protein *via* the Mannich reaction (56). This is a simple reaction (fig. 7) which enables one to use formaldehyde as a bridge between the amino groups of a protein and compounds containing one or more reactive hydrogens.

Among the low molecular weight haptens that have been used as determinant groups are substances which are reactive enough to be coupled to proteins directly. Dinitrofluorobenzene has been used to prepare antigens for the stimulation of antidinitrophenyl antibodies. These have been very useful in studies of the binding characteristics and structure of immunoglobulins. Antibodies that react with deoxyribonucleic acid (DNA) have been elicited by immunization with the product of the reaction of 6-trichloromethylpurine (21, 22) with bovine serum albumin (16). Antipenicillin antibodies have been produced by immunization with penicillin-protein conjugates. The latter were prepared by the reaction of penicillin with protein under slightly alkaline condi-

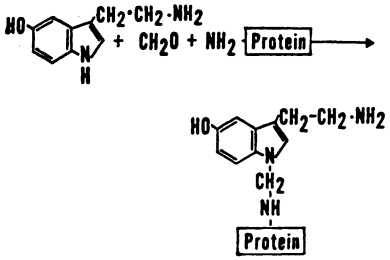

FIG. 7. Preparation of serotonin-protein conjugate by the Mannich reaction. (From N. S. Ranadive and A. H. Sehon: Antibodies to serotonin. Can. J. Biochem. 45: 1701–1710, 1967.)

tions (50, 51). L-Phenylalanine mustard is another example of a reactive hapten (13).

It is not possible to provide complete experimental details for the procedures discussed above. It should be emphasized, however, that a profound knowledge of organic chemistry is not a prerequisite for preparing the conjugates once the structure, or the reactive group, of the hapten is known. Furthermore, very often it is not even necessary to isolate the intermediate compounds.

Characterization of the Conjugates

Generally, the haptenic group has an absorption spectrum which can allow one to differentiate it from the protein carrier. This is particularly true for azo derivatives, which absorb in the visible range. However, even if there is overlap in the two spectra, reasonably accurate determinations of the number of haptenic molecules per carrier protein can be determined from difference spectra.

A more convenient and direct procedure, introduced by Abraham *et al.* (1), is the incorporation of some radioactive hapten in the conjugation procedure. A direct estimation of the extent of substitution can be made by counting undialyzable radioactive material.

A procedure introduced by us for the steroid-protein conjugates (30) was the estimation of the remaining free amino groups with the dinitrophenylation technique of Sanger (59). *epsilon*-Dinitrophenyllysine was not isolated but was estimated directly by spectrophotometry after ether extraction of the acid hydrolysate. A control with unsubstituted carrier was always run concomittantly and the difference between the two taken to be the extent of substitution by hapten.

Recently we have adopted a procedure of Habeeb (40) in which trinitrobenzene sulfonic acid is used as the reagent for estimation of free amino groups in the conjugate. Spectrophotometric comparison of the intact protein-conjugate and the original carrier protein is possible; no acid hydrolysis is required. We found this procedure to be convenient and entirely satisfactory in the case of insect juvenile hormone-protein conjugates (49).

A Comment on Specificity

Landsteiner established, in his early studies, that antibody specificity is directed primarily at that portion of the hapten furthest removed from the functional groups that are linked to the protein carrier. This fact should be kept in mind when an attempt is made to prepare highly specific antisera for use in the estimation of substances present in fluids containing other, structurally related compounds. For example, among the steroids (other than the estrogens), all share a common ring A structure. Thus, it was found that antitestosterone-3-BSA was better able to distinguish among the related steroids than was the antibody to testosterone-17-BSA (9, 10, 53). Other similar examples exist in the literature. A more ideal antigen would be one in which none of the important groups are linked to the protein, but coupling is *via* another position (*e.g.*, C_{11} of a ketosteroid). The intervention of a multicarbon chain between the steroid and the protein would also lead to antisera of higher specificity.

