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The Immunological Assay of Drugs*

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I. Introduction

An important recent contribution of immunology to the biological sciences and to clinical medicine has been the development of sensitive, specific, precise, rapid and convenient immunochemical assay methods for the measurement of serum and tissue concentrations of physiologically important substances which cannot be readily determined by standard analytical laboratory techniques. In the field of pharmacology, in particular, drug-specific antibodies have been used extensively in the past decade in the development of a variety of immunological assay procedures for the measurement of the concentrations of drugs in the serum, urine and tissues of man and of experimental animals (74, 89–92, 110, 138, 185, 398, 410, 422, 449, 485, 629–631). This review will deal with: the general principles of drug immunoassays; a description of each of the individual steps involved in the development of a drug immunoassay; a general consideration of the advantages, limitations and applications of drug immunoassay procedures; and, an individual consideration of the specific drug immunoassay methods which are currently available.

II. General Principles

A. Immunoassay

The development of the radioimmunoassay (RIA) and related competitive binding immunoassay methods stems directly from the classical studies with insulin antibodies by Berson and Yalow (43, 44, 48, 729, 730). Observing that nonradioactive insulin competed with radiolabeled insulin for antibody binding sites, these workers

demonstrated that known quantities of unlabeled insulin interfered with the binding of ^{131}I-insulin by antibody in such a predictable manner that, if assayed under appropriate conditions, the concentration of insulin in a serum or other unknown specimen could be inferred from the extent to which that serum interfered with the binding of ^{131}I-insulin by the same antibody solution (45, 729–731). Not only did Berson and Yalow demonstrate this phenomenon, but they also carefully delineated the general principles underlying this and all other competitive binding immunoassays (46, 47, 49, 727, 728, 731–733), thus enabling the extension of these principles to the development of immunoassay methods for the measurement, first of peptide hormones and of proteins (3, 46, 47, 49, 322, 373, 503, 513, 518, 535, 727, 728) and, subsequently, of drugs (74, 89–92, 110, 138, 185, 398, 410, 422, 449, 485, 629–631), steroid hormones (2, 4, 99, 336, 465, 493, 709), thyroid hormones (84, 134), and other small molecules of physiological and pharmacological importance (34, 93, 518).

All drug immunoassay methods are based on the ability of a given drug to inhibit the reaction between drug-specific antibodies and an appropriately labeled drug or drug derivative; drug labels may be radioactive, as in RIA procedures, but the labels may also be nonradioactive, in the form of an active enzyme (207, 397, 576, 591, 596, 597, 679, 687, 723), of a spin label (416, 417, 476, 596), of red blood cells (7–9, 112, 347, 434), of bentonite particles (347) or of latex particles (572). In all immunoassay procedures, standard solutions containing known concentrations of the drug are incubated with a constant predetermined amount of labeled drug or drug derivative and with a constant, but limited, predetermined amount of drug-specific antibody. If appropriate concentrations of reagents are employed, increasing concentrations of drug will cause increasing degrees of inhibition of binding of the labeled drug or drug derivative. If the biological fluid to be studied does not interfere with the assay procedure, the concentration of drug in that fluid can be determined from the extent to which it inhibits the reaction between antibody and labeled drug, when compared with a simultaneously performed standard curve, a representative example of which is shown in figure 1 (3, 7, 46, 47, 49, 89–91, 185, 200, 202, 253, 276, 322, 333, 513, 518, 519, 535, 596, 629, 634, 699, 727, 728, 730, 732).

Thus, each competitive binding immunoassay procedure is based upon the analytical demonstration of differences in the binding of label by antibody in different individual assay tubes containing various amounts of unlabeled drug. Since all tubes in all such assay procedures initially contain identical amounts of label, it is essential that each method contain a step in

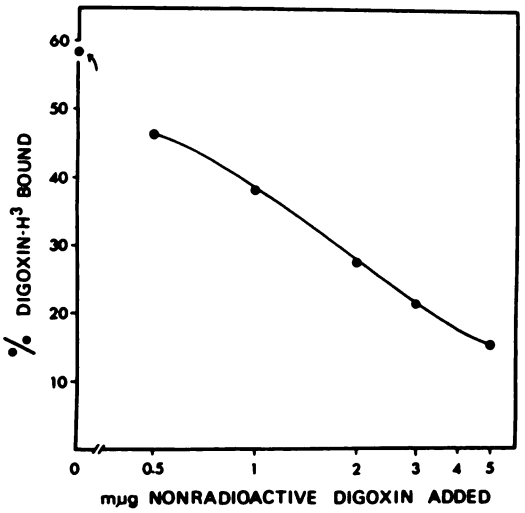

FIG. 1. Representative standard curve for serum digoxin RIA procedure. In the presence of increasing concentrations of nonradioactive digoxin (in known reference standard solutions), the percentage of [^{3}H]digoxin (3 ng) bound by a constant amount of rabbit antidigoxin serum (50 μl of a 1:2500 dilution) decreases from 59% in the absence of nonradioactive digoxin to 16% in the presence of 5 ng (mμg) of nonradioactive digoxin, as determined by the dextran-coated charcoal separation method. If, under identical conditions, 1 ml of a patient's serum reduces binding of [^{3}H]digoxin to 29%, that serum contains 2 ng of digoxin per ml. (Reprinted by permission from V. P. Butler, Jr.: Digoxin: immunologic approaches to measurement and reversal of toxicity. N. Engl. J. Med. 283: 1150–1156, 1970.)

which antibody-bound label can be accurately and quantitatively distinguished from free, unbound label. As shown in table 1, in the RIA procedures, an actual separation of bound from free label is required by such means as electrophoresis (730, 731), dialysis (201, 217), gel filtration (234, 242, 275), selective adsorption of free labeled drug to inert solid particles (295–297, 452, 516, 573, 581) or of antibody-bound drug to membranes (244, 677), coprecipitation of label with antibody in the presence of an antibody precipitant such as ammonium sulfate (128, 218, 470) or a heterologous ("second antibody") anti-immunoglobulin serum (279, 324, 478), or the use of antibody in an easily separable solid phase, coupled to an insoluble support (30, 55, 115, 116, 177, 192, 300, 439, 498, 586, 612, 644, 663, 666, 670, 716, 717). Many of the nonisotopic assay methods do not require such a physicochemical separation of bound from free label. For example, "homogeneous" enzyme immunoassays have been developed in which drug-enzyme conjugates exhibit enzymatic activity only in the free state while bound conjugates are inactive; thus, enzymatic activity upon addition of substrate to the unseparated assay mixture provides a direct index of free label (574, 576, 591, 596, 597, 723). Spin-label immunoassay procedures likewise require no separation step because the mobility of free radicals of spin-labeled drugs in the free state can be distinguished from their mobility when bound to specific antibodies, as measured in an electron spin resonance spectrometer (416, 417, 476, 596).

B. Antibodies to Drugs

The development of immunoassays for proteins and for large peptide hormones is facilitated by the fact that most such substances are capable of eliciting specific antibody production in experimental animals. Small molecules (less than 1000 daltons) such as drugs, steroid hormones, thyroid hormones and small peptide hormones are not usually immunogenic and, thus, repeated injections of such substances will not ordinarily elicit antibody formation. To obtain antibodies specific for a drug, it is usually necessary to conjugate the drug (or a chemical derivative) covalently as a hapten to an antigenic protein or synthetic polypeptide carrier before immunization (34, 74, 90–93, 185, 208, 399, 432, 449, 517, 518). As delineated in the classical studies of Landsteiner (399–402), a hapten may be defined as a low molecular weight substance, too small to be immunogenic by itself, but which, when co-

TABLE 1
Separation of unbound from antibody-bound radiolabeled drug

Method	Mode of Separation	
	Antibody and antibody-bound radioactivity	Unbound (free) radioactivity
Electrophoresis and chromatoelectrophoresis	In γ-globulin electrophoretic fraction	Migrates with its own electrophoretic mobility
Equilibrium dialysis	Retained inside sac	Equal concentration inside and outside sac
Gel filtration	Eluted early	Eluted late
Dextran-coated charcoal	Not adsorbed to charcoal; in supernatant after centrifugation	Adsorbed to charcoal; in precipitate after centrifugation
Membrane filtration	Bound to nitrocellulose membrane*	Washed through membrane*
Ammonium sulfate (Farr)	Precipitated by $(NH_4)_2SO_4$	In supernatant after centrifugation
Double antibody	Precipitated by antibody to animal γ-globulin	In supernatant after centrifugation

* Some radiolabeled drug derivatives, *e.g.*, an adriamycin-^{125}I derivative, have physical properties such that they are retained on the filter. If conditions are employed under which the capacity of the membrane for protein is exceeded, free radioactivity is bound to the membrane, while antibody-bound radioactivity passes through the filter (676).

valently coupled to a large immunogenic protein or polypeptide carrier and injected into an animal, will give rise to the formation of antibodies specific for the chemically coupled small molecule (34, 93, 399, 518).

For proper appreciation of the phenomenon of the formation of hapten-specific antibodies, it is important to be aware of the fact that, while the antigen recognition and processing system of an animal is stimulated only by injection of large molecules such as proteins or hapten-protein conjugates, antibodies formed in response to this stimulation recognize and bind only small segments called "antigenic determinants" (362, 550) of the injected protein or hapten-protein conjugate. Most proteins and hapten-protein conjugates contain many distinct antigenic determinant sites toward which antibodies are formed and thus antisera to such antigens contain a heterogeneous array of antibodies to numerous individual antigenic determinant sites. If a drug constitutes a major portion of a given antigenic determinant site, antibodies with specificity for that site will be capable of binding that drug and can be used in the development of a specific immunoassay. Ordinarily, however, antibodies are also formed to antigenic determinant sites which do not contain hapten. These latter antibodies will react only with the protein or polypeptide carrier. Fortunately, such carrier-specific antibodies do not interfere with properly designed drug immunoassays, but the possibility of such interference must be considered and excluded in the development of any new drug immunoassay method (93).

III. Development of Drug Immunoassays

A. Conjugation of Drug to Protein (or Polypeptide) Carrier

1. Choice of a carrier. In the synthesis of a drug-protein conjugate, a protein carrier must first be chosen. Almost any immunogenic protein may be used as a carrier for an haptenically coupled drug. Because of their ready availability, low cost, excellent solubility, high degree of immunogenicity and relative resistance to denaturation under the somewhat rigorous chemical conditions of some hapten conjugation methods, the serum albumins of various species (notably, bovine and human) have most frequently been employed as the carrier proteins. Various serum globulin fractions, fibrinogen, thyroglobulin, ovalbumin, hemocyanin and synthetic polypeptides have also been widely used. The functional groups in the carrier molecules to which drugs or drug derivatives may be conjugated are listed in table 2. Drugs and their derivatives have been most frequently coupled to the amino, carboxyl or phenolic groups of protein or polypeptide carriers (34, 74, 90–93, 185, 432, 449, 517–519).

2. Choice of a conjugation method. In the method selected for conjugation of a haptenic drug or drug derivative to a carrier, the chemical conditions must entail minimal structural alterations of the hapten and must not cause sufficient denaturation of the carrier molecule to render it insoluble. A number of such relatively gentle conjugation methods have been described. These methods usually, but not exclusively, utilize amino, carboxyl or hydroxyl groups in the drug or drug derivative in the coupling procedure. Although experimental details for these methods

TABLE 2

Functional groups of carrier proteins and polypeptides to which drugs or drug derivatives may be conjugated

Functional Group	Amino Acid(s)
Amino	Lysine
	NH_2-terminal amino acid
Carboxyl	Glutamic acid
	Aspartic acid
	COOH-terminal amino acid
Phenolic	Tyrosine
Imidazo	Histidine
Sulfhydryl	Cysteine
Indolyl	Tryptophan
Guanidino	Arginine

will not be reviewed, it should be emphasized that a profound knowledge of organic chemistry is not required for the use of any of these methods and, in many instances, isolation of intermediate compounds is not required. It should also be emphasized that the most important single consideration in the selection of a conjugation procedure is the availability of potentially reactive groups in the drug. If the drug lacks suitable functional groups or if it is considered desirable to keep certain functional groups intact in the final hapten-protein conjugate, consideration should be given to the use of a drug derivative or analog which contains suitable reactive groups.

Drugs or drug derivatives with free amino groups can be coupled with the carboxyl groups of proteins most readily by the addition to a drug-protein mixture of a water-soluble carbodiimide (12, 66, 131, 256, 423, 426, 441, 673, 676). Alternatively, drugs with amino groups can be conjugated to amino groups of proteins by the addition of glutaraldehyde (238, 547, 551, 673) or of toluene 2,4-diisocyanate (426, 593). Aromatic amino groups of drugs or drug derivatives can be converted, using nitrous acid, to the corresponding diazonium compound which, at a mildly alkaline pH, will react with the tyrosine, histidine and tryptophan residues of the protein carrier (34, 208, 282, 517, 582, 669).

Drugs or drug derivatives with free carboxyl groups can most readily be conjugated to the amino groups of carriers by the addition to a drug-protein mixture of a water-soluble carbodiimide (1, 243, 256, 294, 338, 367, 426, 434, 464, 490, 526, 633, 671) or by the mixed anhydride reaction (156, 186, 188, 210, 211, 425, 494, 680, 693, 695).

Numerous methods have been developed for conjugating compounds with free hydroxyl groups to carrier proteins. Since, in general, direct coupling *via* the hydroxyl group is not possible, it is necessary to prepare intermediate compounds or derivatives which can then be conjugated to the carrier. For example, the hydroxyl groups of steroids and of many drugs react with succinic anhydride to form hemisuccinates which, *via* their free carboxyl groups, can be coupled to the amino groups of proteins by either the carbodiimide or mixed anhydride method, as described above (140, 143, 198, 210, 211, 241, 247, 308, 369, 425, 473, 507). Alternatively, the hydroxyl groups of steroids will react with phosgene to yield a highly reactive chlorocarbonate derivative which reacts directly with the amino groups of protein in the presence of bicarbonate (210, 425). The bifunctional reagent, sebacoyl dichloride, has been used to convert alcohols to acid chlorides which, under mildly alkaline conditions, react with amino groups of proteins (19). Phenols will react readily with diazotized p-aminobenzoic acid; the resulting derivative contains a free carboxyl group, which can be coupled to the amino groups of protein carriers by either the carbodiimide or mixed anhydride method (34, 261). Compounds, such as digitalis glycosides, with vicinal hydroxyl groups can be converted, by the addition of sodium metaperiodate, to dialdehydes which will react directly with the amino groups of protein carriers under mildly alkaline conditions (81, 94, 209, 370). Other methods are also available for the conjugation, to protein carriers, of compounds with hydroxyl groups (34, 208).

Drugs or drug derivatives with keto or aldehyde groups can be converted to O-(carboxymethyl)oximes by adding them to O-(carboxymethyl)hydroxylamine, (carboxymethoxylamine), thus introducing a carboxyl group which can then be coupled to the free amino groups of protein carriers by either the mixed anhydride or the carbodiimide method (100, 161, 210, 211, 308, 350, 425, 461, 463, 473, 499, 540, 703).

B. Characterization of Drug-Protein Conjugates

Incorporation of too few, or of too many, hapten molecules into a hapten-protein conjugate may result in a poor antibody

response. Several workers have stated that substitution of between 5 and 15 haptenic groups per molecule of carrier has seemed ideal when serum albumin was used (208, 399, 519). Thus, before immunization of experimental animals with a drug-protein conjugate, it is useful to determine the average number of drug molecules one has conjugated to each molecule of protein carrier. In table 3 are listed five methods by which the degree of drug incorporation into a conjugate can be characterized.

The extent of haptenic incorporation into a conjugate can, in some instances, be determined by direct chemical analysis of the conjugate (117, 187, 198, 256, 352, 454, 692). If the chemically introduced haptenic group has an absorption spectrum, either in visible or in ultraviolet light, which will enable one to differentiate the hapten from the carrier, the ratio between the molar extinction coefficient (433) of the conjugate and that of the hapten at an appropriate wavelength can be used to calculate the molar incorporation of hapten into the conjugate; even if there is overlap in spectra between hapten and carrier, the molar incorporation of hapten can be estimated with reasonable accuracy by determining the difference in molar extinction coefficients between conjugate and carrier, and then comparing this difference with the molar extinction coefficient of the hapten at the same wavelength (208, 210, 623, 664). Other methods for determining the extent of haptenic incorporation include determination of the extent of incorporation of radiolabeled drug into conjugate (12, 27, 131, 136, 137, 154, 294, 350, 420, 423, 441, 464, 658) and determination of the decrease of free amino groups in the carrier protein, following use of a method involving conjugation of drug to free amino groups of the carrier (125, 151, 208, 210, 247). Finally, if one has already developed an immunoassay for a drug, the extent of drug incorporation into a conjugate can be estimated immunologically; obviously, this latter method is not useful when one is initially synthesizing and characterizing a new drug-protein conjugate.

TABLE 3

Methods for characterization of drug-protein conjugates

1. Chemical analysis
2. Spectrophotometric analysis
3. Incorporation of radiolabeled drug
4. Analysis of receptor groups in carrier
5. Immunological assay

C. Immunization with Drug-Protein Conjugates

Although drug-specific antibodies may be elicited in any mammalian or avian species, animals of some species appear to be capable of greater antibody formation than are animals of other species. Even within a species, however, individual animals vary greatly both in their capacity to form antibodies to a given antigenic determinant and in the specificity of the antibodies formed. It is occasionally necessary to immunize many animals before one encounters a single animal capable of producing antibodies of satisfactory titer, specificity and affinity for use in an immunoassay procedure. Therefore, although large animals such as goats and sheep are good sources of antibodies, it is wise initially to use smaller animals. Some workers have employed guinea pigs and mice with good results. In the experience of most workers, however, rabbits have been most satisfactory from the point of view that they are, in general, good antibody-forming animals and that it is possible to obtain sufficient serum (20 ml) from a single bleeding to perform several hundred thousand individual immunoassay determinations once the specificity and affinity of that serum specimen has been established (91, 92).

Ordinarily, drug-protein conjugates are suspended in Freund's complete adjuvant mixture (129, 236) at a final concentration of 1 mg/ml; lesser concentrations may be employed if the available supply of the

drug-protein conjugate is limited. Numerous immunization schedules, both in terms of antigen dosage and frequency of injections, have been described. In our laboratory, rabbits are injected with 0.4 to 0.6 ml doses weekly for 2 months, followed by injections every 2 weeks for several months and finally by single monthly injections. Longer periods of time (8–16 months, in some instances) than are employed for protein antigens may be required to obtain antihapten antibody of optimal titer, specificity, and affinity (232, 334, 519, 623, 660). Vaitukaitis and her colleagues have described a method which employs a small, divided primary immunizing dose together with *Bordetella pertussis* vaccine and which may be particularly useful when the quantity of hapten or conjugate is limited (492, 668). These and other practical problems connected with raising antisera for use in immunoassays have been reviewed recently (326, 327, 395, 502, 585).

D. Labeling of Drugs and Drug Derivatives

1. Radioactive labeling. The most useful methods for radiolabeling of drugs for use in the detection of drug-specific antibodies involve the incorporation of either tritium (^{3}H) or radioiodine (^{125}I) into drugs or drug derivatives; ^{14}C-labeled drugs can also be used, but the specific activities thus achieved are relatively low. Methods employing ^{14}C labels are thus relatively insensitive both in the detection of antibodies and in the development of radioimmunoassays (11, 12).

Tritium can readily be introduced into most drugs by the Wilzbach technique (720), followed by chromatographic purification. It has a long half-life of 12.3 years and it represents no major external radiation hazard. However, it is not ideal. Firstly, since tritium emits only β-particles, liquid scintillation counting of radioisotopic disintegration must be employed. This can be cumbersome, time-consuming and costly; quench corrections with the addition of internal standards may be necessary, and chemiluminescence can cause delays or misleading results unless proper control procedures are carried out (88, 291). Secondly, in the case of certain drugs, sufficient specific activity cannot be achieved to provide the sensitivity needed for the subsequent development of RIA procedures.

^{125}I-labeled drugs have certain advantages over ^{3}H-labeled drugs. ^{125}I is a γ-emitter, and gamma counting is faster, more convenient, and less expensive than liquid scintillation counting. In addition, higher specific activities can be achieved with ^{125}I than with ^{3}H, with resultant increases in sensitivity, both in antibody detection and in the subsequent development of radioimmunoassay methods. With the use of currently available radioiodination methods, it is difficult to achieve high specific activities by attempting to couple ^{125}I directly to most drugs. To facilitate radioiodination, derivatives may be synthesized which contain histamine (101, 102), desaminotyrosine (254), tyrosine methyl ester (507, 703, 704), tyramine (404, 675) or another phenol-containing group (405, 514, 551, 676); alternatively, drugs and drug derivatives have been conjugated to proteins (1, 339, 466, 697a) or to synthetic polypeptides containing tyrosine residues (32, 419, 491, 674). Such drug derivatives can then be labeled with ^{125}I; radioiodination is usually carried out by the chloramine T method (132, 264, 321–323, 325), although enzymatic radioiodination using bovine lactoperoxidase (181, 445) is also feasible. An alternative method which has been used for the radioiodination of drugs or drug derivatives with free amino groups (76–78) is acylation with the so-called Bolton-Hunter reagent, ^{125}I-labeled 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (61, 578). The steps necessary to radioiodinate a drug or drug derivative may be chemically difficult and, moreover, the resultant change in drug structure may alter its immunological behavior. Other disadvan-

tages of ^{125}I include: a relatively short half-life of 60 days, possible chemical damage to the drug caused by radioidine, the need for frequent iodination with purification and immunological assessment of each newly iodinated batch and the continued exposure of laboratory personnel to ^{125}I (57, 520).

2. Nonradioactive labeling. For use in the development of enzyme-linked immunoassays, certain drugs and other small molecules have been conjugated to such enzymes as horseradish peroxidase (679), lysozyme (597), malate dehydrogenase and glucose 6-phosphate dehydrogenase (591); the extent of hapten incorporation into hapten-enzyme conjugates is deliberately kept low in order that the conjugate may retain enzymatic activity. In an analogous manner, haptens such as prostaglandins and steroid hormones have been coupled to bacteriophage in such a manner that the hapten-bacteriophage conjugate retains its infectivity (15, 16, 277, 442). For use in the development of spin label immunoassays, nitroxide spin labels have been coupled to drugs or drug derivatives by conventional organic syntheses (416, 417, 596). For use in the development of hemagglutination inhibition methods, drug-protein conjugates have been conjugated to red blood cells (7–9, 112, 434).

E. Detection of Drug-Specific Antibodies

Since most animals immunized with drug-protein conjugates form antibodies with specificity for the carrier protein, the method chosen for detection of drug-specific antibodies must be one in which antibodies specific for the carrier will not also interact. The simplest and most direct methods for the detection of drug-specific antibodies without interference by carrier-specific antibodies involve the direct demonstration of binding of radioactively labeled drugs or drug derivatives by antibody. Such binding can be demonstrated by any of the methods listed in table 1.

Since many drugs are bound to a significant degree by normal serum proteins (especially in undiluted serum), it is important to demonstrate that binding of labeled drug is not observed with appropriate dilutions of control sera from nonimmunized animals and from animals immunized with unrelated antigens.

In the absence of radiolabeled drug, drug-specific antibodies can be detected by their ability to inhibit drug-enzyme conjugates, by their capacity to inactivate drug-bacteriophage conjugates, by their ability to agglutinate drug-erythrocyte conjugates or by their ability to alter the signal produced by spin-labeled drugs, as assessed by an electron spin resonance spectrometer. It is also possible to employ classic precipitin (98, 539) or complement fixation (282) methods to demonstrate the interaction of drug-specific antibody with conjugates in which the hapten is attached to a carrier antigenically unrelated to the carrier used for immunization.

Whatever method is selected for the detection of drug-specific antibodies, it is important to ascertain that the observed interaction is specifically inhibited by free, unconjugated drug (90, 91, 93).

F. Characterization of Drug-Specific Antibodies

1. Specificity. Once it has been demonstrated that a labeled drug or drug derivative is bound by an antiserum, it must be shown that this binding is specific. First, one must demonstrate that nonlabeled drug is an effective inhibitor of this binding. Then, one must proceed to assess the degree of inhibition caused by metabolic products of the drug and by other structurally related molecules. To indicate the degree of specificity which can be achieved in selected drug-specific antisera, the structural formula of digoxin is compared in figure 2 with those of digitoxin, a closely related cardiac glycoside, and dihydrodigoxin, a pharmacologically ineffective metabolic breakdown product of digoxin. Despite the minimal chemical differences between digoxin and these two closely related glycosides, it is possible to elicit

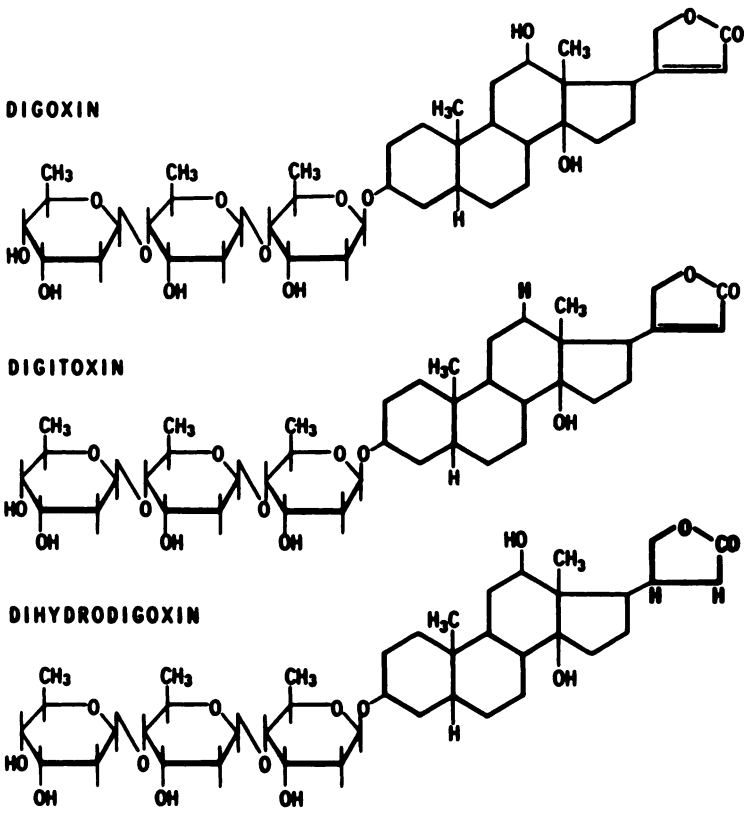

FIG. 2. Structural formulas of digoxin, digitoxin and dihydrodigoxin. The portions of the digitoxin and dihydrodigoxin molecules, respectively, which differ structurally from digoxin are depicted in boldface. (Reprinted by permission from V. P. Butler, Jr., D. H. Schmidt, J. F. Watson and J. D. Gardner: Production and properties of digoxin-specific antibodies. Ann. N.Y. Acad. Sci. 242: 717-730, 1974.)

digoxin-specific antibodies which bind digoxin 20 to 30 times more effectively than they bind either digitoxin or dihydrodigoxin (92, 94, 623). Finally, it is also necessary to demonstrate that structurally more distant compounds, notably other frequently prescribed drugs or substances normally present in human plasma, do not inhibit the binding of drug by antibody. For example, antibodies to digitalis glycosides react with steroid hormones (94); therefore, it was necessary to demonstrate that concentrations of steroid hormones encountered in human sera did not inhibit the binding of ^{3}H-digoxin by antidigoxin sera before the immunoassay method could be used clinically to measure serum digoxin concentrations (622, 623).

In connection with studies of antibody specificity, it is important to recognize that antibodies to a given drug will usually react with metabolites of that drug. With appropriate absorption techniques, it may occasionally be possible to remove some of the antibodies which cross-react in this manner. It should be remembered, however, that absolute specificity of antibodies for a given molecule will rarely, if ever, be observed (34). Cross-reactivity with drug metabolites may not represent a major disadvantage if, in practice, serum concentrations of "immunoreactive" drug correlate well with values obtained by other methods and with the clinical state of the patients studied. For example, such a correlation does exist in the case of digoxin; this correlation, however, does not necessarily apply to other drugs be-

cause digoxin is somewhat unusual in that it is not extensively degraded in man, and in that several of its major metabolites are both pharmacologically and immunologically active (89).

Antibodies to a given drug will often react with related drugs of the same class, but this cross-reactivity should not constitute a major problem clinically if it can be established with certainty that the patient is receiving a given drug and has not recently received a chemically related agent (89).

If problems are encountered in obtaining antibodies of satisfactory specificity for use in an immunoassay procedure, it is important to remember that different individual animals may produce antibodies which vary greatly in specificity; one should, therefore, examine several antisera to select the one with optimal specificity. Moreover, inasmuch as the specificity of antihapten antibodies appears to be directed primarily against that portion of the hapten molecule furthest from the site of conjugation to the carrier, antibodies of different specificity can often be obtained if the hapten is coupled to the carrier *via* a different functional group (31, 63, 93, 208, 266, 340, 425, 495, 630, 683).

2. Affinity. Antiserum to a given drug usually contains a heterogeneous population of drug-specific antibodies with different avidities or association constants. In general, the greater the affinity of antibody for drug, the more sensitive will be the immunoassay which can be developed and the simpler it will be to make assay behavior constant and reproducible. Accordingly, the determination of association constants is useful in the selection of antisera for use in immunoassay work; it is, however, not essential since the avidity of antibodies may be inferred from a variety of measurements of hapten-antibody interactions (322, 327). Recent studies have also emphasized that dissociation constants of drug-antibody complexes may be important when, in an immunoassay procedure, the adsorbent used to separate free from antibody-bound drug competes with antibody for drug molecules which dissociate from antibody during the separation step (460, 616, 625, 626).

It should be pointed out that no two antisera to any antigen may ever be considered to be identical either in specificity or in affinity. Even in antisera obtained at different times from a single animal, striking changes in the affinity and specificity of antidrug antibodies have been observed with time (623). Thus, published results regarding the specificity or affinity of antidrug antibodies should never be extrapolated to one's own antisera; furthermore, it would seem reasonable to require commercial suppliers of drug antisera or of drug immunoassay kits to provide data concerning specificity and affinity with every sample sold.

3. Titer. By antibody titer is meant the greatest dilution of antibody which will produce a given degree of binding of a stated amount of a drug (322). The higher the titer, the more determinations one can perform with a given volume of antiserum. Thus, antidigoxin sera frequently can be used at dilutions theoretically great enough to allow 200,000 digoxin determinations to be performed with 1 ml of antiserum. However, a high-titered antiserum is useless unless both specificity and affinity are satisfactory.

G. Immunoassay Methods

1. Radioimmunoassay. When a radiolabeled drug or drug derivative with satisfactory specific activity and radiochemical purity is available, the use of a radioimmunoassay method may be considered. Any of the separation methods listed in table 1 may be employed (146, 180, 504, 519, 543). Equilibrium dialysis, electrophoresis, chromatoelectrophoresis and gel filtration have proved to be too cumbersome for routine use when large numbers of specimens must be analyzed. The separation methods which are most widely used

at the present time include the dextran-coated charcoal method, the double antibody method, the ammonium sulfate precipitation method and a variety of solid phase antibody procedures.

The dextran-coated charcoal method (295–297) involves the almost instantaneous adsorption of non-antibody-bound drug onto charcoal particles and its rapid centrifugal separation from antibody-bound radioactivity; thus, it is not useful where radiolabeled drugs or drug derivatives are not effectively adsorbed to the charcoal or where, with a high dissociation constant for a drug-antibody complex, results vary with time as dissociated drug is progressively adsorbed to charcoal as a function of the duration of the charcoal incubation step in the immunoassay procedure (460, 625). The principal advantages of the dextran-coated charcoal method are its convenience and rapidity and, when antibody affinities are sufficiently high to permit its use, it is one of the most satisfactory of all RIA procedures.

As noted above, some radiolabeled drugs and drug derivatives, notably radioiodinated drug-protein or drug-polypeptide conjugates, are not readily or completely adsorbed to dextran-coated charcoal. In such instances, the double antibody method (279, 324, 478) may be employed. This method involves the addition of an antiserum to the immunoglobulin of the species from which the drug-specific antibody was obtained; such addition of anti-immunoglobulin serum results in the coprecipitation of drug-specific antibody together with bound labeled drug (or drug derivative), while free radioactivity remains in the supernatant fluid after centrifugation. The double antibody method involves more manipulations than the coated charcoal method, requires 24 to 48 hours to perform and employs large volumes of anti-immunoglobulin serum (as a source of the so-called "second antibody"), which must ordinarily be obtained from a large animal (sheep, goat, horse or cow) at considerable expense. Despite these limitations, it is a specific, precise and highly reproducible method.

The ammonium sulfate precipitation method of Farr (128, 218, 470) involves the coprecipitation of antibody-bound radiolabeled drug with the entire immunoglobulin fraction of antiserum, as the result of the addition of a saturated solution of ammonium sulfate, an effective reagent for the precipitation of immunoglobulins of all species. Like the dextran-coated charcoal method, this method is rapid and convenient and has great utility in the initial detection of drug-specific antibodies, as discussed above in section III E but it is, in general, less precise and less reproducible than the coated charcoal or double-antibody RIA methods.

A large number of RIA methods have been developed in which the separation of bound from free radiolabeled tracer is simplified by the use of antibody coupled to a solid-phase support. Among the particulate supports to which specific antibodies have been coupled are Sephadex (717), polystyrene (115), polypropylene (177), agarose (612), cellulose (644), bromoacetyl cellulose (17), cellulose triacetate beads (157), glass beads (55, 431), polyurethane sponges (670), nylon nets (586) and red blood cells (439); antibodies have also been rendered insoluble by polymerization with ethylchloroformate (192) or have been immobilized by entrapment in polyacrylamide gel (255). In these instances, separation of bound from free radiolabeled tracer is usually achieved by centrifugation. It is also possible to couple antibody to the surface of polypropylene or polystyrene test tubes; in this instance, the separation step consists of simple decantation of unbound tracer after an appropriate incubation period (30, 116, 663). Recently, methods have been described in which antibodies have been conjugated to magnetic supports such as silicone-treated (300) or polymer-coated (498) iron oxide; a magnetic field rather than centrifugation is used to separate unbound tracer from tracer bound to the insolubilized antibody.

One limitation of solid phase RIA methods is that there may be loss of antibody during its coupling to the solid phase; this is not ordinarily a major consideration except in instances in which the available supply of a given antiserum is limited. A more serious limitation of solid phase RIA methods is the fact that nonspecific binding or trapping of tracer by the solid support may occur; the magnitude of such binding or trapping may not become apparent if uncoated insoluble support substances are employed in control tubes; proper controls should include tubes containing unrelated antibodies coupled to the same insoluble support. If such binding and trapping are not significant, solid-phase RIA methods offer advantages over double antibody methods, in that incubation with a second antibody is not required, and over ammonium sulfate precipitation methods, in that results are more accurate and reproducible; solid phase RIA methods are as convenient to employ as the dextran-coated charcoal method and possess significant advantages over this latter method in instances in which either the radiolabeled drug is ineffectively adsorbed to the charcoal or, conversely, dissociates rapidly from the antibody and is adsorbed rapidly to the charcoal.

2. Enzyme immunoassay. Enzyme immunoassays, as initially described, employed antibodies coupled to solid phase supports to enable the separation of bound enzyme-antigen conjugates from free enzyme-antigen conjugates (207, 397, 591, 679, 687). More recently, "homogeneous" enzyme immunoassays have been developed in which drugs have been conjugated to enzymes such as lysozyme, glucose 6-phosphate dehydrogenase and malate dehydrogenase in such a way that, when the enzyme-labeled drug is complexed with drug-specific antibody, the enzyme is rendered inactive; the mechanism by which the enzyme is inactivated is not known, but it has been suggested that the inactivation may involve steric exclusion of the enzyme substrate from the enzymatically active site. Whatever the mechanism may be, physical separation of bound from free drug-enzyme conjugate is unnecessary. When this assay method is used, the addition of increasing amounts of drug to constant amounts of drug-specific antibody and drug-enzyme conjugate will reduce the degree of enzyme inactivation in a reproducible and predictable manner, thus enabling the construction of standard curves and the analysis of test specimens (591, 596, 597). In addition to not requiring a separation step, homogeneous enzyme immunoassays possess a significant advantage over RIA methods in that they do not require the use of radioisotopes, thus rendering them safer and more convenient for laboratory personnel. In addition, the enzyme-labeled drugs and drug derivatives remain stable for longer periods (up to 1 year) than their radioiodinated counterparts, which rarely can be used for longer than 6 or 8 weeks. Finally, assays of enzymatic activity require a spectrophotometer, which is considerably less expensive than the counting equipment required for radioimmunoassays. In view of these advantages the use of enzyme immunoassays for drug measurement has increased significantly in clinical laboratories in recent years.

3. Spin label immunoassay. Spin label immunoassay methods have employed nitroxide radicals which show characteristic electron spin resonance spectra, if able to tumble freely in solution; however, if the mobility of these radicals is reduced, the intensity of their electron spin resonance signals is decreased. Thus, nitroxide-labeled drugs have characteristic spectra, the intensities of which are decreased when the spin-labeled drugs are bound to antibodies. Unlabeled drug will, of course, compete with nitroxide-labeled drug for antibody combining sites, resulting in an increase in the intensity of the electron spin resonance signal; based upon the increases in intensity observed with the addition of increasing amounts of unlabeled

drug to a constant amount of drug-specific antiserum and spin-labeled drug, a standard curve can be constructed and the concentrations of drug in test specimens determined (596). Because this method requires the availability of, and familiarity with, an electron spin resonance spectrometer, its use to date has been limited.

4. Agglutination inhibition. Drug-specific antibodies will agglutinate red blood cells (7), latex particles (572) or bentonite particles (347) which have been "coated" with the corresponding drug, drug derivative or drug-protein conjugate. If drug-specific antibodies are incubated with free drug for a few minutes before the coated red cells or particles are added, these antibodies are no longer available for agglutination of the drug-coated cells. The extent of inhibition of the agglutinating activity of the drug-specific antibodies is, of course, related to the concentration of free drug added and thus the concentration of free drug in test specimens can be determined by the extent of inhibition of agglutination caused by that test specimen in comparison with the inhibition produced by known standard concentrations of free drug (7). Agglutination inhibition assay methods are less sensitive than RIA methods, but can provide useful information when radioactively or enzymatically labeled drugs or drug derivatives are not available.

5. Viroimmunoassay. Although its use in drug measurements has not been reported, the viroimmunoassay method has been used in the assay of estradiol-17β (15) and of prostaglandin $F_{2\alpha}$ (16) and could be used for drug assays. This method employs the ability of hapten-specific antisera to neutralize bacteriophage-hapten conjugates; such inactivation is inhibited by free hapten and this inhibition has formed the basis for the development of viroimmunoassay methods capable of detecting as little as 1 or 2 pg of free hapten (15, 16)

6. Immunoradiometric assay. Immunoradiometric assays have been used mainly in the assay of peptide hormones, and their use for drug assay has not been reported. The immunoradiometric assay method involves the use of solid phase antigen coupled to an insoluble support. Radiolabeled antibodies will ordinarily be bound to solid phase antigen, but this binding will be inhibited if soluble antigen is added to the radiolabeled antibody before its incubation with the solid phase antigen. When different amounts of soluble antigen are added to radiolabeled antibody, the amount of radioactivity remaining in solution is related to the amount of soluble antigen added, thus enabling the formation of a standard curve, from which the concentration of soluble antigen in test specimens can be determined (5, 6, 467–469, 564, 725).

7. Other immunoassay methods. Haptens have also been measured by fluorescence polarization immunoassay (179) and by fluorescence excitation transfer immunoassay (665), but the applicability of these methods to the measurement of drugs or other substances in biological fluids has not yet been established.

H. Application of Immunoassay Methods to Biological Fluids

Before an immunoassay method can be used to measure concentrations of a given drug in serum, plasma, urine or another biological fluid, it must be determined that the fluid to be assayed, when obtained from individuals who have not received the drug, does not contain substances which interfere with the binding of tracer by drug-specific antibodies. Such interference most commonly is caused by naturally occurring substances which cross-react with drug-specific antibodies; it may also be caused by substances which degrade a labeled drug derivative (647) or by substances which bind the tracer (288, 299, 536).

Drug standards for immunoassay determinations should be very carefully prepared (22–24, 162) in the presence of the fluid to be assayed. Such fluid should be obtained from normal individuals who are receiving no drug, and the volume used

should be identical with the volume employed in the assay procedure; it should be established that standards prepared from the fluids of several individuals not receiving the drug yield comparable results before the assay method is employed in the analysis of test specimens from drug-treated individuals.

Many drug immunoassay procedures can be carried out with untreated and unextracted serum, plasma or other biological fluid. It is, however, sometimes necessary to carry out extractions or other separatory procedures before the immunoassay in order to concentrate the test substance, to remove it from a normal binding site on a protein in the test serum, or to separate it from certain of its metabolic derivatives or from other structurally related substances which are capable of inhibiting the reaction between drug-specific antibody and the labeled drug or drug derivative. Occasionally, a prior treatment or separatory procedure is required to inactivate or remove substances which degrade or metabolize the labeled drug or drug derivative.

The stability of the drug in each biological fluid to be assayed must be established and appropriate conditions for storage determined by assaying identical standard specimens at various time intervals after the addition of drug and by the repetitive analysis of test specimens at varying time intervals after the specimen is obtained; such assessment of stability is particularly important in the case of urine immunoassays.

I. Analysis of Results

Numerous methods are available for the analysis of data obtained by immunoassay procedures. Most commonly, a binding parameter is expressed graphically on the ordinate as a function of drug concentrations in standard specimens as plotted (usually, but not exclusively, logarithmically) on the abscissa; then, the drug concentrations in identical volumes of simultaneously analyzed test specimens are determined from this standard curve on the basis of the extent of binding of labeled drug or drug derivative observed in the presence of each test specimen. The binding parameters which have been expressed graphically include the ratio of antibody-bound (B) to free (F) labeled drug or drug derivative (B/F ratio), the percentage of label bound (fig. 1) or the percentage of inhibition of binding of the labeled compound; in the case of RIA, results have also been expressed as units of radioactivity bound, whereas, in the instances of homogeneous enzyme immunoassays and spin label immunoassays, results have been expressed as enzymatic activity or as the peak heights of the electron spin resonance signal. The mathematical significance and the computerized processing of immunoassay data, particularly those obtained by RIA procedures, has been the subject of numerous treatises; in the course of these studies and analyses, methods have been devised to develop empirical quality control systems to obtain estimates of the stability, precision and reproducibility of RIA systems (127, 195, 220, 221, 225, 302, 451, 556–563, 700, 724).