Summary

A brief review has been presented of the many procedures applicable for the preparation of hapten-protein conjugates that can be used to elicit antibodies to biologically interesting compounds. This review has been organized in a way to make it easier for the working scientist to recognize and thus utilize methods that would be suitable for his own research problems. Despite the recent upsurge in the application of immunological methods for the investigation of small biological molecules, there are still many more applications possible, and it is hoped that this review will encourage and facilitate some of them.

Acknowledgment. Among the scientific findings described in this paper are some made in the au-

thor's laboratory over a period of many years. They were made with the financial assistance of a number of granting agencies, private and public, who are acknowledged in the original papers. However, I should like to cite them again and to emphasize that without their help, science, at least in my laboratory, would have progressed very little: National Institutes of Health, National Science Foundation, Office of Naval Research, American Heart Association, Population Council, Inc., Irene Heinz Given and John La Porte Given Foundation, Inc., and the Raymond and Beverly Sackler Foundation.

Finally, I would like to acknowledge a 20-year collaboration with the late Dr. Sam M. Beiser, without whom much of the material for this review would not have been possible.

REFERENCES

1. Abraham, G. E., Swerdloff, R., Tulchinsky, D. and Odell, W. D.: Radioimmunoassay of plasma progesterone. J. Clin. Endocrinol. Metab. 32: 619–624, 1971.
2. Africa, B. and Haber, E.: The production and characterization of specific antibodies to aldosterone. Immunochemistry 8: 479–486, 1971.
3. Anderer, F. A.: Preparation and properties of an artificial antigen immunologically related to tobacco mosaic virus. Biochim. Biophys. Acta 71: 246–248, 1963.
4. Anderer, F. A. and Schlumberger, H. D.: Cross-reactions of antisera against the terminal amino acid and dipeptide of tobacco mosaic virus. Biochim. Biophys. Acta 115: 222–224, 1966.
5. Anderer, F. A. and Schlumberger, H. D.: Properties of different artificial antigens immunologically related to tobacco mosaic virus. Biochim. Biophys. Acta 97: 503–509, 1965.
6. Anderson, G. W., Zimmerman, J. E. and Callahan, F. M.: The use of esters of N-hydroxysuccinimide in peptide synthesis. J. Amer. Chem. Soc. 86: 1839–1842, 1964.
7. Bailey, J. M. and Butler, J.: Synthetic cholesterol-ester antigens in experimental atherosclerosis. *In* the Reticuloendothelial System and Atherosclerosis, ed. by N. R. DiLuzio and R. Paoletti, pp. 433–441, Plenum Press, New York, 1967.
8. Bassiri, R. M. and Utiger, R. D.: The preparation and specificity of antibody to thyrotropin releasing hormone. Endocrinology 90: 722–727, 1972.
9. Beiser, S. M. and Erlanger, B. F.: Estimation of steroid hormones by an immunochemical technique. Nature (London) 214: 1044–1045, 1967.
10. Beiser, S. M., Erlanger, B. F., Agate, F. J. and Lieberman, S.: Antigenicity of steroid-protein conjugates. Science 129: 564–565, 1959.
11. Benesch, R. and Benesch, R. E.: The introduction of new sulfhydryl groups and disulfide bonds into proteins. *In* Sulfur in Proteins, ed. by R. Benesch, R. A. Benesch, P. D. Boyer, I. M. Klotz, W. R. Middlebrook, A. G. Szent-Gyorgyi and D. R. Schwartz, pp. 15–24, Academic Press, New York, 1959.
12. Berson, S. A. and Yalow, R. S.: Radioimmunoassay: A status report. *In* Immunobiology: Current Knowledge of Basic Concepts in Immunology and Their Clinical Application, ed. by R. A. Good and D. W. Fisher, pp. 287–293, Sinauer Associates, Stamford, Conn., 1971.