IV. General Considerations

A. Advantages of Immunoassay Methods

In the past decade, the development and refinement of spectrophotofluorometric, gas chromatographic, mass spectrometric and high pressure liquid chromatographic methods have provided clinical and investigative laboratories with the capacity to measure drug concentrations, at the picomolar level, in tissues and biological fluids (548, 682). Many such assay methods are sensitive and highly specific but they are often cumbersome and, in most instances, require one or more extraction steps, thus making the analysis of large numbers of specimens difficult; furthermore, milliliter volumes of serum are usually required for analysis. Immunoassay methods are also sensitive and specific but, in addition, they often provide the

rapidity and convenience needed for the daily performance of large numbers of drug analyses on small volumes of serum in clinical laboratories. It is not difficult for one technician to perform blood drug level determinations on microliter volumes of serum from 40 patients within a few hours (92) and the development of automated immunoassay methods (72, 152, 203) will further increase the capacity of the clinical laboratory. The rapid analysis of large numbers of samples is often highly desirable clinically in the evaluation of drug therapy and in the prompt determination of appropriate dosage schedules for individual patients being treated with a given drug; for example, the availability of digoxin immunoassay methods has provided aid to physicians in properly individualizing digoxin dosage schedules (89, 95, 414, 528, 619, 621) and appears to have contributed to a temporally related decrease in the incidence of digoxin intoxication in digoxin-treated patients (197, 381). The simplicity and convenience of drug immunoassay methods also render them highly suitable for large-scale studies of drug bioavailability (196, 427, 429, 457, 538, 549, 604, 689), pharmacokinetics (41, 51, 156, 188, 212, 304, 343, 461, 599, 601, 659), compliance (38, 609, 708) and abuse (110, 138, 485).

B. Limitations of Immunoassay Methods

1. Specificity. Since antibodies to a given drug may cross-react to varying degrees with metabolic derivatives of the drug, drug metabolites may contribute to observed serum concentrations of "immunoreactive" drug. Although immunoreactive drug concentrations are often proportional to tissue concentrations of biologically active drug, it should be noted that immunoassay methods are sometimes less specific than other assay methods in that immunoreactive drug concentrations may reflect not only the drug but also certain active and/or inactive metabolites. It is therefore useful to assess the significance of serum drug concentrations as measured by immunoassay by comparison of the observed results with values obtained by other methods of analysis in specimens obtained from individuals (or from experimental animals) receiving this drug; alternatively, serum concentrations of immunoreactive drug may be compared with a quantifiable pharmacological or toxic effect of the drug, or empirically with the clinical response to the drug (89).

2. Stability of reagents. Although antibodies are stable for many years if properly stored in concentrated form, deterioration of antisera may occur if appropriate precautions are not taken (249, 373). Deterioration of labeled drugs or drug derivatives may also occur with time, particularly in the case of radioiodinated drug derivatives (373). The stability of standards must also be assessed if stored, rather than freshly prepared, standards are to be employed in immunoassay procedures (22–24, 29, 162, 571).

C. Applications of Drug Immunoassays

Drug immunoassay methods have been used most extensively in the clinical assessment of patients, with particular respect to the determination of optimal dosage schedules for individual patients being tested with a given drug. As with all drug assay procedures, drug immunoassay methods will yield clinically useful information only if it can be established, experimentally or on clinical grounds, that the drug concentrations in the specimens being analyzed bear some relationship to a pharmacological effect of, or the clinical response to, that drug (71, 182, 379, 380, 682). In this connection, the time at which the specimen is best obtained with respect to the last dose of the drug is quite important and must be established before the immunoassay procedure can be used properly (89, 379, 380). With the increasing availability of such information, much has been learned about heretofore obscure processes which contribute to the long-recognized but hitherto poorly understood individual variability in drug dosage re-

quirements and in susceptibility to the toxic effects of drugs (95, 546, 681). For example, immunoassay methods have been used to identify and characterize certain drug-drug interactions(80, 358, 428, 529, 686), and to define clinically important differences in the bioavailability of different preparations of the same drug (427, 429, 457, 538, 549, 604, 689). Drug immunoassay methods have also provided much useful pharmacokinetic information concerning drug absorption (14, 80, 216, 290, 318, 320, 341, 343, 344, 358, 428, 443, 482, 501, 505, 603, 686, 711, 713), compartmental distribution (388, 650), metabolic degradation (96, 230, 409, 463) and excretion (33, 79, 223, 239, 262, 269, 280, 328, 360, 385, 455, 640).

V. Specific Drug Immunoassays

Table 4 lists drugs for which immunoassays and/or antibodies are currently available. This section contains descriptions of the methods used to elicit drug-specific antibodies, of the use of drug-specific antibodies in the development of immunoassay methods and of the clinical and investigative applications of drug-specific immunoassay procedures.

A. Adrenergic Blocking Agents

1. Propranolol. To conjugate the *beta* adrenergic blocking agent, propranolol, to protein carriers, Kawashima *et al.* (369) first prepared a hemisuccinate derivative of propranolol by the interaction between the drug and succinic anhydride, resulting in the formation of an ester bond between the succinyl group and the 2-hydroxyl group in the propranolol sidechain. The propranolol hemisuccinate was then coupled *via* the hemisuccinate carboxyl group to the amino groups of bovine serum albumin by the mixed anhydride method; conjugates containing either *dl*-propranolol or *l*-propranolol were prepared in this manner. Rabbits immunized with either of these conjugates formed antibodies capable of binding *dl*-propranolol-^{3}H, as assessed by the ammonium sulfate precipitation method. As determined by inhibition of binding of *dl*-propranolol-^{3}H, antiserum against *dl*-propranolol recognized both *d*- and *l*-propranolol to the same degree, while antiserum against *l*-propranolol bound *l*-propranolol more selectively than it bound the *d*-isomer, thus enabling the development of stereospecific ammonium sulfate RIA methods capable of detecting as little as 10 pg of propranolol isomers in 10 μl of unextracted rat serum. Metabolites of propranolol do not interfere with the assay unless concentrations are very high and thus the method has proved to be useful in the measurement of propranolol in the serum and tissues of rats and mice (369, 421).

More recently, a propranolol derivative, desisopropylpropranolol has been conjugated *via* the amino group in its side chain to bovine serum albumin, using a water-soluble carbodiimide as the coupling reagent. Rabbits immunized with the resulting conjugates have formed antibodies capable of binding propranolol-^{3}H or an ^{125}I-labeled propranolol derivative, as determined by the dextran-coated charcoal and double antibody methods, as well as by a solid-phase antibody approach. With the use of these antibodies and the ^{125}I-labeled propranolol derivative, a sensitive dextran-coated charcoal radioimmunoassay method has been developed for the measurement of propranolol concentrations in 50 μl aliquots of unextracted human serum.‡

B. Anti-Anxiety Drugs

1. Benzodiazepines. *a. Chlordiazepoxide.* To conjugate chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1,4 benzodiazepine 4-oxide) to a protein carrier, an analog, the 4-hydrazinocarbonylmethoxy-5-phenyl derivative, was converted to a reactive acyl azide and then coupled to bovine serum albumin by addition to this protein carrier at pH 8.5.

‡ V. P. Butler, Jr., D. T. Eng, J. P. Bilezikian and N. H. Wassermann, unpublished observations.

TABLE 4
Available drug immunoassays

Adrenergic blocking agents
- Propranolol

Anti-anxiety drugs
- Benzodiazepines
  - Chlordiazepoxide
  - Diazepam
- Butyrophenones
  - Haloperidol
- Dibenzodiazepines
  - Clozapine
- Flupenthixol
- Phenothiazines
  - Chlorpromazine

Anti-arrhythmic agents
- Aprindine
- Cardiac glycosides
- Diphenylhydantoin
- Procaine amide*
- Propranolol
- Quinidine

Antibiotics
- Aminoglycosides
  - Amikacin
  - Gentamicin
  - Netilmicin
  - Sisomicin
  - Tobramycin
- Chloramphenicol
- Clindamycin
- Dapsone
- Isoniazid
- Penicillin
- Rifampin
- Sulfonamides*
- Viomycin

Anticoagulants
- Warfarin

Anticonvulsants
- Carbamazepine
- Clonazepam
- Diphenylhydantoin
- Ethosuximide
- Phenobarbital
- Primidone

Antidepressants
- Non-tricyclic
  - Nomifensine
- Tricyclic
  - Amitriptyline and nortriptyline
  - Clomipramine
  - Desmethylimipramine

Antidiarrheal drugs
- Loperamide

Antihypertensive drugs
- Clonidine
- Debrisoquin
- Hydralazine*
- Minoxidil
- Propranolol
- Reserpine
- Saralasin

Anti-inflammatory agents
- Acetylsalicylic acid*
- Antipyrine
- Colchicine
- Corticosteroids
- Indomethacin
- Phenylbutazone

Antimuscarinic drugs
- Atropine

Anti-tumor drugs
- Actinomycin D
- Adriamycin (doxorubicin)
- 1-β-D-Arabinofuranosylcytosine
- 1-β-D-Arabinofuranosyluracil
- Bleomycin
- Daunorubicin
- Macromycin
- Methotrexate
- L-Phenylalanine mustard*
- Vinca alkaloids

Anti-ulcer drugs
- Carbenoxolone

Bronchodilators
- Theophylline

Cardiac glycosides
- Acetylstrophanthidin
- Digitoxin
- Digoxin
- Gitaloxin
- Gitoxin
- Lanatoside C
- β-Methyldigoxin
- Ouabain
- Proscillaridin

CNS stimulants and hallucinogens
- Amphetamine
- Caffeine
- Cocaine
- 2,5-Dimethoxy-4-methylamphetamine
- Lysergic acid diethylamide
- Mescaline
- Nicotine
- Pimozide
- Tetrahydrocannabinol
- Theophylline

Cholinergic agents
- Nicotine

Diuretics
- Bumetanide

Local anesthetic agents
- Cocaine

Narcotic antagonists
- Cyclazocine

TABLE 4—*Continued*

(+)-2-Hydroxy-N-cyclopropylmethylmorphinan	Dexamethasone
Naloxone	Ethynylestradiol
Narcotics and analgesics	Fluoxymesterone
Acetylsalicylic acid*	Medroxyprogesterone acetate
Anileridine	Mestranol
Antipyrine	Methylprednisolone
Codeine	Metyrapone
Etorphine	Nandrolone
Fentanyl	Norethindrone
Hydromorphone	Norgestrel
Meperidine	Prednisolone
Methadone	Prednisone
Morphine	Triamcinolone acetonide
Pentazocine	Trienbolone acetate
Neuromuscular blocking agents	*Toxins*
d-Tubocurarine	Amanitin
Oral hypoglycemic agents	Arsenicals*
1-Butylbiguanide	Carcinogens
Glibenclamide	Genistein
Glisoxepide	Insecticides
Oxytocic agents	Ochratoxin A
9,10-Dihydroergotamine	Paralytic shellfish poison
Renal tubular transport inhibitors	Paraquat
Probenecid	Strychnine*
Sedatives and hypnotics	Tartrazine*
Barbiturates	*Vitamins*
Glutethimide	Pantothenic Acid
Methaqualone	Vitamin A*
Synthetic steroids	Vitamin B_{12}
Betamethasone	Vitamin D
Betamethasone 17-benzoate	Vitamin K*
Cyproterone acetate	

* Antibodies available, but immunoassay not described to date.

Approximately 8 molecules of hapten were coupled to each molecule of albumin, as estimated by differential pulse polarography against a known amount of chlordiazepoxide. Rabbits immunized with the hapten-protein conjugate formed antibodies which bound chlordiazepoxide-^{14}C, as determined by the ammonium sulfate precipitation method. Nonradioactive chlordiazepoxide specifically inhibited the binding of radiolabeled drug, while other benzodiazepines were ineffective inhibitors. There was slight cross-reactivity of the chlordiazepoxide antibodies with N-desmethylchlordiazepoxide, a metabolite of the parent drug. By using these antibodies together with chlordiazepoxide-^{14}C, an ammonium sulfate RIA method was developed which was capable of detecting as little as 20 ng of drug per ml in 0.1 ml samples of unextracted plasma from human subjects who had received chlordiazepoxide; the maximum error in the determination of this drug due to the presence of its metabolite, N-desmethylchlordiazepoxide, was felt to be no greater than 10% (187).

b. Diazepam. Derivatives of diazepam and of its N-demethylated metabolite, N-desmethyldiazepam, have been diazotized and then directly conjugated to the tyrosine, histidine and tryptophan groups of bovine serum albumin. Rabbits immunized with each of these conjugates have formed antibodies capable of binding diazepam-^{14}C, as determined by the ammonium sulfate precipitation method. Both diazepam and N-desmethyldiazepam are capable of inhibiting the binding of labeled diazepam by antiserum to the N-desmethyldiazepam derivative, while antiserum to the diazepam binds only the parent drug

and not its N-desmethylated metabolite. By using antisera to both antigens together with diazepam-^{14}C, an ammonium sulfate RIA procedure has been developed for the measurement of diazepam and of N-desmethyldiazepam. This RIA method is capable of detecting concentrations of these compounds as low as 20 ng/ml in 50 μl of human blood (527). Greater sensitivity can be achieved if an extraction step is carried out on a 0.5 ml serum specimen before the RIA procedure; results obtained by this approach correspond well with serum concentrations determined by a gas chromatographic method (240a).

2. **Butyrophenones.** *a. Haloperidol.* To couple haloperidol to bovine serum albumin, a hydrazide derivative of bovine serum albumin was prepared by adding hydrazine hydrate to the albumin in the presence of water-soluble carbodiimide. The albumin-hydrazide and haloperidol were mixed and allowed to react for 7 days in the dark. On the basis of incorporation of haloperidol-^{3}H into the conjugate, it was estimated that from 2 to 6 molecules of haloperidol were covalently attached to each molecule of bovine serum albumin (137). Alternatively, an O-(carboxymethyl)oxime derivative was prepared by reacting the drug with O-(carboxymethyl)-hydroxylamine, and this derivative was coupled to bovine serum albumin by the mixed anhydride procedure, yielding a product containing 14 haloperidol residues per mole of conjugates, as estimated spectrophotometrically (611a). Rabbits immunized with either of these conjugates formed antibodies capable of binding haloperidol-^{3}H; these antibodies were specific for haloperidol and, in general, none of the known major metabolites of haloperidol exhibited significant crossreactivity (137, 611a). With the use of haloperidol-specific antibodies and haloperidol-^{3}H, a dextran-coated charcoal immunoassay has been developed for the measurement of concentrations of haloperidol in 50 μl aliquots of unextracted human serum (137).

3. **Dibenzodiazepines.** *a. Clozapine.* A derivative of clozapine, *viz.* 11-(4-carboxymethyl-1-piperazinyl)-5H-dibenzo[b,e] [1,4]-diazepine, was conjugated to bovine serum albumin by the carbodiimide method. A goat immunized with this conjugate formed antibodies capable of binding ^{3}H-clozapine as determined by a gelatin-coated charcoal separation method. These antibodies exhibited greater affinity for clozapine than for its known metabolites, thus permitting the use of these antibodies, together with ^{3}H-clozapine, in the development of a gelatin-coated charcoal RIA method which has been used to determine plasma clozapine concentrations in patients given this drug (570a).

4. **Flupenthixol.** To elicit antibodies to the neuroleptic drug, flupenthixol, a derivative of the drug, 7-carboxyflupenthixol, was conjugated to ovalbumin by the carbodiimide method, with the resulting conjugate containing approximately 45 haptenic molecules coupled to each carrier protein molecule, as estimated by ultraviolet spectrophotometry. A sheep immunized with this conjugate formed antibodies capable of binding flupenthixol-^{3}H, as determined by a dextran-coated charcoal separation method. Flupenthixol and the closely related drug, fluphenazine, were effective inhibitors of this binding; two known flupenthixol metabolites were moderately effective as inhibitors but other tricyclic drugs were ineffective inhibitors. The ability of nonradioactive flupenthixol to inhibit the binding of flupenthixol-^{3}H by antibody has enabled the development of a dextran-coated charcoal RIA capable of detecting flupenthixol concentrations as low as 2 ng/ml in 0.1 ml of plasma. This RIA has been used in studies of the pharmacokinetics of flupenthixol in man (555a).

5. **Phenothiazines.** *a. Chlorpromazine.* Antibodies to the phenothiazine drug, chlorpromazine, have been successfully elicited by immunization with each of three different phenothiazine-protein conjugates. Desdimethylchlorpromazine has been conjugated *via* its amino group to the

free carboxyl groups of bovine serum albumin by the carbodiimide method, approximately 11 moles of hapten being coupled to each mole of albumin as estimated spectrophotometrically. The hemisuccinate of the phenothiazine, perphenazine, has been synthesized and coupled *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method; an estimated 15 moles of the phenothiazine derivative were conjugated to each mole of protein, as determined spectrophotometrically. Rabbits immunized with either of these conjugates formed antibodies capable of binding ^{14}C-chlorpromazine, as determined by the dextran-coated charcoal method (611). Utilizing a different approach, Kawashima *et al.* (368) coupled diazotized *p*-aminobenzoic acid to chlorpromazine. The resulting derivative was conjugated *via* the carboxyl group in the benzoic acid moiety to the free amino groups of bovine serum albumin with the mixed anhydride method; the approximate number of haptenic molecules per albumin molecule was 9, as estimated spectrophotometrically. Rabbits immunized with the chlorpromazine-albumin conjugate formed antibodies capable of binding ^{3}H-chlorpromazine, as determined by an ammonium sulfate precipitation method. Chlorpromazine and certain structurally related derivatives are effective inhibitors of the binding of tritiated chlorpromazine by antibody but three major chlorpromazine metabolites, chlorpromazine sulfoxide, nor$_1$-chlorpromazine and nor$_2$-chlorpromazine are ineffective inhibitors of this binding, thus enabling the development of an ammonium sulfate RIA method capable of detecting as little as 10 pg of chlorpromazine. This RIA method has been used in studies of the pharmacokinetics of chlorpromazine in rat plasma and tissues (368).

C. Anti-Arrhythmic Agents

1. Aprindine. Aprindine, (N-[3-diethylamino]propyl)-N-phenyl-2-indanamine, is a new antiarrhythmic agent used in the treatment of disorders of ventricular irritability. Aprindine was diazotized in position 4 of its phenyl ring and then conjugated directly to the tyrosine, tryptophan and histidine residues of human serum albumin; it was estimated that 11 molecules of aprindine were conjugated to each albumin molecule. Rabbits immunized with aprindine-albumin conjugates formed antibodies capable of binding aprindine-^{3}H, as demonstrated by the dextran-coated charcoal separation method. Nonradioactive aprindine was an effective inhibitor of this binding; two of four aprindine metabolites studied, namely the desethyl and parahydroxy derivatives, also inhibited this binding but the cross-reactivity of the other two metabolites, the dephenyl and the desindanyl derivatives, was undetectable. Antiserum from one rabbit has been used, together with aprindine-^{3}H, in the development of a dextran-coated charcoal RIA method capable of detecting aprindine concentrations as low as 10 ng/ml in canine plasma and in extracts of canine cardiac tissue. Results obtained by RIA were very similar to those obtained by gas chromatographic analysis of the same plasma and tissue specimens (415a).

2. Cardiac Glycosides. *See* section V O.

3. Diphenylhydantoin. *See* section V F.

4. Procaine amide. Procaine amide has been diazotized and conjugated to protein carriers *via* the newly introduced diazonium group. Rabbits immunized with procaine amide-protein conjugates have formed antibodies to procaine amide as detected by hemagglutination of procaine amide-coated sheep erythrocytes and by precipitation of heterologous procaine amide-protein conjugates; the specificity of these hemagglutination and precipitation reactions was established by demonstrating the capacity of procaine amide to inhibit both reactions (251, 579). The use of these antibodies in the development of a procaine amide immunoassay has not been reported.

5. Propranolol. *See* section V A.

6. Quinidine. The antiarrhythmic drug, quinidine, has been demethylated to yield a phenol, which was then alkylated with methyl 5-bromovalerate. This alkyl ester was then hydrolyzed to form the corresponding carboxylic acid, which could then be conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method; 29 molecules of the quinidine derivative were found to be coupled to each molecule of albumin. Rabbits and sheep immunized with these conjugates formed antibodies capable of binding dihydroquinidine-^{3}H. An antiserum selected for use in a quinidine RIA was reasonably specific for quinidine in comparison with its 2′-oxo and 3-hydroxy metabolites. With the use of this antiserum and dihydroquinidine-^{3}H, a solid phase RIA system has been developed which employs antibody coupled to cellulose triacetate beads and which is capable of detecting as little as 50 pg of quinidine (157).

D. Antibiotics

1. Aminoglycoside antibiotics. *a. Amikacin.* Amikacin has been coupled *via* its amino groups to the free carboxyl groups of porcine thyroglobulin by the carbodiimide reaction, with 115 amikacin molecules being coupled to each molecule of thyroglobulin, as estimated by the incorporation of amikacin-^{3}H into the conjugate. Rabbits immunized with amikacin-thyroglobulin conjugates formed antibodies capable of binding amikacin-^{3}H, as determined by a double antibody method, employing sheep anti-rabbit γ-globulin serum. Amikacin and another aminoglycoside antibiotic, kanamycin, were effective inhibitors of the binding of labeled amikacin by antibody but other aminoglycoside antibiotics, including gentamicin, neomycin, streptomycin and tobramycin, all exhibited negligible cross-reactivity with the antibodies to amikacin. The specificity of these antibodies has enabled their use, together with amikacin-^{3}H in the development of a double antibody method capable of detecting as little as 5 ng of amikacin in specimens of human serum, cerebrospinal fluid or urine (424).

b. Gentamicin. Gentamicin has been conjugated *via* its amino groups to the free carboxyl groups of several different protein antigens by the carbodiimide method; a representative gentamicin-human serum albumin conjugate contained 15 molecules of gentamicin per molecule of albumin, as estimated by incorporation of gentamicin-^{3}H. Rabbits immunized with gentamicin-protein conjugates have formed antibodies capable of binding gentamicin-^{3}H, as determined by the double antibody and dextran-coated charcoal methods. Gentamicin was found to be an effective inhibitor of the binding of labeled gentamicin by antibody (423, 441). Sisomicin also competes effectively with labeled gentamicin for antibody binding sites (77, 471) but other antibiotics tested to date, including several aminoglycoside antibiotics, exhibit insignificant cross-reactivity. Gentamicin antibodies have been used, together with gentamicin-^{3}H in the development of sensitive and specific double antibody and dextran-coated charcoal RIA methods, capable of determining concentrations of gentamicin as low as 3 ng/ml in human serum (423, 437, 441, 471, 642). Recently, gentamicin has been labeled with ^{125}I by the procedure of Bolton and Hunter (61), enabling the development of a dextran-coated charcoal RIA procedure capable of detecting 80 pg of gentamicin in human serum (76).

c. Netilmicin. Netilmicin, a new semisynthetic aminoglycoside antibiotic, has been conjugated by its amino groups to the free carboxyl groups of porcine thyroglobulin, using the carbodiimide method. Rabbits immunized with netilmicin-thyroglobulin conjugates formed antibodies capable of binding an ^{125}I-labeled netilmicin derivative, as determined by the dextran-coated charcoal separation method. Netilmicin was an effective inhibitor of this binding, whereas other aminoglycoside antibiotics, such as gentamicin, sisomicin, tobramycin and amikacin exhibited little or no inhibi-

tory capacity; the effectiveness of netilmicin as an inhibitor of binding has enabled the use of netilmicin antibodies, together with the radioiodinated netilmicin, in the development of a sensitive and specific RIA procedure capable of detecting as little as 500 pg of netilmicin (643).

d. Sisomicin. Antibodies to gentamicin often cross-react significantly with the structurally related aminoglycoside antibiotic, sisomicin (77, 471), and are capable of binding an ^{125}I-derivative of sisomicin, radioiodinated by the method of Bolton and Hunter (61). Thus, gentamicin antibodies and the ^{125}I-sisomicin derivative have been used in the development of a dextran-coated charcoal RIA system which has been used to measure concentrations of sisomicin in the serum of patients receiving this drug (77).

e. Tobramycin. Tobramycin has been conjugated *via* its amino groups to the free carboxyl groups of bovine serum albumin by the carbodiimide method. For the detection of antibodies, tobramycin was labeled with ^{125}I by the method of Bolton and Hunter (61). Rabbits immunized with the tobramycin-albumin conjugate formed antibodies capable of binding the ^{125}I-tobramycin derivative, as assessed by the dextran-coated charcoal method. Tobramycin was the most effective inhibitor of this binding. Kanamycin, an aminoglycoside antibiotic which differs from tobramycin by one substituent group, showed partial cross-reactivity, while other aminoglycosides exhibited minimal or no cross-reactivity at the highest concentrations tested. However, although carbenicillin does not interfere with the binding of the radioiodinated tobramycin derivative by antibody, this antibiotic (but not two others containing the β-lactam ring, *viz.*, methicillin and cephaloridine) does interfere with the immunoreactivity of tobramycin *in vitro* (78) perhaps by forming a complex with tobramycin (459, 696). Antibodies to tobramycin, together with the ^{125}I-labeled tobramycin derivative, have been used in the development of a dextran-coated charcoal RIA system capable of detecting 280 pg of tobramycin in specimens of human serum (78).

2. Chloramphenicol. To permit conjugation of chloramphenicol, its nitro group was reduced to form a *p*-aminophenyl derivative. This aromatic amine was then converted to the diazonium derivative and coupled, principally to tyrosine, but also to histidine and tryptophan, residues in several different protein carriers. Spectrophotometric analysis for azo groups indicated an average of 5 molecules of the chloramphenicol derivative per molecule of carrier protein. Rabbits immunized with chloramphenicol-bovine γ-globulin conjugates produced antibodies which reacted with chloramphenicol-rabbit serum albumin conjugates, as detected by the complement fixation method (282). The capacity of free chloramphenicol to inhibit this complement fixation reaction (282, 283) has served as the basis for the development of an immunoassay method capable of detecting picogram quantities of chloramphenicol in biological specimens (282), but the use of this assay to measure serum concentrations of this antibiotic has not been described to date.

3. Clindamycin. Clindamycin-2-hemisuccinate was synthesized, isolated and conjugated *via* the carboxyl group in the succinyl moiety to the free amino groups of bovine serum albumin, using a water-soluble carbodiimide as the coupling reagent. The resulting conjugate contained an average of 30 clindamycin residues per molecule of protein as determined by the reduction in the number of free amino groups in the albumin carrier. Rabbits immunized with this conjugate formed antibodies capable of binding clindamycin-^{3}H, as determined by a double antibody method. Clindamycin and its derivatives and metabolites were capable of inhibiting the binding of clindamycin-^{3}H; lincomycin was a weak inhibitor of this binding, but no other antibiotic tested exhibited significant inhibition. Clindamycin antibodies and the tritiated drug have been used in

the development of a double antibody RIA capable of detecting clindamycin concentrations of 100 ng/ml in human serum, with the possibility of achieving a 20-fold increase in sensitivity with the synthesis of labeled clindamycin of higher specific activity (247).

4. **Dapsone.** Dapsone has been diazotized and conjugated to the tyrosine, histidine and tryptophan groups of bovine serum albumin. Rabbits immunized with this conjugate formed antibodies which could be bound by a solid-phase dapsone-hemocyanin conjugate as determined by an enzyme-linked immunosorbent assay. Dapsone inhibited the binding of antibody, thus enabling the development of an assay system capable of detecting dapsone concentrations as low as 0.3 μg/ml. This assay has been used to detect dapsone in the serum and urine of leprosy patients being treated with this drug (320a).

5. **Penicillin.** Penicilloyl-protein conjugates have been synthesized. Rabbits immunized with penicilloyl-protein conjugates have formed antibodies capable of causing immune hemolysis of penicillin-coated erythrocytes in the presence of complement (718), or capable of binding an ^{125}I-bovine serum albumin conjugate (697a) or an ^{35}S-penicillin derivative (363). The ability of penicillin to inhibit these interactions between penicillin derivatives and antipenicilloyl sera has formed the basis for the development of immunoassay procedures for the detection and measurement of penicillins and their derivatives in biological fluids (363, 697, 718).

6. **Rifampin.** The rifampin derivative, 3-formylrifampicin-SV, contains a free aldehyde group which will react with the free amino groups of proteins at a mildly alkaline pH. With the use of this reaction, 3-formylrifampicin-SV has been conjugated to bovine serum albumin, with an estimated 7 to 8 residues of the rifampin derivative being incorporated into the carrier protein, as determined spectrophotometrically. Rabbits immunized with these conjugates form antibodies capable of binding rifampin-^{3}H, as determined by equilibrium dialysis. Rifampin and its derivatives are effective inhibitors of this binding, suggesting that this assay system may be useful in the future development of a rifampin RIA procedure (354).

7. **Isoniazid.** The antituberculosis drug isoniazid, or isonicotinic acid hydrazide, was coupled *via* its hydrazide group to the carboxyl groups of human serum albumin by the carbodiimide method, with the resulting conjugates containing 13 isoniazid residues per molecule of carrier, as determined by a spectrophotometric method (664). Rabbits immunized with isoniazid-albumin conjugates formed antibodies capable of binding isoniazid-^{3}H, as assessed by the ammonium sulfate precipitation method. Isoniazid and the structurally related compound, isonicotinamide, were effective inhibitors of this binding, but the two major metabolites of isoniazid in man, isonicotinic acid and acetylisoniazid, exhibited minimal cross-reactivity, thus enabling the use of these antibodies together with isoniazid-^{3}H in the development of an ammonium sulfate RIA method capable of detecting serum isoniazid concentrations as low as 50 ng/ml. This RIA method has been used to measure isoniazid concentrations in the serum of patients with tuberculosis who are being treated with this drug (598). Patients who acetylate isoniazid slowly may be more susceptible to the development of adverse reactions to this drug (617), and so the RIA method has also been used to classify isoniazid-treated patients into two groups according to the genetically predetermined rate at which they convert this drug to the inactive metabolite, acetylisoniazid (598).

8. **Sulfonamides.** Sufanilamide, sulfapyridine, sulfathiazole and sulfanilic acid contain aromatic amino groups which have enabled the conversion of these compounds to their corresponding diazonium derivatives and the subsequent conjugation of these diazo compounds to the tyrosine, histidine and tryptophan groups of carrier proteins. Rabbits immunized with

sulfonamide-protein conjugates form antibodies capable of reacting with sulfonamide-protein conjugates as determined by precipitation (705) and passive hemagglutination (577) reactions which were inhibited by the corresponding sulfonamide. These antibodies have not been employed to date in drug immunoassays.

9. Viomycin. Viomycin has been converted to N_{β}-monoacetylviomycin, which has then been treated with succinic anhydride to form the corresponding hemisuccinyl derivative. The free carboxyl group in the succinyl residue provided a means by which this hemisuccinyl derivative could be coupled to the free amino groups of bovine serum albumin by the mixed anhydride method. It was estimated that approximately 8.6 molecules of the viomycin derivative had been introduced into each molecule of albumin. Rabbits immunized with viomycin-bovine serum albumin formed antibodies capable of binding a viomycin-β-D-galactosidase conjugate, as detected by precipitation of enzymatic activity. Since the binding of the conjugate by antibody is inhibited by free viomycin, it has been possible to develop an enzyme immunoassay capable of detecting as little as 100 pg of viomycin (375).

E. Anticoagulants

1. Warfarin. The 4′-carboxyethyl analog of RS-warfarin was resolved and the enantiomeric acids were coupled to bovine serum albumin. Rabbits immunized with either of these conjugates formed antibodies which selectively bound the predicted ^{3}H-warfarin enantiomer. Cross-reactions with various warfarin metabolites were low (0.2–3%) as were cross-reactions with the opposite enantiomer (approximately 7%), enabling the development of an RIA procedure which has been used to study the pharmacokinetics of warfarin enantiomers in rats given racemic warfarin (153a).

F. Anticonvulsants

1. Carbamazepine. A commercial firm has developed an enzyme immunoassay for carbamazepine which can be used to measure its concentration in the serum of patients receiving this drug. Results obtained by this method were similar to those obtained by gas chromatography, which suggests that the two methods can be used interchangeably (648a).

2. Clonazepam. Clonazepam is a member of the 1,4-benzodiazepine class of compounds which has recently been found to be clinically effective in controlling petit mal seizures. The 3-hemisuccinyloxy derivative of clonazepam was synthesized and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method. It was estimated spectrophotometrically that the conjugate contained 35 moles of the clonazepam derivative per mole of albumin. Rabbits immunized with this conjugate formed antibodies capable of binding clonazepam-^{3}H, as determined by an ammonium sulfate precipitation technique. Clonazepam and its 3-hydroxy derivative (a minor metabolite) were effective inhibitors of this binding of clonazepam-^{3}H by antibody, while the two major known metabolites of clonazepam, the 7-amino and the 7-acetylamino derivatives, exhibited minimal cross-reactivity; similarly, certain structurally related compounds such as diazepam, nitrazepam and oxazepam displayed insignificant cross-reactivity, as did five structurally unrelated anticonvulsant drugs (189). Accordingly, these antibodies were used, initially with clonazepam-^{3}H (189), and subsequently with an ^{125}I-labeled clonazepam derivative (185b), in the development of specific and rapid ammonium sulfate and polyethylene glycol precipitation RIA methods for the measurement of clonazepam concentrations in the plasma of patients receiving this drug.

3. Diphenylhydantoin. Diphenylhydantoin (phenytoin) was allowed to react with methyl 5-bromovalerate, followed by hydrolysis of the resulting ester to yield 5,5-diphenylhydantoin-3-(ω-valeric acid), which was coupled *via* the carboxyl group in the newly introduced valeric acid side

chain to the free amino groups of bovine serum albumin by the mixed anhydride reaction. The resulting conjugate contained 29 residues of diphenylhydantoin per molecule of bovine serum albumin, as estimated from the extent of incorporation of diphenylhydantoin-^{14}C into the conjugate (154). Diphenylhydantoin-bovine serum albumin conjugates containing 32 moles of diphenylhydantoin per mole of albumin have been prepared by direct coupling, using a water-soluble carbodiimide (554). Rabbits immunized with either of these conjugates have formed antibodies capable of binding diphenylhydantoin-^{3}H, as assessed by the dextran-coated charcoal method (154, 554), or of binding diphenylhydantoin-^{14}C (522) or an ^{125}I-labeled diphenylhydantoin derivative (523), as studied by the double antibody method. Diphenylhydantoin is an effective inhibitor of this binding but 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, its major metabolite, is ineffective; structurally related hydantoins and barbiturates are likewise ineffective inhibitors of this binding (154, 522, 554). Other methods of eliciting antibodies to diphenylhydantoin have been described (522, 662), but in general are less satisfactory. For example, the reaction of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin with chloroacetic acid results in the formation of the 5-(*p*-carboxymethoxyphenyl) derivative which can be conjugated to the amino groups of proteins by the mixed anhydride reaction. Antisera produced by rabbits in response to immunization with this immunogen cannot distinguish diphenylhydantoin from its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (662).

Several immunoassays have been developed for the determination of diphenylhydantoin concentrations in serum and other body fluids. These include dextran-coated charcoal RIA methods using diphenylhydantoin-^{3}H (153, 154, 512, 554), an ammonium sulfate precipitation RIA method using diphenylhydantoin-^{14}C (662), double antibody radioimmunoassays using diphenylhydantoin-^{14}C (522) or an ^{125}I-labeled diphenylhydantoin derivative (523), a spin-label immunoassay (476, 477) and an enzyme immunoassay (62, 595, 637); the enzyme immunoassay has recently been adapted for use in the centrifugal analyzer (83, 224). Diphenylhydantoin immunoassay methods have been very useful in monitoring serum concentrations of this drug in patients with convulsive disorders.

4. Ethosuximide. A commercially available enzyme immunoassay has been used to measure concentrations of ethosuximide in the serum of patients receiving this drug. Results obtained with this immunoassay and results obtained by gas chromatography were comparable, which suggests that the two methods can be used interchangeably (648a).

5. Phenobarbital. *See* section V Z.

6. Primidone. An enzyme immunoassay for primidone has recently become available commercially. Experimental details concerning the developments of antibodies and of the immunoassay method are not available in the literature, but the antibodies are reported to be highly specific for primidone, with minimal cross-reactivity with barbiturates and other anticonvulsants. Results obtained by this immunoassay procedure on 94 serum specimens obtained from patients on primidone therapy agreed well with results obtained by a gas-liquid chromatographic assay, with a correlation coefficient of 0.98 (649).

G. Antidepressants

1. Non-tricyclic. *a. Nomifensine.* In order to develop a nomifensine RIA, N-(8-nomifensine) succinamic acid was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; acid hydrolysis of the conjugate revealed that approximately 20 moleucles of hapten had been conjugated to each molecule of albumin, as estimated by high pressure liquid chromatography of the hydrolysate. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-nomifen-

sine, as determined by a dextran-coated charcoal separation method. Nomifensine was an effective inhibitor of this binding, while its known metabolites were relatively ineffective inhibitors, enabling the development of a dextran-coated charcoal RIA method capable of detecting nomifensine concentrations of 300 pg/ml in 0.1 ml aliquots of unextracted human plasma; acid-labile nomifensine metabolites in the plasma of nomifensine-treated subjects can be quantified by determining the difference in immunoreactive nomifensine in plasma specimens from such subjects before and after acid treatment of the plasma. The nomifensine RIA method has been used in studies of the pharmacokinetics of this drug in man (294a).

2. Tricyclic. *a. Amitriptyline and nortriptyline.* To develop radioimmunoassays for amitriptyline and nortriptyline, a derivative, N-4-aminobutyl nortriptyline was synthesized and coupled *via* its amino group to the free carboxyl groups of bovine serum albumin by the carbodiimide method. Approximately 2 or 3 residues of nortriptyline were conjugated to each molecule of albumin, as estimated by the extent of incorporation of ^{14}C-nortriptyline into the conjugate. Sheep immunized with this conjugate formed antibodies capable of binding ^{14}C-nortriptyline, as determined by the dextran-coated charcoal separation method. Nortriptyline, amitriptyline, imipramine and other tricyclic antidepressants were effective inhibitors of this binding. Because of the low specific activity of the ^{14}C-nortriptyline, the dextran-coated charcoal RIA method originally developed with these antibodies was not very sensitive, being capable of detecting no less than 200 ng/ml (12). Recently, with the availability of ^{3}H-imipramine, a significant increase in the sensitivity of the RIA method has been achieved, so that concentrations of nortriptyline or of amitriptyline as low as 500 pg/ml can be measured (11). Alternatively, a bovine serum albumin-N-succinyl nortriptyline conjugate has been prepared, and rabbit antibodies to this conjugate have been shown, by the ammonium sulfate precipitation method, to be capable of binding ^{3}H-nortriptyline or ^{3}H-amitriptyline. These antibodies reacted effectively both with amitriptyline and with nortriptyline (367a). This cross-reactivity creates no problem in plasma assays of nortriptyline-treated patients. However, since nortriptyline is a major metabolite of amitriptyline, it is necessary to extract each of these compounds selectively from plasma at a different pH if one wishes to measure concentrations of both compounds in the plasma of patients treated with amitriptyline (438a).

b. Clomipramine. The 10/11-hydroxy derivative of clomipramine was allowed to react with succinic anhydride and the resulting hemisuccinate was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin, using the mixed anhydride reaction. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-10/11-dehydroclomipramine, as determined by the dextran-coated charcoal separation method. Clomipramine was an effective inhibitor of this binding and other tricyclic antidepressants were moderately effective inhibitors. However, known metabolites of clomipramine as well as other drugs commonly given with clomipramine were relatively ineffective inhibitors, thus enabling the use of clomipramine antibodies together with ^{3}H-10/11-dehydroclomipramine in the development of a dextran-coated charcoal RIA method which is capable of detecting clomipramine concentrations as low as 200 pg/ml in extracts of human plasma (546a).

c. Desmethylimipramine. To elicit antibodies specific for desmethylimipramine, the active metabolite of imipramine, diazotized *p*-aminobenzoic acid was coupled to desmethylimipramine by a diazonium linkage. The resulting desmethylimipramine derivative was coupled *via* its benzoic acid group to the free amino groups of bovine serum albumin by the carbodiimide method. Rabbits immunized with the desmethylimipramine conjugate formed anti-

bodies capable of binding ^{3}H-desmethylimipramine, as demonstrated by the ammonium sulfate precipitation method. Compounds such as imipramine and desmethylimipramine, which contain an unsubstituted dibenzylazepine ring configuration, are effective inhibitors of this binding, thus enabling the development of an ammonium sulfate RIA method which has been used to measure desmethylimipramine concentrations in extracts of rat plasma and brain tissue (635).

H. Antidiarrheal Drugs

1. Loperamide. To enable the conjugation of loperamide, 4-(4-chlorophenyl)-4-hydroxy-*N*,*N*-dimethyl-α,α-diphenyl-1-piperidinebutanamide, to protein carriers, this drug was converted to a hemisuccinyl derivative by the addition of succinic anhydride; the resulting loperamide derivative was then conjugated *via* its carboxyl group to the amino groups of bovine serum albumin by the carbodiimide method. Rabbits immunized with the resulting loperamide-albumin conjugate formed antibodies capable of binding ^{3}H-loperamide, as determined by a dextran-coated charcoal separation method. Nonradioactive loperamide was an effective inhibitor of this binding, as was fluperamide, a structurally related compound; known and suspected metabolites of loperamide were ineffective inhibitors of this binding, as were other structurally related drugs. The specificity of these antibodies for loperamide has enabled their use, together with ^{3}H-loperamide, in the development of a dextran-coated charcoal RIA method, capable of detecting loperamide concentrations as low as 100 pg/ml in 0.5 ml of human plasma. This RIA method has been used in studies of the pharmacokinetics of loperamide in man (464a).