13. Burke, J. F., Mark, V. H., Soloway, A. H. and Leskowitz, S.: The effect of antibody to L-phenylalanine mustard conjugate on malignant cells selectively marked through "early inflammatory-like" vascular permeability. Cancer Res. 26: 1893–1904, 1966.
14. Butler, G. C., Harington, C. R. and Yuill, M. E.: Studies in synthetic immunochemistry. 5. Observations on antisera against aspirin-protein complexes Biochem. J. 34: 838–845, 1940.
15. Butler, V. P., Jr. and Beiser, S. M.: Antibodies to small molecules: Biological and clinical applications. Advan. Immunol. 17: in press.
16. Butler, V. P., Jr., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S. and Bendich, A.: Purine-specific antibodies which react with deoxyribonucleic acid (DNA). Proc. Nat. Acad. Sci. U.S.A. 48: 1597–1602, 1962.
17. Butler, V. P. and Chen, J. P.: Digoxin-specific antibodies. Proc. Nat. Acad. Sci. U.S.A. 57: 71–78, 1967.
18. Churchill, W. H. and Tapley, D. F.: Antibodies specific for thyroxine and its analogues. Nature (London) 202: 29–31, 1964.
19. Clutton, R. F., Harington, C. R. and Yuill, M. E.: Studies in synthetic immunochemistry. III. Preparation and antigenic properties of thyroxyl derivatives of proteins, and physiological effects of their antisera. Biochem. J. 32: 1119–1132, 1938.
20. Coat, J. P., David, S. and Fischer, J. C.: Préparation de combinaisons protéines-nucléosides en rue d'études immunochimiques. Bull. Soc. Chim. Fr. 21: 2489–2493, 1965.
21. Cohen, S., Thom, E. and Bendich, A.: Amides and peptides derived from 6-trichloromethylpurine and amino acids. Biochemistry 2: 176–181, 1963.
22. Cohen, S., Thom, E. and Bendich, A.: The preparation and properties of 6-halomethylpurines. J. Org. Chem. 27: 3545–3549, 1962.
23. Cordoba, F., Gonzalez, C. and Rivera, P.: Antibodies against pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate. Biochim. Biophys. Acta 127: 151–158, 1966.
24. Creech, H. J.: Chemical and immunological properties of carcinogen-protein conjugates. Cancer Res. 12: 557–564, 1952.
25. Creech, H. J. and Franks, W. R.: Compounds synthesized from proteins and carcinogenic hydrocarbons. Amer. J. Cancer 30: 555–562, 1937.
26. Deodhar, S. D.: Immunologic production of anti-angiotensin. I. Preparation of angiotensin-protein complex antigen. J. Exp. Med. 111: 419–427, 1960.
27. Deodhar, S. D.: Immunologic production of anti-angiotensin. II. Production and detection of anti-angiotensin. J. Exp. Med. 111: 429–439, 1960.
28. Dietrich, F. M.: Immunochemical analysis of rabbit antibodies against angiotensin. II. Immunochemistry 4: 65–76, 1967.
29. Erlanger, B. F. and Beiser, S. M.: Antibodies specific for ribonucleosides and ribonucleotides and their reaction with deoxyribonucleic acid (DNA). Proc. Nat. Acad. Sci. U.S.A. 52: 68–74, 1964.
30. Erlanger, B. F., Borek, F., Beiser, S. M. and Lieberman, S.: Steroid-protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and cortisone. J. Biol. Chem. 228: 713–727, 1957.
31. Erlanger, B. F., Borek, G., Beiser, S. M. and Lieberman, S.: Steroid-protein conjugates. II. Preparation and characterization of conjugates of bovine serum albumin with progesterone, deoxycorticosterone and estrone. J. Biol. Chem. 234: 1090–1094, 1959.
32. Erlanger, B. F., Senitzer, D., Miller, O. J. and Beiser, S. M.: Nucleic acid-reactive antibodies specific for nucleosides and nucleotides. Acta Endocrinol. suppl. 168, 206–221, 1972.
33. Frohman, L. A., Reichlin, H. and Sokal, J. E.: Immunologic and biologic properties of antibodies to a glucagon-serum albumin polymer. Endocrinology 87: 1055–1061, 1970.
34. Goebel, W. F.: Chemo-immunological studies on conjugated carbohydrate-proteins. X. The immunological properties of an artificial antigen containing glucuronic acid. J. Exp. Med. 64: 29–38, 1936.
35. Goebel, W. F.: Chemo-immunological studies on conjugated