I. Antihypertensive Drugs

1. Clonidine. The 4-hydroxy derivative of clonidine has been conjugated to bovine serum albumin *via* a diazo linkage. Rabbits immunized with this conjugate formed antibodies capable of binding ^{14}C-clonidine. Clonidine and 4-hydroxyclonidine were equally effective in inhibiting the binding of ^{14}C-clonidine by antibody. However, extraction of clonidine from plasma and tissues made possible the selective measurement of clonidine by RIA. This clonidine assay method has been used in studies of the pharmacokinetics of clonidine in rats (337).

2. Debrisoquin. A butanoic acid derivative of debrisoquin has been synthesized and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride reaction. Approximately 22 moles of the debrisoquin derivative were conjugated to each mole of albumin carrier, as estimated by the decrease in the number of free amino groups in the carrier after the conjugation procedure. Rabbits immunized with the conjugate formed antibodies capable of binding debrisoquin-^{3}H, as assessed by the ammonium sulfate precipitation method. Debrisoquin was an effective inhibitor of this binding, whereas various debrisoquin metabolites and other antihypertensive agents were ineffective inhibitors, thus permitting the use of these antibodies, together with debrisoquin-^{3}H in an ammonium sulfate RIA system capable of measuring debrisoquin concentrations as low as 1 ng/ml in 100 μl aliquots of human plasma (186).

3. Hydralazine. Rabbits and guinea pigs immunized with hydralazine-albumin conjugates, prepared by various methods, have formed antibodies to hydralazine (204, 237, 734), but the use of such antibodies in immunoassay procedures has not been described.

4. Minoxidil. To elicit antibodies specific for minoxidil, 2,4-diamino-6-piperidinopyrimidine-3-oxide, the N-4-glutaryl derivative has been prepared by addition of glutaric anhydride to minoxidil and has been conjugated to bovine serum albumin by the carbodiimide method. Alternatively, a dipiperidine derivative has been synthesized and attached to a modified

sepharose gel which, in turn, was conjugated to bovine serum albumin. Rabbits immunized with either of these conjugates formed antibodies capable of binding minoxidil-^{3}H, as determined by a double antibody precipitation method. Minoxidil was an effective inhibitor of this binding while known metabolites of minoxidil were, in general, ineffective inhibitors, thus enabling the use of minoxidil antibodies, together with minoxidil-^{3}H, in the development of a double antibody RIA method which has been used in the measurement of minoxidil concentrations in the serum of patients receiving this drug (575a).

5. Propranolol. *See* section V A.

6. Reserpine. The antihypertensive alkaloid, reserpine, has been conjugated to bovine serum albumin by two different procedures. In the first procedure, diazotized *p*-aminobenzoic acid was reacted with reserpine, and the product was then coupled *via* the carboxyl group in the benzoic acid moiety to the free amino groups of albumin, using the mixed anhydride reaction; as estimated by incorporation of ^{3}H-reserpine into the conjugate, an average of 23 haptenic molecules were conjugated to each albumin molecule. In the second method, the Mannich reaction was used to conjugate reserpine through its indolic nitrogen to the free amino groups of the protein carrier; the number of reserpine molecules coupled to each albumin molecule by this method was estimated to be 5. Rabbits immunized with each of the conjugates formed reserpine-specific antibodies, as determined by their ability to bind ^{3}H-reserpine, as demonstrated by a polyethylene glycol technique for the separation of free from bound tracer. Reserpine inhibited this binding specifically while one major reserpine metabolite, methyl reserpate, cross-reacted to a slight extent; the cross-reactivity of the other major reserpine metabolite, reserpic acid, was barely detectable. With the use of ^{3}H-reserpine and antibodies elicited by the diazo-coupled immunogen (which were higher in titer than those elicited by the Mannich reaction immunogen), a polyethylene glycol RIA for reserpine has been developed. This RIA is capable of detecting reserpine concentrations of 15 ng/ml in unextracted serum, enabling its use in pharmacokinetic studies of reserpine in rats (420).

7. Saralasin. The synthetic peptide angiotensin II analog and antagonist, (Sar^1, Ala^8)-angiotensin II (saralasin), was conjugated to bovine γ-globulin by the carbodiimide method. Rabbits immunized with saralasin-protein conjugates formed antibodies capable of binding ^{125}I-saralasin, as determined by a dextran-coated charcoal separation technique. Saralasin was an effective inhibitor of this binding, while angiotensins I, II and III were relatively ineffective, thus enabling the development of a dextran-coated charcoal RIA for the measurement of plasma saralasin concentrations (530). This RIA has been used in studies of the pharmacokinetics of saralasin in rats and man (530, 531).

J. Anti-Inflammatory Agents

1. Acetylsalicylic acid. Several methods have been employed to conjugate derivatives of acetylsalicylic acid (aspirin) to protein carriers. Acetylsalicyl (aspiryl) azide has been synthesized and conjugated to horse serum globulin at an alkaline pH (87). Aspiryl chloride has been synthesized and conjugated to ovalbumin, bovine serum albumin, bovine γ-globulin, and keyhole limpet hemocyanin by a modified Schotten-Baumann reaction (13, 309, 707, 715). Aspirin anhydride, a frequent contaminant of commercial aspirin preparations (185a), has been shown to be capable of acetylating proteins directly. Rabbits and guinea pigs immunized with these aspiryl-protein conjugates have formed aspiryl-specific antibodies, as demonstrated by positive precipitation (87, 309, 707, 715) and passive hemagglutination (13, 707, 715) reactions with heterologous aspiryl-protein conjugates, by inactivation of aspiryl-bacteriophage (13), and by the bind-

ing of a ^{14}C-aspiryl derivative, as demonstrated by equilibrium dialysis (309); in most instances, aspirin was shown to be capable of inhibiting the reaction between antibodies and aspiryl proteins. The use of antibodies in the development of an immunoassay for aspirin has not been reported to date.

2. **Antipyrine.** Succinic anhydride was coupled to the amino group of 4-aminoantipyrine, producing N-(4-antipyrinyl)-succinamic acid which was coupled *via* its free carboxyl groups to the free amino groups of bovine serum albumin with the mixed anhydride procedure. From the decrease in free amino groups available to react with fluorescamine, it was estimated that 18 moles of hapten were present in each mole of hapten-albumin conjugate. A rabbit immunized with this conjugate formed antibodies capable of binding antipyrine-^{3}H, as determined by the ammonium sulfate precipitation method. Antipyrine inhibited this binding of antipyrine-^{3}H, but its major metabolic product, 4-hydroxyantipyrine, also was an effective inhibitor. With the use of these rabbit antibodies together with antipyrine-^{3}H, an ammonium sulfate RIA method has been developed which is capable of measuring antipyrine concentrations as low as 10 ng/ml in 0.1 ml samples of human plasma or saliva. This assay method has been used in studies of the pharmacokinetics of antipyrine in man (125).

3. **Colchicine.** A colchicine derivative, N-desacetylthiocolchicine, which possesses a free amino group, has been conjugated to the free carboxyl groups of bovine serum albumin, using the carbodiimide method; spectrophotometric measurements at 375 nm indicated a ratio of 7 moles of hapten per mole of albumin carrier (66). Alternatively, colchicine has been conjugated to bovine serum albumin through the keto group on ring C, using a colchicine oxime as an intermediate (212). Rabbits immunized with either of these conjugates formed antibodies capable of specifically binding colchicine-^{3}H, as determined by the double antibody method, thus enabling the development of sensitive and specific RIA methods for the measurement of colchicine (66, 212). One of these assay methods has been used extensively in studies of the pharmacokinetics of colchicine in man (212).

4. **Corticosteroids.** *See* section V AA.

5. **Indomethacin.** Indomethacin has been conjugated *via* its carboxyl group to the free amino groups of antigenic protein carriers, utilizing either the N-hydroxysuccinimide active ester procedure (284a) or direct carbodiimide conjugation, without the preparation of an active intermediate (490); average incorporations of 17 molecules of indomethacin per molecule of bovine serum albumin (284a) and 19 drug residues per porcine thyroglobulin molecule (490) were obtained by these methods. Rabbits immunized with either of these conjugates formed antibodies capable of binding ^{14}C-indomethacin (284a) or ^{3}H-indomethacin (490). The ability of nonradioactive indomethacin to inhibit this binding has enabled the development of dextran-coated charcoal (284a) and double antibody RIA methods (490) for the measurement of indomethacin concentrations in human serum or urine. However, the specificity of indomethacin antisera tested to date indicates that certain indomethacin metabolites, notably its glucuronide conjugate, a major metabolite, are more effective inhibitors of the binding of ^{14}C-indomethacin by antibody than is indomethacin itself. Thus, the RIA method is not totally specific; however, if urine extracts are prepared for RIA before and after glucuronidase hydrolysis, concentrations of free and conjugated indomethacin can be calculated by difference (284a).

6. **Phenylbutazone.** An acid analog of phenylbutazone has been prepared and conjugated to bovine serum albumin by means of the N-hydroxysuccinimide ester. The resulting conjugate, containing 27 moles of hapten per mole of albumin, was used to immunize rabbits, which formed antibodies capable of binding phenylbuta-

zone-^{3}H. As little as 20 pg of phenylbutazone effectively inhibited this binding, while the *p*-hydroxyphenyl metabolite was a weak inhibitor. The application of these antibodies to the assay of phenylbutazone in biological fluids has not yet been reported (155).

K. Antimuscarinic Drugs

1. Atropine. To introduce a carboxyl group which would facilitate the conjugation of atropine to a protein carrier, atropine has been converted to atropine-9-O-hemisuccinate by treatment with succinic anhydride (218a); alternatively, *p*-aminobenzoic acid has been diazotized and coupled to the benzene group in the atropine molecule (725a). These atropine derivatives have been conjugated *via* their carboxyl groups to the free amino groups of bovine serum albumin by the carbodiimide method; from 15 to 21 haptenic molecules were coupled to each molecule of albumin, as estimated by the extent of incorporation of ^{3}H-atropine into the albumin-atropine conjugates. Rabbits immunized with these conjugates formed antibodies capable of binding ^{3}H-atropine, as determined by the ammonium sulfate precipitation technique. Atropine and certain structurally related antimuscarinic alkaloids were effective inhibitors of this binding (218a, 725a), but, in the case of sera elicited by the hemisuccinate derivative, atropine breakdown products were relatively ineffective inhibitors (725a). Atropine antibodies have been used, together with the ^{3}H-labeled drug, in the development of ammonium sulfate RIA methods (218a, 725a) capable of detecting atropine concentrations as low as 6 ng/ml in 10 μl specimens of unextracted plasma (725a). These RIA methods have been used in studies of atropine pharmacokinetics in mice (218a) and in dogs 725a).

L. Anti-Tumor Drugs

1. Actinomycin D. Actinomycin D has been conjugated, presumably *via* its free amino group, to bovine serum albumin by the carbodiimide method. Rabbits immunized with the albumin-actinomycin D conjugate formed antibodies capable of binding ^{3}H-actinomycin D, as determined by the double antibody precipitation method. Actinomycin D and actinomycin C_3 were equally potent inhibitors of this binding, but more extensive studies of specificity and use of actinomycin D antibodies in an RIA procedure have not yet been reported (542).

2. Adriamycin. The anthracycline antibiotic, Adriamycin (doxorubicin), has been conjugated *via* its amino group to the free carboxyl groups of human serum albumin and of keyhole limpet hemocyanin, using the carbodiimide method; Adriamycin has also been coupled *via* this amino group to the free amino groups of human serum albumin by the glutaraldehyde method. The various conjugates contained between 8 and 15 moles of Adriamycin per mole of carrier as determined spectrophotometrically. Rabbits, monkeys and a goat immunized with Adriamycin-protein conjugates formed antibodies capable of binding an ^{125}I-labeled Adriamycin derivative, as determined by the double antibody method. The ability of Adriamycin and structurally related compounds to inhibit this binding has formed the basis for the development of double antibody and nitrocellulose membrane filtration assay methods capable of detecting as little as 2 pmol of Adriamycin per ml in the unextracted plasma or urine of man and of experimental animals (676). The double antibody RIA method has been used to study the pharmacokinetics of Adriamycin in man (18) and in experimental animals (676).

Since the Adriamycin RIA detects the drug and its metabolites, this RIA method has been used in conjunction with high-pressure liquid chromatography (HPLC) to measure the concentrations of Adriamycin and its metabolites in the urine of patients receiving this drug; HPLC was used to separate Adriamycin, adriamycinol and Adriamycin aglycone(s) and the concentrations of the isolated compounds in HPLC fractions were determined by

RIA. This combined HPLC-RIA system appears to offer an efficient and sensitive assay procedure which is not subject to the interference from nonrelated endogenous substances which may affect results obtained with other methods (409).

3. **1-β-D-Arabinofuranosylcytosine.** The 5'-hydroxyl group of 1-β-D-arabinofuranosylcytosine (ara-C) was selectively acylated with 2,2,2-trichloroethyl succinyl chloride yielding the 2,2,2-trichloroethyl hemisuccinate. The addition of glacial acetic acid converted the trichloroethyl succinyl ara-C to succinyl ara-C which was coupled *via* the succinyl carboxyl group to the amino groups of human serum albumin by the carbodiimide method. The resulting conjugate contained approximately 8 residues of ara-C per molecule of albumin. Rabbits immunized with ara-C-albumin conjugates formed antibodies capable of binding ^{3}H-ara-C as determined by a Millipore membrane technique. The ability of ara-C to inhibit this binding has enabled the development of a Millipore membrane RIA method capable of detecting 20 ng of ara-C per ml of plasma. This assay method is practically free from interference by cytidine, deoxycytidine, 1-β-D-arabinofuranosyluracil (an ara-C metabolite), other nucleosides and various antibiotics (506a).

4. **1-β-D-Arabinofuranosyluracil.** At mildly alkaline pH, 1-β-D-arabinofuranosyluracil (ara-U) shows marked pH-dependent cross-reactivity with antibodies directed toward 1-β-D-arabinofuranosylcytosine (ara-C). It is felt that this ability of ara-U to inhibit the binding of ^{3}H-ara-C by ara-C antibodies, which is not observed at pH 6.2, may be due to base-catalyzed tautomerism of ara-U to its enolic form, thereby rendering it more similar structurally to ara-C. By performing RIA determinations at both pH 6.2 and 8.6, ara-C and ara-U could be determined simultaneously in mouse plasma. (506)

With the use of methods described previously in the synthesis of albumin-ara-C conjugates (506a), the 5'-hydroxyl group of ara-U was selectively acylated with 2,2,2-trichloroethyl succinyl chloride to form the 2,2,2,-trichloroethyl hemisuccinate which was, in turn, converted to the succinate by treatment with glacial acetic acid in the presence of zinc dust. Succinyl ara-U was conjugated *via* its carboxyl group to the free amino groups of human serum albumin by the carbodiimide method; the resulting conjugate contained about 4.7 ara-U residues per molecule of albumin, as estimated spectrophotometrically. Rabbits immunized with albumin-ara-U conjugates formed antibodies capable of binding ^{3}H-ara-U as determined by a Millipore membrane assay method. Ara-U was an effective inhibitor of this binding and two of four antisera studied in detail exhibited sufficiently high specificity for ara-U to permit their use in the development of a sensitive and reliable RIA method, which has been used in the determination of ara-U concentrations in the plasma (506b) of mice treated with ara-C.

5. **Bleomycin.** Bleomycin has been conjugated to bovine and human serum albumins by the carbodiimide method (75, 205). The molar ratio of bleomycin to albumin in one such conjugate was estimated spectrophotometrically to be approximately 28:1 (75). Rabbits immunized with bleomycin-albumin conjugates formed antibodies capable of binding ^{125}I-bleomycin, as demonstrated by the dextran-coated charcoal technique (75) or of binding ^{57}Co-labeled bleomycin, as demonstrated by the polyethylene glycol separation method (205). Bleomycin was capable of inhibiting this binding, while other antineoplastic drugs were ineffective inhibitors (75, 176, 205), thus enabling the development of sensitive and specific RIA methods capable of measuring bleomycin concentrations as low as 250 pg/ml (75, 205). The bleomycin RIA method has been used to study the pharmacokinetics of bleomycin in experimental animals (656) and in man (175, 656).

6. **Daunorubicin.** The anthracycline antibiotic, daunorubicin, differs structur-

ally from Adriamycin only in that it lacks the hydroxyl group present in Adriamycin at the carbon-14 position. Because of this structural similarity, daunorubicin is almost as effective as Adriamycin in inhibiting the binding of an ^{125}I-labeled Adriamycin derivative by anti-Adriamycin sera. Accordingly, the ^{125}I-labeled Adriamycin derivative and anti-Adriamycin sera have been used in the development of a double antibody RIA procedure capable of detecting daunorubicin concentrations as low as 2 pmol/ml in unextracted plasma or urine (18, 676).

7. **Macromycin**. The polypeptide antitumor agent, macromycin, has been conjugated to hemocyanine by the carbodiimide reaction, with the resulting derivative containing approximately 4 moles of drug per mole of hemocyanin, as estimated by the extent of incorporation of ^{125}I-labeled macromycin into the conjugate. Rabbits immunized with this conjugate formed antibodies capable of binding ^{125}I-labeled macromycin, as determined by the dextran-coated charcoal separation method. Macromycin was an effective inhibitor of this binding whereas other antitumor drugs such as bleomycin and actinomycin D exhibited minimal cross-reactivity, thus enabling the development of a dextran-coated charcoal RIA method capable of detecting as little as 100 pg of macromycin (722).

8. **Methotrexate**. With the carbodiimide method, the folic acid antagonist, methotrexate, has been coupled via its carboxyl group to synthetic polypeptides (338, 435), hemocyanin (418), methylated bovine serum albumin (60, 292, 542a), and human serum albumin (435). Rabbits and a goat immunized with methotrexate-polypeptide or methotrexate-protein conjugates have formed antibodies capable of binding ^{3}H-methotrexate as demonstrated by the double antibody method (60, 418). Methotrexate is an effective inhibitor of this binding of ^{3}H-methotrexate by antibody while folic acid and folinic acid are relatively ineffective inhibitors, thus enabling the use of ^{3}H-methotrexate and methotrexate-specific antibodies in the development of double antibody (60, 418), nitrocellulose membrane (542a), polyethylene glycol (292) and dextran-coated charcoal (12a, 435) RIA methods for the measurement of methotrexate in biological fluids.

9. **L-Phenylalanine mustard**. L-Phenylalanine mustard, when added to human γ-globulin at pH 6.6, alkylates free amino groups in the protein, thereby forming a protein-L-phenylalanine mustard conjugate. Rabbits immunized with this conjugate formed antibodies capable of precipitating with a bovine serum albumin-L-phenylalanine mustard conjugate, but inhibition studies with L-phenylalanine mustard or its derivatives were not described (85). The use of antibodies to L-phenylalanine mustard in the development of an immunoassay for this drug has not yet been described.

10. **Vinca alkaloids**. The Vinca alkaloid, vinblastine, was conjugated *via* its indole group to the free amino groups of bovine serum albumin in the presence of formaldehyde by the Mannich reaction. The resulting conjugates contained between 8 and 11 moles of vinblastine per mole of albumin carrier, as estimated spectrophotometrically. Rabbits immunized with vinblastine-albumin conjugates formed antibodies capable of binding ^{3}H-vinblastine, as demonstrated by the dextran-coated charcoal separation method. Vinblastine and the structurally related Vinca alkaloid, vincristine, were effective inhibitors of this binding, while the other alkaloids and cytotoxic agents tested exhibited no cross-reactivity. Since little is known about the metabolic derivatives of Vinca alkaloids, their cross-reactivity with antivinblastine serum could not be assessed. Nevertheless, the ability of unlabeled vinblastine and vincristine, respectively, to inhibit the binding of ^{3}H-vinblastine by antibody has enabled the development of a dextran-coated charcoal RIA system capable of detecting less than

5 ng of immunoreactive Vinca alkaloid per ml in the plasma of patients receiving either vinblastine or vincristine (655).

M. Anti-Ulcer Drugs

1. **Carbenoxolone.** Carbenoxolone has been conjugated *via* its carboxyl groups to the free amino groups of bovine serum albumin by the carbodiimide method. Approximately 10 molecules of carbenoxolone were coupled to each molecule of albumin, as estimated spectrophotometrically. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-carbenoxolone, as determined by a charcoal separation method. Carbenoxolone and certain closely related derivatives were effective inhibitors of the binding of ^{3}H-carbenoxolone by antibody, thus enabling the development of a charcoal RIA method capable of detecting carbenoxolone concentrations as low as 1 ng/ml (526).

N. Bronchodilators

1. **Theophylline.** Two methods have been described for the experimental production of antibodies to theophylline. The 8-(3-carboxypropyl) derivative of theophylline (1,3-dimethylxanthine) has been conjugated to bovine serum albumin, 30 to 40 theophylline residues being coupled to each albumin molecule, as estimated spectrophotometrically (159). Alternatively 8-carboxytheophylline has been synthesized and conjugated to methylated bovine serum albumin by the carbodiimide method (488). Rabbits immunized with these conjugates formed antibodies which bound ^{3}H-theophylline as determined by ammonium sulfate precipitation methods. Theophylline was an effective inhibitor of this binding; caffeine (1,3,7-trimethylxanthine) cross-reacted weakly and other xanthines exhibited little or no cross-reactivity with antitheophylline serum. Antitheophylline serum has been used together with ^{3}H-theophylline to develop RIA methods for the measurement of theophylline in biological fluids. These RIA methods have been used to measure theophylline concentrations in the plasma of adult patients (159) and premature infants (488) receiving this drug.

O. Cardiac Glycosides

1. **Acetylstrophanthidin.** Acetylstrophanthidin is not a glycoside, but represents the synthetic C-3 acetyl ester of the aglycone, strophanthidin. Strophanthidin is structurally similar to ouabagenin, the aglycone of the cardiac glycoside, ouabain, which is also derived from *Strophanthus* seeds, and hence acetylstrophanthidin is a very effective inhibitor of the binding of ouabain-^{3}H by antiouabain sera (620). Accordingly, ouabain antisera have been used, together with ouabain-^{3}H, in the development of a dextran-coated charcoal RIA method capable of detecting as little as 100 pg of acetylstrophanthidin in 1 ml of unextracted plasma, urine or bile. This method has been used to study the pharmacokinetics of acetylstrophanthidin in dogs and in human subjects (599).

2. **Digitoxin.** To elicit antibodies for the cardiac glycoside, digitoxin, its aglycone, digitoxigenin, was allowed to react with succinic anhydride to yield the reaction product, 3-O-succinyl digitoxigenin. This latter derivative was then conjugated *via* its carboxyl group to the free amino groups of human or bovine serum albumin, using either the carbodiimide or the mixed anhydride coupling method, yielding conjugates which contained between 5 and 13 digitoxigenin residues per molecule of albumin. Rabbits immunized with these conjugates formed antibodies capable of binding ^{125}I-labeled succinyl digitoxigenin tyrosine methyl ester, as demonstrated by a double antibody method. Nonradioactive digitoxin was an effective inhibitor of this binding. A closely related glycoside, digoxin, or 12-hydroxydigitoxin, is about one-tenth as effective an inhibitor as digitoxin, while steroid hormones are ineffective inhibitors at physiological concentrations. Thus, the ability of nonradioactive digitoxin to inhibit the binding of the ^{125}I-labeled digitoxigenin derivative by digi-

toxigenin-specific antibodies has enabled the use of these antibodies, together with the radioiodinated digitoxigenin derivative, in the development of a sensitive and specific double antibody RIA method capable of detecting as little as 1 ng of digitoxin per ml in extracts of human serum (507). Since digoxin is structurally so similar to digitoxin, antibodies to digoxin bind digitoxin-^{3}H with high affinity and have been used together with digitoxin-^{3}H in the development of a rapid dextran-coated charcoal RIA method capable of detecting as little as 2 ng of digitoxin per ml of unextracted human serum (618). A solid phase RIA method employing an ^{125}I-labeled digitoxigenin derivative and digitoxin-specific antibodies coupled to sepharose has also been described (59). Digitoxin RIA methods detect digitoxin metabolites in addition to detecting digitoxin (281, 645), but, nonetheless, measurements of serum concentrations of immunoreactive digitoxin have been found to be of value in determining optimal digitoxin dosage schedules for adult patients receiving this drug (35, 53, 89, 124, 133, 194, 223, 414, 507, 528, 618, 619, 621). Similar studies with a serum digitoxin RIA in infants and children have provided evidence that infants and children tolerate higher serum digitoxin concentrations than do adults without developing toxic manifestations and that infants and children may require more digitoxin on a milligrams per kilogram basis than do adults for a therapeutic effect (246). Digitoxin RIA methods have also been used in studies of digitoxin bioavailability (645) and of the effects of rifampicin on digitoxin metabolism in man (529), as well as in studies of digitoxin pharmacokinetics in dogs (51).

3. **Digoxin.** Digoxin, the most widely used cardiac glycoside in the United States, consists of an aglycone, digoxigenin, and 3 glycosidic digitoxose residues linked to the aglycone at the C-3 position. The terminal digitoxose residue contains vicinal hydroxyl groups which, following oxidation with sodium periodate, yield a dialdehyde derivative of digoxin which can be coupled to the free amino groups of bovine or human serum albumin; approximately 2 to 7 molecules of digoxin are ordinarily conjugated to each molecule of albumin, as estimated spectrophotometrically. Rabbits immunized with these conjugates have formed antibodies capable of binding digoxin-^{3}H, as determined by equilibrium dialysis and by the dextran-coated charcoal separation method (94, 623); the specificity and affinity of these antibodies for digoxin were found to be greater after several months of immunization than after a few weeks of immunization (623, 625).

The specificity of digoxin antisera has been studied extensively. Antidigoxin sera are capable of binding cortisone-^{3}H hydrocortisone-^{3}H and dehydroepiandrosterone-^{3}H, as demonstrated by equilibrium dialysis (94). However, in comparison with digoxin, much higher concentrations of these and other endogenous steroids of man are required to produce significant inhibition of the binding of radiolabeled digoxin by antidigoxin sera (94, 623, 625). If antisera of sufficient specificity are selected for use in the development of digoxin RIA methods, the concentrations of endogenous steroids encountered in human serum will not interfere significantly (622, 625). However, if antidigoxin sera with significant cross-reactivity are employed, endogenous steroids (625) and prednisone (736) may interfere with digoxin RIA procedures. Similarly, the aldosterone antagonist, spironolactone, may also interfere with the binding of radiolabeled digoxin by some antidigoxin sera (532, 736), but the presence of this drug in clinical serum samples should not constitute a problem if antisera of proper specificity are employed (316, 545).

Metabolic derivatives of digoxin, or digoxigenin tridigitoxoside, cross-react with different digoxin antibodies to varying degrees. Since the immunogenic digoxin-protein conjugate contains an intact digoxi-

genin moiety, most antidigoxin sera cross-react extensively with digoxin derivatives which contain the intact aglycone. Digoxigenin and its mono- and bisdigitoxosides are therefore highly immunoreactive in most digoxin RIA systems (36, 228, 396, 411, 447, 646). Since these three metabolic derivatives are cardioactive as well as immunoreactive, the presence of significant quantities of these compounds in serum may not constitute a practical disadvantage if measurements of immunoreactive digoxin provide an accurate clinical reflection of pharmacologically active digoxin derivatives present in a patient's serum (89). Other digoxin metabolites which lack significant pharmacological activity, such as dihydrodigoxin, may also inhibit the binding of radiolabeled digoxin by antibody (387), but the presence of such cardioinactive metabolites should not constitute a significant problem if antibodies of appropriate specificity are employed (95, 396).

Other cardiac glycosides containing digoxigenin, such as deslanoside, lanatoside C, α-acetyldigoxin, β-acetyldigoxin and β-methyldigoxin are very effective inhibitors of the binding of radiolabeled digoxin by antidigoxin sera (17, 227, 287, 343, 364, 623) to an extent that digoxin RIA systems can be used to measure serum and urinary concentrations of these cardiac glycosides in individuals who are being treated with one of these medications, but who are not also receiving digoxin (52, 54, 56, 190, 227, 274, 287, 343, 364, 448, 552).

Digitoxin differs structurally from digoxin only in that it lacks the C-12 hydroxyl group present in digoxin. It is therefore often 20 to 30 times less effective than digoxin in inhibiting the binding of radiolabeled digoxin by antidigoxin sera. This cross-reactivity will not constitute a problem in serum digoxin measurements if it is known with certainty that a patient has received no cardiac glycoside other than digoxin. However, serum digitoxin concentrations in patients receiving digitoxin or digitalis leaf are ordinarily much higher than serum digoxin concentrations; therefore, if a serum digoxin measurement is erroneously performed on such a patient, a meaningless serum digoxin level will be reported to the physician. Accordingly, when a serum digoxin measurement is performed, it is essential that the patient be receiving only digoxin (89).

The affinity of digoxin antibodies for digitoxin, while less than their affinity for digoxin, is sufficiently great to allow their use together with digitoxin-^{3}H in the development of digitoxin RIA procedures (618). Similarly, antibodies to digoxin have been employed, together with radiolabeled digitoxin, in the development of RIA methods for the measurement of two other digitalis glycosides, gitaloxin and gitoxin (413).

The average intrinsic affinity constants of antidigoxin sera are often in the range of 10^{9} to 10^{10} M^{-1}, particularly after several months of immunization. Affinity constants may, however, be lower during the early weeks of immunization. If antiserum of relatively low affinity is used in a dextran-coated charcoal RIA procedure, extensive dissociation of digoxin from antibody accompanied by adsorption to charcoal may occur during incubation with dextran-coated charcoal; as much as 60% of the bound digoxin may dissociate within 15 minutes. Since the extent of such dissociation is related to the duration of exposure to dextran-coated charcoal, the apparent percentages of radiolabeled digoxin bound in various individual tubes will vary significantly if there is a slight tube-to-tube variability in the duration of the charcoal incubation step; such variability in bound radiolabeled digoxin may cause significant errors in assay results (460, 625). If antiserum of high affinity is used, however, the rate of dissociation is relatively slow and does not constitute a significant source of error in the RIA procedure (625, 626).

The original digoxin RIA method employed digoxin-^{3}H and the dextran-coated charcoal separation method (622). Subsequently, digoxin RIA procedures have been described which employ ^{125}I-labeled

digoxin derivatives (118, 123, 193, 265, 271, 313, 396, 533, 653) and a variety of methods for the separation of antibody-bound from free radiolabeled digoxin, including Somogyi precipitation (460), ammonium sulfate precipitation (193), polyethylene glycol precipitation (26, 199, 608), gel equilibration (265), double antibody separation (199, 396), Sephadex separation (135), membrane filtration (244) and several solid-phase RIA methods (17, 58, 386, 431, 735). Recently, use of an enzyme immunoassay for digoxin has been reported (569, 648).

Kits for the measurement of digoxin by RIA have been marketed by many manufacturers and comparison of results obtained using various kits have appeared in the literature (28, 36, 86, 284, 391–394, 440, 486, 487, 592, 602, 688). Some inaccuracies have been encountered by these and other (82, 311, 515) authors when commercial kits are employed. However, if antisera of high affinity and specificity are provided (625) and if proper experimental technique is employed (310, 602, 607), reliable and reproducible results can be obtained with commercial RIA kits.

Over the past decade, considerable clinical experience has been obtained with radioimmunological measurements of serum or plasma digoxin concentrations in adult patients with and without clinical evidence of digoxin toxicity. Although most toxic patients have serum digoxin levels in excess of 2 ng/ml, a significant overlap exists between serum digoxin concentrations in patients with and without clinical evidence of toxicity. Thus, an elevated serum digoxin level alone can never be used to make a diagnosis of digoxin intoxication. However, a knowledge of the serum digoxin concentration, when considered together with other clinical and laboratory parameters, is often of great value to physicians in determining optimal digoxin dosage schedules for patients requiring this drug who: 1) are suspected, on clinical grounds to be digoxin-toxic; 2) are at high risk for the development of digoxin intoxication, due to impaired renal function; 3) are unable to give a reliable history of recent digoxin ingestion; or, 4) respond poorly to a given dose of digoxin on the basis of impaired absorption or accelerated metabolic degradation of the drug (35, 53, 89, 95, 97, 108, 120–122, 130, 191, 194, 213, 214, 231, 252, 273, 307, 315, 319, 332, 351, 356, 414, 508, 511, 537, 553, 568, 588, 615, 619, 621, 622, 624, 625, 714, 736).

A major goal in the development of the digoxin RIA was the safe and more effective use of this digitalis glycoside. In this connection it should be noted that, although the compliance of some patients with the instructions of their physicians regarding digoxin administration has been found by radioimmunological serum digoxin measurements to be suboptimal (38, 609, 708), the finding of a low serum digoxin level has enabled physicians to identify and improve the compliance of patients who have previously failed to take their digoxin regularly (38). Another study has provided evidence which suggests that the use of the serum digoxin RIA for adjustment of digoxin dosage reduces the frequency of digoxin intoxication in digoxin-treated patients (197, 381).

Infants in the first year of life are often given digoxin doses up to 3 times as great as doses given to adults, on a milligrams per kilogram basis (260, 289, 489, 580) and several studies have revealed that serum digoxin concentrations in infants without clinical evidence of digoxin intoxication are significantly higher than in older children and adults. These studies provide strong evidence that digoxin-treated infants with cardiac disease are more resistant to the development of toxic arrhythmias than are digoxin-treated adults with cardiac disease (289, 328, 390, 403, 456, 510, 567, 584, 710, 712, 721).

In addition to their widespread clinical use in the monitoring of serum digoxin levels, digoxin RIA methods have been used extensively in investigative studies, both in man and in experimental animals. Digoxin RIA methods have been employed in studies of the bioavailability (54, 216, 235, 262, 263, 318, 342, 344, 365, 427, 429,

430, 443, 446, 457, 497, 500, 538, 549, 603–606, 689, 738), pharmacokinetics (50, 70, 260, 305, 360, 384, 388, 496, 641) and salivary excretion (317) of digoxin in man. Digoxin RIA methods have also been used to study the intestinal absorption of digoxin (14, 68, 290, 320, 341, 359, 482, 501, 505, 661, 711, 713) as well as the interference, by other drugs, with digoxin absorption in man (80, 358, 428, 686). Digoxin RIA methods have been used to study the effects of thyroid disease (178), altered renal function (33, 36, 79, 223, 239, 269, 280, 331, 377, 385, 455, 640, 650, 690), maintenance hemodialysis (329) and cardiopulmonary bypass (107, 148, 389, 481) on serum levels and urinary excretion of digoxin in man. In other studies, serum immunoreactive digoxin concentrations have been correlated with the acetylstrophanthidin tolerance of digoxin-treated patients (378) and with the effects of digoxin on echocardiographically assessed left ventricular performance (163), systolic time intervals (103, 306) and several electrocardiographic parameters of digoxin action (355, 357). Digoxin RIA methods have been used to determine concentrations of digoxin in the myocardium and other tissues obtained surgically or at autopsy from digoxin-treated individuals (104, 147, 149, 150, 259, 270, 286, 361, 366, 372, 587) or from patients who ingested toxic amounts of digoxin with suicidal intent (330).

The serum digoxin RIA method has also been used to measure digoxin concentrations in the serum, urine and tissues of experimental animals (25, 69, 126, 215, 233, 245, 684) and in crude extracts of *Digitalis* leaf (706).

4. Gitaloxin. Gitaloxin, 16-formylgitoxin, is a glycoside derived from *Digitalis* leaf. The ability of gitaloxin to inhibit the binding of digitoxin-^{3}H by antidigoxin sera has formed the basis for a dextran-coated charcoal RIA of gitaloxin in human plasma (413).

Recently, the aglycone of gitaloxin, gitaloxigenin, has been combined with succinic anhydride to produce gitaloxigenin hemisuccinate; this gitaloxigenin derivative has been coupled *via* its carboxyl group to the free amino groups of human serum albumin, using the carbodiimide method. Rabbits immunized with this conjugate have formed antibodies capable of binding digitoxin-^{3}H, as determined by the dextran-coated charcoal separation method. Gitoxin was a more effective inhibitor of this binding than was gitaloxin, suggesting that hydrolysis of the formyl group may have occurred soon after the injection of the conjugate into the rabbit. Thus, although the antibodies elicited in response to the gitaloxigenin-protein conjugate must be considered as being of the antigitoxin, rather than antigitaloxin, type, preliminary studies suggest that these antibodies can be used together with digitoxin-^{3}H in the radioimmunological measurement of gitaloxin after a prior deformylation step (415).

5. Gitoxin. Like gitaloxin, gitoxin is also a glycoside derived from *Digitalis* leaf. It is more effective than gitaloxin in inhibiting the binding of digitoxin-^{3}H by antidigoxin sera (413) or by antibodies elicited in response to immunization with a gitaloxigenin-protein conjugate (415). The ability of gitoxin to inhibit such binding has enabled the immunological measurement of gitoxin in human plasma by a dextran-coated charcoal assay method (413, 415).

6. Lanatoside C. Lanatoside C is a cardiac glycoside derived from the leaves of *Digitalis lanata* and it differs from digoxin only in that its terminal digitoxose contains additional glucose and acetyl moieties. Since its aglycone is digoxigenin, it is a very effective inhibitor of the binding of radiolabeled digoxin by antidigoxin sera thus enabling its measurement in a dextran-coated charcoal RIA system, employing digoxin antisera and radiolabeled digoxin. This RIA method has been used to measure concentrations of lanatoside C in the serum of patients being treated with this drug (54, 364).

7. β-Methyldigoxin. β-Methyldigoxin

differs from digoxin only in that it contains an additional methyl group in the terminal digitoxose residue. It is an effective inhibitor of the binding of radiolabeled digoxin by antidigoxin sera, thus enabling its measurement in RIA systems using radiolabeled digoxin and antidigoxin antibodies. β-Methyldigoxin RIA methods have been used to measure serum and urine concentrations of this drug in pharmacokinetic studies in human subjects (52, 56, 274, 287, 343, 448, 552).

8. **Ouabain.** Ouabain has been conjugated *via* its glycosidic rhamnose residue to the free amino groups of poly (DL-alanyl)-human serum albumin by the periodate oxidation method. Rabbits immunized with this conjugate formed antibodies which were capable of binding ouabain-^{3}H as determined by the dextran-coated charcoal separation method (616, 620). Ouabain and a structurally related compound, acetylstrophanthidin, were very effective inhibitors of the binding of ouabain-^{3}H, while digoxin and digitoxin were only 1/15 as effective as ouabain in inhibiting this binding. Endogenous steroids did not cross-react to a measurable extent even when present in concentrations more than 1000-fold in excess of inhibitory ouabain concentrations (620). The specificity of ouabain antibodies has enabled their use, together with ouabain-^{3}H, in the development of a sensitive and specific RIA method capable of detecting less than 100 pg per ml of plasma or urine. This RIA method has been used in studies of the myocardial uptake of ouabain in man (600) and in studies of the pharmacokinetics of ouabain in dogs and in man (601). Ouabain antibodies have also been used together with ouabain-^{3}H, in the development of an RIA method for the study of the pharmacokinetics of acetylstrophanthidin (599).

9. **Proscillaridin.** Proscillaridin, a squill glycoside, has been conjugated *via* its rhamnose moiety, to the free amino groups of human serum albumin by the periodate oxidation method. Rabbits immunized with this conjugate formed antibodies capable of binding proscillaridin-^{3}H, as demonstrated by the dextran-coated charcoal separation method. Proscillaridin and certain related derivatives were effective inhibitors of this binding while more distantly related cardiac glycosides, such as digoxin, digitoxin and ouabain were very weak inhibitors; cholesterol and testosterone exhibited no inhibition at the highest concentrations tested. Thus, proscillaridin-^{3}H and antiproscillaridin sera can be used to measure nonradioactive proscillaridin. Unfortunately, the sensitivity of the presently available RIA system (15 ng/ml) is not high enough to determine plasma proscillaridin levels in man without a prior extraction step; radiolabeled proscillaridin of higher specific activity will be required to increase the sensitivity of this RIA method (37).

P. Central Nervous System Stimulants and Hallucinogens

1. **Amphetamine.** To develop an immunoassay for amphetamine, a derivative of methamphetamine was synthesized by the addition of N-(4-bromobutyl)phthalimide to methamphetamine. The derivative, N-(4-aminobutyl)methamphetamine, was conjugated *via* its amino group to the free carboxyl groups of bovine serum albumin by the carbodiimide. Rabbits immunized with this conjugate formed antibodies which bound ^{3}H-amphetamine, as determined by an ammonium sulfate precipitation method. Amphetamine and methamphetamine were effective inhibitors of the binding of ^{3}H-amphetamine by antibody; mephenterine and norephedrine were moderately effective inhibitors, whereas the inhibition produced by methylbenzylamine, tyramine and isoproterenol was minimal (131). The specificity of antibodies elicited by this approach has been extensively characterized, enabling the development of an RIA method in which canine plasma and tissue amphetamine concentrations are determined by the ability of plasma or of tissue homogenates to

inhibit the binding of ^{3}H-amphetamine by antibody, as determined by an equilibrium dialysis method (217). Recently, an ammonium sulfate RIA method employing an ^{125}I-labeled amphetamine derivative has been described (65) as has an enzyme-linked immunoassay for amphetamine (65, 67, 483, 596).

2. Caffeine. A caffeine derivative, 7-(5-carboxypentyl)-1,3-dimethylxanthine was synthesized and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method, an estimated 24 residues of caffeine being incorporated into each molecule of conjugate, as determined by differential spectrophotometry. Rabbits immunized with caffeine-albumin conjugates formed antibodies capable of binding an ^{3}H-labeled 7-propyl analog of caffeine, as determined by a dextran-coated charcoal separation method. Caffeine was an effective inhibitor of the binding of the ^{3}H-caffeine derivative; theophylline was a weak inhibitor and the inhibition of binding caused by uric acid and other xanthine derivatives was negligible. The ability of caffeine to inhibit the binding of the ^{3}H-labeled derivative by antibody has enabled the development of a dextran-coated charcoal RIA method which has been used in pharmacokinetic studies of caffeine concentrations in the plasma and saliva of human subjects (156).