carbohydrate-proteins. XII. The immunological properties of an artificial antigen containing cellobiuronic acid. J. Exp. Med. 68: 469–484, 1938.

36. Goebel, W. F. and Hotchkiss, R. D.: Chemo-immunological studies on conjugated carbohydrate-proteins. XI. The specificity of azoprotein antigens containing glucuronic and galacturonic acids. J. Exp. Med. 66: 191–205, 1937.
37. Goodfriend, T. L., Fasman, G., Kemp, D. and Levine, L.: Immunochemical studies of angiotensin. Immunochemistry 3: 223–231, 1966.
38. Goodfriend, T. L., Levine, L. and Fasman, G.: Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. Science 143: 1344–1346, 1964.
39. Greenstein, J. P. and Winitz, M.: Chemistry of the Amino Acids, vol. II, New York, John Wiley & Sons, 1961.
40. Habeeb, A. F. S. A.: Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14: 328–336, 1966.
41. Haber, E., Page, L. B. and Jacoby, G. A.: Synthesis of antigenic branch-chain copolymers of angiotensin and poly-L-lysine. Biochemistry 4: 693–698, 1965.
42. Halloran, M. J. and Parker, C. W.: The preparation of nucleotide-protein conjugates: Carbodiimides as coupling agents. J. Immunol. 96: 373–378, 1966.
43. Halloran, M. J. and Parker, C. W.: The production of antibodies to mononucleotides and DNA. J. Immunol. 96: 379–385, 1966.
44. Hamburger, R. N.: Chloramphenicol-specific antibody. Science 152: 203–205, 1966.
45. Jaffe, B. M., Smith, J. W., Newton, W. T. and Parker, C. W.: Radioimmunoassay for prostaglandins. Science 171: 494–496, 1971.
46. Kabat, E. A.: Kabat and Mayer's Experimental Immunochemistry, 2nd ed., Charles C Thomas, Springfield, Ill., 1961.
47. Karol, M. H. and Tanenbaum, S. W.: Antibodies to hapten-conjugated proteins which cross-react with RNA. Proc. Nat. Acad. Sci. U.S.A. 57: 713–720, 1967.
48. Landsteiner, K.: The Specificity of Serological Reactions, Harvard Univ. Press, Cambridge, Mass., 1945.
49. Lauer, R. C., Solomon, P., Nakanishi, K. and Erlanger, B. F.: Preparation and specificity of antibodies to insect C16-juvenile hormone (Abstract). Fed. Proc. 32: 500, 1972.
50. Levine, B. B.: Immunologic mechanisms of penicillin allergy. N. Engl. J. Med. 275: 1115–1125, 1966.
51. Levine, B. B. and Ovary, Z.: Studies on the mechanism of the mechanism of the formation of the penicillin antigen. III. The N-(D-α-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. J. Exp. Med. 114: 875–904, 1961.
52. Levine, L. and Van Vunakis, H.: Antigenic activity of prostaglandins. Biochem. Biophys. Res. Comm. 41: 1171–1177, 1970.
53. Lieberman, S., Erlanger, B. F., Beiser, S. M. and Agate, F. J.: Steroid-protein conjugates: their chemical, immunochemical and endocrinological properties. Recent Progr. Hormone Res. 15: 165–200, 1959.
54. Oliver, G. C., Parker, B. M., Brasfield, D. L. and Parker, C. W.: The measurement of digitoxin in human serum by radioimmunoassay. J. Clin. Invest. 47: 1035–1042. 1968.
55. Peskar, B. A., Peskar, B. M. and Levine, L.: Specificities of antibodies to normetanephrine. Eur. J. Biochem. 26: 191–195, 1972.
56. Ranadive, N. S. and Sehon, A. H.: Antibodies to serotonin. Can. J. Biochem. 45: 1701–1710, 1967.
57. Reichlin, M., Schnure, J. J. and Vance, V. K.: Induction of antibodies to porcine ACTH in rabbits with nonsteroidogenic polymers of BSA and ACTH. Proc. Soc. Exp. Biol. Med. 128: 347–350, 1968.
58. Ricker, R. and Stollar, B. D.: Antibodies reactive with specific folic acid determinants. Biochemistry 6: 2001–2005, 1967.
59. Sanger, F.: The terminal peptides of insulin. Biochem. J. 45: 563–574, 1949.
60. Sela, M. and Ungar-Waron, H.: Nucleoside-specific antibodies elicited by synthetic antigens. Fed. Proc. 24: 1438–1445, 1965.
61. Sela, M., Ungar-Waron, H. and Schechter, Y.: Uridine-specific antibodies obtained with synthetic antigens. Proc. Nat. Acad. Sci. U.S.A. 52: 285–292, 1964.
62. Sheehan, J. C., Cruickshank, P. A. and Boshart, G. L.: A. convenient synthesis of water-soluble carbodiimides. J. Org. Chem. 26: 2525–2528, 1961.
63. Sheehan, J. C. and Hlavka, J. S.: The use of water-soluble and basic carbodiimides in peptide synthesis. J. Org. Chem. 21: 439–441, 1956.
64. Spragg, J., Austen, K. F. and Haber, E.: Production of antibodies against bradykinin: demonstration of specificity by complement fixation and radioimmunoassay. J. Immunol. 96: 865–871, 1966.
65. Steiner, A. L., Kipnis, D. M., Utiger, R. and Parker, C.: Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proc. Nat. Acad. Sci. U.S.A. 64: 367–373, 1969.
66. Szafran, H., Beiser, S. M. and Erlanger, B. F.: The use of egg albumin conjugates for the purification of antibody. J. Immunol. 103: 1157–1158, 1969.
67. Ungar-Waron, R. and Sela, M.: Pyridoxal-specific antibodies obtained with a synthetic pyridoxal-polypeptide conjugate. Biochim. Biophys. Acta 124: 147–153, 1966.
68. Van Vunakis, H., Farrow, J. T., Gjika, H. B. and Levine, L.: Specificity of the receptor site to D-lysergamide: model of a physiological receptor for lysergic acid diethylamide. Proc. Nat. Acad. Sci. U.S.A. 68: 1483–1487, 1971.
69. Van Vunakis, H., Wasserman, E. and Levine, L.: Specificities of antibodies to morphine. J. Pharmacol. Exp. Ther. 180: 514–521, 1972.
70. Vaughan, J. R., Jr. and Osato, R. L.: Preparation of peptides using mixed carbonic-carboxylic acid anhydrides. J. Amer. Chem. Soc. 74: 676–678, 1952.
71. de Weck, A. L. and Eisen, H. N.: Some immunochemical properties of penicillenic acid. J. Exp. Med. 112: 1227–1247, 1960.
72. Weiner, L. M., Rosenblatt, M. and Howes, H.: The detection of humoral antibodies directed against salicylates in hypersensitive states. J. Immunol. 90: 788–792, 1963.
73. Weliky, N. and Weetall, H. H.: The chemistry and use of cellulose derivatives for the study of biological systems. Immunochemistry 2: 293–322, 1965.
74. Young, J. D., Byrnes, D. J., Chisholm, D. J., Griffiths, F. B. and Lazarus, L.: Radioimmunoassay of gastrin in human serum. J. Nucl. Med. 10: 746–748, 1969.