3. Cocaine. *See* section V S.

4. 2,5-Dimethoxy-4-methylamphetamine. The hallucinogen, 2-5-dimethoxy-4-methylamphetamine (DOM), has been conjugated *via* its methylamino group to the free amino groups of rabbit serum albumin by the glutaraldehyde method, 14 residues of hapten having been coupled to each molecule of albumin, as estimated spectrophotometrically. Rabbits immunized with this conjugate formed hapten-specific antibodies which reacted with the DOM-rabbit albumin conjugate as detected by a complement fixation reaction which was inhibited by DOM, but not by amphetamine or by 3,4-dimethoxyphenylethylamine (673). Guinea pigs immunized with a DOM-human serum albumin conjugate prepared in a similar manner formed antibodies capable of binding ^{3}H-DOM, as determined by an ammonium sulfate precipitation method, thus enabling the development of an RIA method for the measurement of serum concentrations of DOM (371). An ^{125}I-labeled derivative of DOM has been synthesized and used, together with DOM-specific antibodies, in the development of a double antibody RIA method capable of detecting as little as 2 ng of DOM (551).

5. Lysergic acid diethylamide. To elicit antibodies to lysergic acid diethylamide (LSD), lysergic acid has been coupled by its carboxyl groups to the free amino groups of poly (L-lysine), using the carbodiimide method. The extent of substitution in the resulting conjugate, as estimated from spectrophotometric measurements, was approximately 1 molecule of lysergic acid per 6 to 10 molecules of lysine. An electrostatic complex of lysergic acid-polylysine with succinylated hemocyanin was used to immunize rabbits and guinea pigs. Antisera from these animals fixed complement in the presence of lysergic acid-polylysine, but not in the presence of polylysine alone (674). Antisera from rabbits immunized with conjugates prepared in a similar manner bound ^{3}H-LSD as determined by equilibrium dialysis and by an ammonium sulfate precipitation method (438); antisera from rabbits immunized with lysergic acid-human serum albumin conjugates prepared by the carbodiimide method also bound ^{3}H-LSD as demonstrated by the dextran-coated charcoal separation technique (436). Antisera from animals immunized with lysergic acid-polylysine conjugates complexed with succinylated hemocyanin were shown to be capable of binding an ^{125}I-labeled lysergic acid-polyamino acid conjugate, as detected by the double antibody method; D-lysergic acid, LSD and four ergot alkaloids were

effective inhibitors. Serotonin and several other compounds whose structures differ appreciably from LSD were also strong inhibitors of the binding of the ^{125}I-derivative by antibody, thus limiting the use of antibodies to lysergic acid in the development of a specific RIA for LSD (674).

LSD itself has been conjugated through its indole nitrogen to the amino groups of human and bovine serum albumins by the Mannich reaction, 10 to 40 LSD residues being coupled to each molecule of albumin, as estimated spectrophotometrically. Rabbits immunized with these conjugates formed antibodies which bound an ^{125}I-labeled LSD-polyamino acid conjugate, as demonstrated by the double antibody method (654), or which bound ^{3}H-LSD, as demonstrated by the dextran-coated charcoal (109) and double antibody (544) methods. Antibodies elicited by LSD-protein conjugates prepared by the Mannich reaction exhibited much greater specificity for LSD, as demonstrated by hapten inhibition experiments, than did the previously elicited antibodies to lysergic acid. The specificity of these antibodies has enabled their use in the development of double antibody (544, 654) and dextran-coated charcoal (109) RIA methods for the measurement of picogram quantities of LSD in the serum and urine of users of this drug.

Recently, a conjugate has been prepared in which a four-carbon "spacer" chain has been substituted for an ethyl group in LSD and has been used to couple LSD to bovine serum albumin. A sheep immunized with this lysergic acid N-ethyl-N-(5-carboxyamyl)amide-bovine serum albumin conjugate (which contained between 12 and 15 hapten molecules per molecule of albumin, as estimated by ultraviolet absorption and spectrofluorometry) formed antibodies capable of binding ^{3}H-LSD, as determined by the double antibody method. These antibodies have been used, together with ^{3}H-LSD, in the development of a sensitive double antibody RIA method for the measurement of LSD in human serum and urine. This RIA method exhibits excellent specificity for LSD and was not as significantly affected by immunoreactive LSD metabolites as was a similar RIA method employing antibodies elicited by an LSD-albumin conjugate prepared by Mannich condensation (544).

6. Mescaline. Mescaline (3,4,5-trimethoxyphenylethylamine) has been conjugated to the free carboxyl groups of polyglutamic acid by the carbodiimide method, with incorporation of about 3 or 4 hapten residues per 10 residues of glutamic acid, as estimated spectrophotometrically. Rabbits were immunized with electrostatic complexes formed between these conjugates and methylated bovine serum albumin. After absorption of antibodies to polyglutamic acid, antisera from these rabbits reacted with mescaline-polyglutamic acid conjugates, as detected by complement fixation. Mescaline and its analogs with methoxy groups on positions 3,4 and 5 on the benzene ring were effective inhibitors of this complement fixation reaction, but removal of one methoxy group from position 4 or 5 reduced the inhibitory effectiveness at least 50-fold (673). Mescaline antibodies of similar specificity have been raised in rabbits immunized with mescaline-human serum albumin or N-succinylmescaline-human serum albumin conjugates prepared by the carbodiimide method. These antibodies have been used, together with an ^{125}I-labeled mescaline derivative, in the development of a double antibody RIA method which is capable of detecting as little as 100 pg of mescaline and which has been used in studies of mescaline metabolism in rabbits (551).

7. Nicotine. *See* section V Q.

8. Pimozide. An acetic acid homolog of the neuroleptic drug, pimozide, was prepared and conjugated to bovine serum albumin by the carbodiimide method; the degree of hapten substitution was estimated to be 2 to 3 moles of pimozide per mole of protein, as measured by incorporation of ^{3}H-labeled pimozide homolog into

the conjugate. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-pimozide, as determined by a dextran-coated charcoal separation method. Pimozide and related neuroleptic drugs of the diphenylbutylamine series were effective inhibitors of this binding, but the major metabolites of pimozide were very ineffective inhibitors. Although this RIA method has not yet been applied to the measurement of pimozide in biological fluids, this assay is theoretically capable of detecting as little as 50 pg of pimozide (464).

9. Tetrahydrocannabinol. Tetrahydrocannabinol (THC), the main pharmacologically active constituent of cannabis, has been conjugated to protein carriers by three different methods. THC has been coupled with diazotized *p*-aminobenzoic acid and the product has been coupled *via* its benzoyl carboxyl group to the free amino groups of keyhole limpet hemocyanin by the carbodiimide method; the minimum hapten/protein molar ratio was 30:1, as determined spectrophotometrically (261). The chlorocarbonate derivative of THC was prepared by the reaction of THC with phosgene, and the derivative was then conjugated to bovine serum albumin by the Schotten-Baumann reaction; by including ^{3}H-THC in the process, it was shown that an average of 12 THC molecules were conjugated to each albumin molecule (658). The hemisuccinate ester of THC has been prepared and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; measurement of tracer ^{3}H-THC present in the conjugate showed that an average of 25 THC molecules were attached to each albumin molecule (660).

Animals immunized with each of these conjugates formed THC-specific antibodies, as demonstrated by fluorescence quenching with THC-azobenzoic acid (261) and by binding of ^{3}H-THC in uncoated charcoal (658) and dextran-coated charcoal (268, 627, 659, 660) separation systems; THC was capable of inhibiting the fluorescence quenching and the binding of ^{3}H-THC. Most antisera cross-reacted extensively with 11-hydroxy-THC, a major THC metabolite (268, 660).

Anti-THC sera have been used together with ^{3}H-THC in the development of dextran-coated charcoal RIA methods for the measurement of cannabinoids in extracts of plasma and urine (268, 627, 657, 660). These RIA methods have been used in the detection of cannabis use in man (450, 656a) and in studies of the pharmacokinetic behavior of cannabinoids in man (627, 659) and in experimental animals (657, 660).

Recently, cannabinoid acids have been conjugated *via* their carboxyl groups to the free amino groups of ovalbumin and of bovine serum albumin by the mixed anhydride method; from 4 to 26 molecules of these haptens were coupled to each molecule of the carrier proteins, as estimated spectrophotometrically. Rabbits immunized with cannabinoid acid-bovine albumin conjugates formed hapten-specific antibodies which precipitated with cannabinoid acid-ovalbumin conjugates; the precipitation reaction was inhibited by free hapten. Three spin-labeled cannabinoid derivatives have been synthesized and are being used in the development of spin label immunoassays for the detection and measurement of cannabinoids in human urine (96).

10. Theophylline. *See* section V N.

Q. Cholinergic Agents

1. Nicotine. Three methods have been used to date to elicit antibodies specific for the major tobacco alkaloid, nicotine. In the first method described, 3′-succinylmethylnicotine was isolated after the reaction of equimolar quantities of succinic anhydride and 3′-hydroxymethylnicotine. The 3′-succinylmethylnicotine was conjugated *via* its free succinyl carboxyl group to the amino groups of protein and poly(L-lysine) carriers by the carbodiimide method. The degree of coupling varied between 6 and 18 molecules of hapten per molecule of conjugate, as estimated spectrophotometrically

(405). In the second method, 6-(*p*-nitrobenzamido)nicotine was synthesized by the addition of *p*-nitrobenzoyl chloride to 6-aminonicotine. After catalytic reduction of the nitro group, the resulting 6-(*p*-aminobenzamido)nicotine was diazotized and conjugated to the tyrosyl, histidine and tryptophan residues of bovine serum albumin; approximately 30 residues of nicotine were conjugated to each molecule of albumin, as determined by acid hydrolysis of the conjugate and high pressure liquid chromatographic assay of released 6-aminonicotine (454). Finally, 6-aminonicotine has been treated with succinic anhydride to yield N-succinyl-6-aminonicotine which was coupled *via* the free succinyl carboxyl group to the free amino groups of protein carriers by the carbodiimide method; the extent of conjugation was determined by ultraviolet spectroscopy, which indicated that 4 to 10 molecules of nicotine were coupled to each protein molecule (278). Rabbits immunized with each of these conjugates formed antibodies capable of binding ^{3}H-nicotine, as determined by the double antibody (278, 405) and ammonium sulfate precipitation (453) methods. Nicotine is an effective inhibitor of this binding, whereas cotinine and other nicotine metabolites are very ineffective inhibitors. This ability of nicotine antibodies to distinguish nicotine from its major metabolites has enabled the development of specific and sensitive double antibody (278, 405) and ammonium sulfate (453) RIA methods capable of detecting as little as 350 pg of nicotine in unextracted plasma or urine.

Nicotine RIA methods have been used to characterize the formation of a nicotine analog of triphosphopyridine nucleotide in rat liver microsomal preparations (610) and to study nicotine concentrations in the plasma and urine of tobacco smokers (278, 405, 406).

In order to elicit antibodies to cotinine, a major metabolite of nicotine, trans-4'-carboxycotinine was conjugated *via* its carboxyl group to the free amino groups of protein and poly(L-lysine) carriers by the carbodiimide method; between 6 and 18 molecules of hapten were incorporated into each molecule of conjugate, as estimated spectrophotometrically. Rabbits immunized with the conjugate formed antibodies capable of binding ^{125}I-labeled N-(*p*-hydroxyphenethyl)-*trans*-cotinine carboxamide, as determined by the double antibody method. Cotinine is 2000 times more effective than its parent compound, nicotine, in inhibiting this binding, thus enabling the development of a sensitive and specific double antibody RIA method capable of detecting as little as 200 pg of cotinine in unextracted plasma or urine (405). Since nicotine is metabolized rapidly, it has been suggested that it may be advantageous to measure cotinine, its longer lived and more abundant metabolite, in studies designed to relate the effects of smoking to health (406). The measurement of 24-hour urinary cotinine excretion by RIA has been used to document the absorption of nicotine by workers harvesting green tobacco and to implicate absorption of nicotine as the likely cause of green tobacco sickness, an occupational illness of tobacco harvesters (240).

To develop an immunoassay for γ-(3-pyridyl)-γ-oxo-N-methylbutyramide (oxoamide), a metabolite of cotinine, γ-(3-pyridyl)-γ-oxobutyric acid was conjugated *via* its carboxyl group to the free amino groups of human serum albumin and of hemocyanin by the carbodiimide method. Rabbits immunized with these conjugates formed antibodies capable of binding an ^{125}I-labeled analog of oxoamide, as demonstrated by the double antibody method. Oxoamide was an effective inhibitor of this binding, whereas nicotine, cotinine and several other metabolites were poor inhibitors, thus making it possible to determine oxoamide concentrations at the picomole level by RIA. This RIA method has been used to study the enzyme system responsible for the conversion of cotinine to oxoamide in rabbit liver extracts and to measure oxoamide levels in the serum, urine and amniotic fluid of tobacco smokers. (404).

To study other metabolites of nicotine and cotinine, the mononucleotides of nicotine and of cotinine were conjugated to bovine serum albumin. Animals immunized with these conjugates formed antibodies which were highly specific for the hapten nucleotide. For example, the diphosphopyridine analogs and the mononucleotides were 1000-fold more effective than nicotine, cotinine or diphosphopyridine nucleotide in inhibiting the binding of radiolabeled antigen by specific antibodies, thus enabling the development of radioimmunoassay methods which have been used to demonstrate the existence of the nucleotide analogs in tissues of rabbits injected with nicotine or cotinine (610a).

R. Diuretics

1. Bumetanide. A glycine derivative of the sulfonamide diuretic, bumetanide, has been conjugated *via* its glycyl carboxyl group to the amino groups of bovine serum albumin by the mixed anhydride method; it was estimated spectrophotometrically that the resulting conjugate contained approximately 40 moles of drug coupled to 1 mole of albumin. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-bumetanide, as determined by an ammonium sulfate precipitation method; bumetanide was an effective inhibitor of this binding while the N-desbutyl derivative, a known metabolite of bumetanide, exhibited minimal cross-reactivity. Antibumetanide sera have been used together with ^{3}H-bumetanide in the development of an ammonium sulfate RIA method for the measurement of bumetanide concentrations in extracts of plasma or urine; using 0.1 ml of plasma or urine, bumetanide concentrations as low as 1 ng/ml can be determined by this technique. This method has been used in studies of the pharmacokinetics of bumetanide in man (188).

S. Local Anesthetic Agents

1. Cocaine. Cocaine (benzoyl methylecgonine) is rapidly metabolized in man; the principal metabolite appearing in the urine is benzoylecgonine, with smaller amounts of ecgonine also being present. Cocaine lacks the proper groups which would enable it to be conjugated directly to protein by currently available methods, and it was not possible to conjugate benzoylecgonine to protein carriers. Accordingly, ecgonine was conjugated *via* its carboxyl group to the free amino groups of sheep γ-globulin by the carbodiimide method. Rabbits and sheep immunized with the ecgonine-sheep γ-globulin conjugate formed antibodies capable of binding an ^{125}I-labeled benzoylecgonine derivative, as determined by the ammonium sulfate precipitation method. Benzoylecgonine was the most effective inhibitor of this binding, with ecgonine being about half as effective; cocaine showed 15% cross-reactivity and benzoylnorecgonine exhibited 10% cross-reactivity. Other related compounds, other local anesthetic agents and other drugs of abuse exhibited little or no cross-reactivity in this system (367), enabling the development of an ammonium sulfate RIA method for the measurement of immunoreactive cocaine metabolites in the urine of human subjects and in the plasma, urine and tissues of rabbits receiving an intravenous dose of cocaine (367, 484). Ecgonine antibodies have recently been used in the development of a hemagglutination inhibition assay which is capable of detecting the major immunoreactive metabolites of cocaine in urine or serum in concentrations as low as 1 ng/ml (183b). An enzyme immunoassay for cocaine metabolites has also been described (67, 483, 484, 596, 672).

T. Narcotic Antagonists

1. Cyclazocine. An azobenzoic acid derivative of cyclazocine was conjugated to poly(L-lysine) by a carbodiimide condensation; the resulting conjugate was found to have a cyclazocine content of 6%. Rabbits immunized with this conjugate formed antibodies which bound ^{3}H-cyclazocine as determined by an unspecified method. Cyclazocine and certain other benzazocines were effective inhibitors of this binding (534). The use of these antibodies in a

plasma cyclazocine RIA has not yet been described.

2. (+)-2-Hydroxy-N-cyclopropylmethylmorphinan. The (+)-2-[4-(4-aminobenzoyl)-aminobutoxy] derivative of the narcotic antagonist, (+)-2-hydroxy-N-cyclopropylmethylmorphinan has been synthesized, diazotized and coupled to the tyrosine, tryptophan and histidine groups of bovine serum albumin. Rabbits immunized with the albumin-drug conjugate formed antibodies capable of binding ^{3}H-labeled drug. Nonradioactive drug was an effective inhibitor of this binding, while several narcotics and other narcotic antagonists were ineffective inhibitors, thus enabling the development of an RIA method capable of detecting drug concentrations as low as 2 ng/ml in 0.1 ml plasma samples of canine plasma (735a).

3. Naloxone. In order to conjugate naloxone, a potent and specific antagonist of narcotic analgesics, to bovine serum albumin, ethyl-N-carbamyl-cyanomethylacetimidate was first conjugated to bovine albumin. The albumin derivative was diazotized and immediately mixed with naloxone, reacting with its phenolic ring to form a naloxone-albumin conjugate. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-naloxone, as determined by an ammonium sulfate precipitation technique. Naloxone was an effective inhibitor of this binding. A naloxone analog, naltrexone, and the reduced metabolite of naloxone were also effective inhibitors. However, the cross-reactivity of morphine and of two other naloxone metabolites, naloxone-3-glucuronide, the major metabolite, and N-dealkylnaloxone, was weak, thus enabling the development of an ammonium sulfate RIA method capable of detecting as little as 100 pg of naloxone in specimens of serum or of brain tissue. This assay method has been used to study the pharmacokinetics of naloxone in rats (40).

U. Narcotics and Analgesics

1. Acetylsalicylic acid. *See* section V J.

2. Anileridine. The N-succinyl derivative of anileridine, a synthetic phenylpiperidine analgesic agent, has been prepared and "activated" by the addition of N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide. The activated N-hydroxysuccinimide ester of N-succinylanileridine was then conjugated directly to protein carriers. Rabbits and a goat immunized with N-succinylanileridine-protein conjugates formed antibodies capable of binding an ^{125}I-tyramine derivative of N-succinylanileridine, as determined by the double antibody method. Anileridine and N-acetylanileridine, a metabolite of the drug in some species, were very effective inhibitors of this binding. Two other synthetic phenylpiperidine analgesics, meperidine and piminodine, were moderately effective inhibitors while normeperidine, an anileridine metabolite, was a weaker inhibitor. No significant cross-reactivity of anti-N-succinylanileridine sera was observed with anileridine acid (an anileridine metabolite), α-prodine (a synthetic phenylpiperidine analgesic), morphine or methadone. These antisera have been used together with the ^{125}I-tyramyl derivative of N-succinylanileridine in the development of a double antibody RIA which has been used to measure concentrations of anileridine and of meperidine in the plasma of experimental animals (675).

3. Antipyrine. *See* section V J.

4. Codeine. To elicit antibodies with a high degree of specificity for codeine, codeine-6-hemisuccinate was synthesized and conjugated *via* its succinyl carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method; alkaline hydrolysis of the resulting conjugate revealed that the molar incorporation ratio of hapten was 28 moles of codeine per mole of bovine serum albumin. Rabbits immunized with codeine-albumin conjugates formed antibodies capable of binding ^{14}C-codeine, as determined by an ammonium sulfate precipitation method. Codeine was a very effective inhibitor of this binding while heroin and morphine were moderately effective; nalorphine, me-

peridine, naloxone and methadone were relatively ineffective inhibitors. The use of these antibodies in a codeine RIA has not yet been described (692).

By using another approach, N-butyroylnorcodeine has been synthesized and conjugated to bovine serum albumin by the carbodiimide method. Rabbits immunized with this conjugate have formed antibodies capable of binding ^{3}H-codeine as determined by an albumin-coated charcoal separation method. Codeine is an effective inhibitor of this binding; of the other inhibitors tested, only ethylmorphine, dihydrocodeine and norcodeine cross-reacted significantly. No significant cross-reactivity was observed with heroin, morphine, 6-monoacetylmorphine or codeine-6-glucuronide, thus enabling the development of an albumin-coated charcoal RIA capable of detecting codeine concentrations of 1 ng/ml or less in biological fluids (222).

Antibodies to morphine often cross-react extensively with codeine (10, 382, 628, 631, 678, 693). Morphine immunoassay procedures employing antibodies which cross-react extensively with codeine have been used to study the pharmacokinetics of codeine in rat plasma and brain; in this study, antibodies to N-carboxymethylnormorphine which do not react with codeine were also used to detect and quantify the extent of the biotransformation of codeine to morphine (248). Morphine antibodies which cross-react with codeine can be used to detect codeine in human urine; however, unless it is known with certainty that an individual has ingested no other opiate, it is necessary to combine immunoassay with a subsequent chromatographic step for identification of the immunoreactive substance as codeine (335).

5. Etorphine. To elicit antibodies specific for etorphine, an analgesic agent 1,000 to 80,000 times more potent than morphine, etorphine-3-hemisuccinate was synthesized and conjugated to bovine serum albumin by the carbodiimide method. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-etorphine, as determined by a dextran-coated charcoal separation method. Etorphine was an effective inhibitor of this binding; there was no significant cross-reactivity of the etorphine antibodies with other opiate alkaloids, including morphine, codeine, nalorphine, diamorphine and normorphine, thus enabling the use of these antibodies in the development of a dextran-coated charcoal RIA suitable for clinical application and capable of detecting serum etorphine concentrations as low as 1 ng/ml (555).

6. Fentanyl. To elicit antibodies to the synthetic opioid, fentanyl, an analog, carboxyfentanyl, was synthesized and conjugated to bovine γ-globulin by the carbodiimide method; the resulting conjugate contained approximately 12 moles of fentanyl per mole of γ-globulin, as determined by the incorporation of ^{14}C-fentanyl into the conjugate. Rabbits immunized with carboxyfentanyl-bovine γ-globulin formed antibodies capable of binding ^{3}H-fentanyl, as determined by the ammonium sulfate precipitation method. Fentanyl was a highly specific inhibitor of this binding, thus enabling the development of an ammonium sulfate RIA method capable of detecting as little as 10 ng of fentanyl in serum (293, 294). This RIA method has been used to study fentanyl pharmacokinetics in dogs (294) and in man (594).

7. Hydromorphone. Hydromorphone-6-carboxymethyl oxime was synthesized and conjugated to bovine serum albumin by the mixed anhydride method; the extent of incorporation of hydromorphone into this conjugate was not determined. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-dihydromorphine, as determined by an ammonium sulfate precipitation method. Hydromorphone, heroin and morphine were the most effective inhibitors of this binding, while nalorphine, naloxone and meperidine were weaker inhibitors of this binding. The use of these antibodies in a hydromorphone RIA has not yet been described (692), but it has been reported that a

morphine RIA method can be used to detect hydromorphone concentrations in the 10 to 40 ng/ml range in 0.1 ml of plasma (311a).

8. **Meperidine.** Two methods have been used to elicit antibodies specific for meperidine, a synthetic narcotic analgesic drug. In one of these methods, meperidinic acid was prepared by alkaline hydrolysis of meperidine. Meperidinyl chloride was formed by refluxing meperidinic acid with thionyl chloride; this acyl chloride was then reacted with ethyl glycollate to form ethyl O-meperidinyl glycollate which was subsequently hydrolyzed in KOH to yield O-meperidinyl glycollic acid. This latter derivative was covalently attached to bovine serum albumin by the mixed anhydride procedure; with the use of a variety of analytical methods, it was estimated that approximately 49 molecules of O-meperidinyl glycollic acid had been conjugated to each molecule of albumin. Antisera obtained from rabbits immunized with this conjugate contained antibodies which bound ^{3}H-meperidine as determined by an ammonium sulfate precipitation method. Meperidine was an effective inhibitor of the binding of ^{3}H-meperidine by antibody. Two major metabolites of meperidine, meperidinic acid and normeperidinic acid, were very weak inhibitors but a third major metabolite, normeperidine, cross-reacted to some degree; the degree of normeperidine interference was, however, found to be comparable to the degree of its interference in other assay systems (695). In the other method, a hydrazide derivative of meperidine was prepared by the reaction of meperidine with hydrazine hydrate; the hydrazide derivative was converted to an azide derivative which was then coupled directly to bovine serum albumin. By gas chromatographic analysis of the acid-hydrolyzed conjugate, it was estimated that an average of 17 molecules of meperidine had been conjugated to each molecule of the albumin carrier. Rabbits immunized with this conjugate formed antibodies which bound ^{3}H-meperidine as determined by an ammonium sulfate precipitation method. Meperidine was an effective inhibitor of this reaction; meperidinic acid was a weak inhibitor and the inhibition by morphine, codeine, naloxone and fentanyl was minimal. The sensitivity of this assay method is approximately 30 ng of meperidine per ml (114). The clinical and experimental use of these two meperidine RIA systems has not yet been reported.

Meperidine is an effective inhibitor of the binding of an ^{125}I-tyramyl derivative of N-succinylanileridine by anileridine antibodies. Hence, this tracer and these antibodies have been employed in the development of a double antibody RIA method which is capable of detecting as little as 100 pg of meperidine and which has been used to measure plasma meperidine concentrations in experimental animals and in man (675).

To develop a radioimmunoassay for normeperidine, a metabolic derivative of meperidine, normeperidine was hydrolyzed to form normeperidinic acid which was, in turn, coupled to protein carriers by the carbodiimide method. Rabbits immunized with the normeperidinic acid-protein conjugate formed antibodies which bound ^{125}I-labeled O-tyramyl normeperidinic acid, as determined by a double antibody method. Normeperidine and normeperidinic acid were effective inhibitors of this binding while meperidine and anileridine were ineffective inhibitors, thus enabling the development of a sensitive and specific double antibody RIA method which has been used to study the conversion of meperidine or anileridine to normeperidine in experimental animals and in man (235a).

9. **Methadone.** A methadone derivative, 4-dimethylamino-2,2-diphenylvaleric acid was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; it was subsequently estimated by immunoassay that from 3 to 6 moles of hapten had been conjugated to each mole of albumin carrier. Antisera from rabbits immu-

nized with the conjugate contained antibodies which bound ^{14}C-methadone, as determined by an ammonium sulfate precipitation method. After absorption of antisera with carbodiimide-treated bovine serum albumin to remove carrier-specific antibodies, the residual hapten-specific antibodies agglutinated sheep erythrocytes coated with the hapten-albumin conjugate. Methadone was an effective inhibitor of this hemagglutination. Dextropropoxyphene was a weak inhibitor of hemagglutination but morphine, codeine, cocaine, amphetamine and naloxone exhibited no significant inhibition, thus enabling the development of a hemagglutination inhibition test which can detect methadone in human urine in concentrations of 1 ng/ml; the simultaneous use of heroin with methadone does not interfere with this immunoassay procedure (434). Alternatively, methadol has been prepared by reduction of methadone and allowed to react with succinic anhydride to form methadol hemisuccinate. The methadol hemisuccinate was conjugated *via* its carboxyl group to the free amino groups of bovine thyroglobulin by the carbodiimide method; it was estimated that 135 residues of hapten had been conjugated to each molecule of thyroglobulin, as determined by incorporation of ^{3}H-methadone. Rabbits immunized with methadol-thyroglobulin conjugates formed antibodies capable of binding ^{3}H-methadone, as determined by the dextran-coated charcoal separation method. Methadone and methadol inhibited this binding but morphine, propoxyphene and meperidine exhibited no significant effect, thus enabling the development of a dextran-coated charcoal RIA method capable of detecting 1.4 pmol of methadone in 100 μl of human serum (27). A methadone RIA employing an ^{125}I-labeled methadone derivative has been described (566), as has an enzyme immunoassay for methadone (73, 458, 596). Methadone immunoassay procedures have been used to detect and quantify methadone in the serum and urine of patients receiving this drug (27, 434, 458, 566). Methadone antibodies have also been used to demonstrate specific neuronal staining for methadone by the immunofluorescent technique in the brain tissue of 10 drug addicts who had died from drug overdosage (525).

10. Morphine. Antibodies to morphine have been elicited by several different methods. For example, the reaction of the phenolic group of morphine with sodium-β-chloroacetate yielded 3-O-carboxymethylmorphine which, in turn, was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; approximately 3 to 4 carboxymethylmorphine groups were coupled to each molecule of albumin, as estimated spectrofluorometrically (633). Alternatively, the reaction of the phenolic group of morphine with succinic anhydride yielded morphine-6-hemisuccinate (613, 694), which was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method; spectrophotometric analysis of an alkaline hydrolysate of morphine-6-succinyl-albumin revealed that approximately 6.5 residues of morphine had been conjugated to each molecule of albumin (693). Oxymorphone 6-(O-carboxymethyl)oxime has been synthesized by heating oxymorphone with O-(carboxymethyl)hydroxylamine in alcoholic alkali solution and has been conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; approximately 5 moles of hapten were coupled to each mole of albumin carrier, as estimated spectrophotometrically (382). Diazotized ethyl *p*-aminobenzoate has been coupled to morphine and the azomorphine product has been conjugated to bovine serum albumin by the carbodiimide method (267). Another morphine derivative, 2-(*p*-aminophenylazo)-morphine, has been synthesized and conjugated to bovine serum albumin (631), but the details have not been described. To conjugate morphine to carrier proteins through its heterocyclic

nitrogen, morphine has been converted to normorphine (248, 298, 479) which was then N-acylated with succinic acid to form N-succinyl-normorphine (479) or was N-alkylated with ethylbromoacetate to form N-ethoxycarbonylmethylnormorphine which was, in turn, hydrolyzed to form N-carboxymethylnormorphine (248, 298). N-succinylnormorphine and N-carboxymethylnormorphine were then conjugated *via* their carboxyl groups to the free amino groups of bovine serum albumin by the carbodiimide or mixed anhydride method (248, 298, 479). Rabbits immunized with each of these conjugates have formed antibodies capable of binding morphine. The presence of such antibodies has been demonstrated by the capacity of antisera from these rabbits to bind ^{3}H-dihydromorphine or ^{14}C-morphine, as demonstrated by the ammonium sulfate precipitation method (248, 267, 298, 382, 631, 633, 691, 693) or by equilibrium dialysis (298). Antisera elicited by similar techniques have also been shown to be capable of causing: hemagglutination of tanned sheep erythrocytes coated with a carboxymethylmorphine-rabbit albumin conjugate (8); binding of an ^{125}I-carboxymethylmorphine-polyamino acid conjugate, as demonstrated by a double antibody method (678); inhibition of the enzymatic activity of a carboxymethylmorphine-lysozyme conjugate (576); and decreased mobility of the nitroxide radical in a spin-labeled morphine derivative (417). Morphine has been a very effective inhibitor of each of these reactions between morphine derivatives and the antibodies elicited by various morphine-protein conjugates. However, the capacity of morphine metabolites and of other opiates to inhibit this binding has varied considerably from antiserum to antiserum, the principal determinant in this variability appearing to be the mode of linkage between the morphine hapten and the albumin carrier in conjugates prepared by different coupling methods (631). In general, antibodies elicited by the 3-O-carboxymethylmorphine conjugate, by the morphine-6-succinyl conjugate, and by the 2-(*p*-aminophenylazo)-morphine conjugate formed antibodies which were not effective in distinguishing morphine from codeine or heroin (10, 382, 628, 631, 678, 691, 693). However, antibodies from rabbits immunized with N-succinylnormorphine-protein or N-carboxymethylnormorphine-protein conjugates exhibit little or no detectable cross-reactivity with codeine (248, 479).

Antibodies elicited by the various morphine-protein conjugates have been used together with ^{3}H-dihydromorphine (10, 113, 628, 677) or ^{125}I-labeled morphine derivatives (183, 634, 667, 678) in the development of ammonium sulfate (113, 183, 628, 634, 667), double antibody (678) and nitrocellulose membrane (677) RIA procedures for the detection and measurement of morphine in serum and urine. Morphine antibodies have also been employed in the development of a number of nonisotopic morphine immunoassay methods, including hemagglutination inhibition (7–9), enzyme immunoassay (67, 73, 483, 574, 576, 596, 597), spin-label immunoassay (67, 160, 416, 417, 475, 476, 596) and latex agglutination-inhibition (572).

RIA and nonisotopic immunoassay methods have been used extensively in studies of the pharmacokinetics of morphine in the serum and urine of man and experimental animals (39, 41, 111, 628, 636, 651) and in the detection of morphine and other opiates in the serum and urine of users of these drugs (112, 113, 257, 258, 335, 678).

11. Pentazocine. Carboxymethyl and azobenzoic acid derivatives of pentazocine have been coupled by carbodiimide condensation through their carboxylic acid groups to the free amino groups of poly(L-lysine); the conjugates contained between 0.2 and 1 molecule of hapten for each 10 molecules of lysine, as estimated spectrophotometrically. Rabbits immunized with either of these conjugates formed antibodies capable of binding ^{3}H-pentazocine, as determined by an ammonium sulfate precipitation method. Pentazocine was an

effective inhibitor of this binding, but none of the known metabolites of the drug cross-reacted to any appreciable extent with the pentazocine antibodies. This specificity enabled the development of an ammonium sulfate RIA method for pentazocine. Plasma and urine interfered significantly with the RIA, but it was possible to use this RIA method to measure pentazocine concentrations in benzene extracts of human plasma and urine (719).

V. Neuromuscular Blocking Agents

1. *d*-Tubocurarine. Diazotized *p*-aminobenzoic acid was coupled to *d*-tubocurarine *via* its diazonium group and the resulting *d*-tubocurarine-*p*-aminobenzoic acid derivative was coupled *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method. Rabbits immunized with the tubocurarine-albumin conjugate formed antibodies capable of binding ^{3}H-*d*-tubocurarine, as determined by an ammonium sulfate precipitation method. *d*-Tubocurarine was a highly specific inhibitor of this binding; high concentrations of three curare analogs, curine, isochondodendrine and chondocurine produced only a small degree of inhibition and there was essentially no cross-reaction with other drugs known to act on the neuromuscular junction. The excellent specificity of the binding of ^{3}H-*d*-tubocurarine by antibodies has enabled the development of an ammonium sulfate RIA method capable of detecting *d*-tubocurarine concentrations as low as 5 ng/ml in 10 μl of serum or urine. This RIA method has been used in studies of the pharmacokinetics of *d*-tubocurarine in cats and in man (314).

W. Oral Hypoglycemic Agents

1. 1-Butylbiguanide. To elicit antibodies to 1-butylbiguanide, an analog, *p*-carboxyphenethylbiguanide was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method; the extent of incorporation of biguanide groups into the conjugate was 33 moles per mole of albumin, as estimated by a chemical method. Rabbits immunized with these hapten-protein conjugates formed antibodies capable of binding ^{125}I-*p*-hydroxyphenethylbiguanide, as determined by a charcoal separation method. Phenethylbiguanide and *p*-hydroxyphenethylbiguanide were the most effective inhibitors of this binding, but 1-butylbiguanide was sufficiently effective as an inhibitor to enable the development of a charcoal RIA capable of detecting as little as 1.5 ng of 1-butylbiguanide in aliquots of human serum (514).

2. Glibenclamide. The hemisuccinate derivative of a glibenclamide metabolite, 4-hydroxyl-glibenclamide, has been conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method. The resulting conjugate contained approximately 28 molecules of the glibenclamide metabolite per molecule of albumin, as estimated spectrophotometrically. Antisera obtained from rabbits immunized with this conjugate contained antibodies which bound ^{14}C-glibenclamide, as determined by the dextran-coated charcoal separation method. Glibenclamide and two of its metabolites were effective inhibitors of this binding, while other oral hypoglycemic agents were ineffective inhibitors, thus enabling the development of a dextran-coated charcoal RIA procedure capable of detecting immunoreactive glibenclamide concentrations as low as 20 ng/ml in the serum of individuals receiving this drug (250, 250a).

3. Glisoxepide. A piperidine carboxylic acid analog of glisoxepide has been conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method. The carboxylic acid analog coupled to ^{3}H-tyrosine methyl ester was used as the tracer; antisera from rabbits immunized with the hapten-albumin conjugate formed antibodies capable of binding the tritiated tracer, as determined by the dextran-coated charcoal separation method. Glisoxepide specifi-

cally inhibited this binding, thus enabling the development of a dextran-coated charcoal RIA method which has been used to determine plasma glisoxepide concentrations and has been employed in studies of the pharmacokinetics of glisoxepide in man (494).

X. Oxytocic Agents

1. 9,10-Dihydroergotamine. The 6-nor-6-carboxymethyl derivative of 9,10-dihydroergotamine has been prepared and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method. Rabbits immunized with the dihydroergotamine-albumin conjugate produced antibodies capable of binding ^{3}H-9,10-dihydroergotamine, as determined by a charcoal separation method. This binding was effectively inhibited by 9,10-dihydroergotamine; since its known metabolites were ineffective inhibitors, this binding of ^{3}H-9,10-dihydroergotamine by antibody formed the basis for the development of a specific RIA for the detection of picomolar quantities of 9,10-dihydroergotamine in human serum (570).

Y. Renal Tubular Transport Inhibitors

1. Probenecid. Probenecid was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method. It was estimated that between 12 and 20 molecules of drug were coupled to each molecule of albumin, as determined by the extent of incorporation of ^{14}C-probenecid into the conjugate. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-probenecid, as determined by the dextran-coated charcoal separation method. Probenecid and certain structurally related analogs were effective inhibitors of this binding while probenecid metabolites were, in general, relatively ineffective inhibitors. (482a) The ability of probenecid to inhibit the binding of ^{3}H-probenecid by specific antibodies has enabled the development of a dextran-coated charcoal RIA method which has been used to study the pharmacokinetics of probenecid in the plasma and cerebrospinal fluid of experimental animals (608a).

Z. Sedatives and Hypnotics

1. Barbiturates. Three general methods have been used to elicit antibodies which can be employed to detect various barbiturate sedatives. In the first of these methods, 5-allyl-5-(1-carboxyisopropyl)barbituric acid was allowed to react with *p*-nitrophenol to yield 5-allyl-5-(1-*p*-nitrophenyloxycarbonylisopropyl)barbituric acid; this latter derivative was conjugated to the free amino groups of bovine γ-globulin in the presence of dicyclohexylcarbodiimide. The barbiturate-bovine γ-globulin conjugate was found to contain 2 to 3 moles of barbiturate per mole of protein, as estimated spectrophotometrically (632). In the second method, a phenobarbital analog, 5-phenyl-5-(4-aminobutyl)barbituric acid was conjugated *via* its amino group to the free carboxyl groups of bovine serum albumin by the carbodiimide method; on the basis of incorporation of ^{14}C-labeled barbiturate, it was estimated that each molecule of conjugate contained 33 barbiturate residues (136). Finally, phenobarbital was converted to *p*-nitrophenobarbital and then catalytically reduced to form *p*-aminophenobarbital; the *p*-aminophenyl derivative was then diazotized and the diazonium derivative was coupled directly to the tyrosine and histidine residues of acetylated bovine serum albumin. On the basis of the decrease in the number of unsubstituted tyrosine and histidine residues found on amino acid analysis of the conjugate, it was estimated that 20 moles of hapten had been conjugated to each mole of protein carrier (582).

Rabbits immunized with barbiturate-protein conjugates prepared by each of these three methods have formed antibodies capable of binding various radiolabeled barbiturates, including ^{14}C-pentobarbital (632), ^{14}C-barbital (229), ^{14}C-phenobarbital (583) and ^{3}H-phenobarbital (136, 230, 583), as determined by the am-

monium sulfate precipitation method. Antibodies elicited by the 5-allyl-5-(1-carboxyisopropyl)barbituric acid-protein conjugate reacted equally well with barbital, pentobarbital, phenobarbital and secobarbital but their cross-reactivity with metharbital, hexobarbital and barbituric acid was insignificant (229, 632). In contrast, antibodies elicited by the other two conjugates, both of which contained phenobarbital analogs, reacted much more effectively with phenobarbital than with other barbiturate derivatives, lacking the 5-phenyl ring (136, 583).

Barbiturate-specific antibodies have been used, together with ^{14}C-labeled (229), ^{3}H-labeled (136, 153, 230, 583) and ^{125}I-labeled (139, 667, 685) barbiturates in the development of RIA methods for the determination, in man and in experimental animals, of serum, urine and tissue concentrations of barbital, pentobarbital (229) and phenobarbital (583). Barbiturate-specific antibodies have also been used in the development of an enzyme immunoassay for barbiturates (62, 73, 595, 596, 637, 698).

Barbiturate immunoassay methods have been used in studies of the pharmacokinetics of barbital, pentobarbital (229) and phenobarbital (583). Since antibodies elicited by 5-allyl-5-(1-carboxyisopropyl)barbituric acid-protein conjugates recognize barbital but not metharbital, such antibodies have been used to study the metabolic conversion, by N-demethylation, of metharbital to barbital by mouse liver enzymes (230). Barbiturate immunoassay methods have been used extensively in the detection of barbiturates in the serum and urine of patients being treated with barbiturates for seizure disorders and in the serum and urine of comatose patients and other individuals suspected of drug abuse (62, 139, 565, 667, 698).

2. Glutethimide. To elicit antibodies specific for the piperinedione hypnotic, glutethimide, or 2-phenyl-2-ethylglutarimide, an analog, 2-(*p*-aminophenyl)-2-ethylglutarimide, was diazotized and conjugated to the tyrosine and histidine residues of bovine serum albumin. Rabbits immunized with this conjugate formed antibodies which agglutinated tanned sheep red blood cells coated with a glutethimide-ovalbumin conjugate. Glutethimide, but not phenobarbital or diphenylhydantoin, was capable of inhibiting this hemagglutination reaction, thus enabling the development of a hemagglutination-inhibition assay capable of detecting 50 ng of glutethimide in 0.1 ml of serum or urine (669).

3. Methaqualone. The use of an ammonium sulfate RIA method, employing an ^{125}I-labeled methaqualone derivative and methaqualone antibodies, has been reported. The reagents needed for this RIA are commercially available, but the details of the methods used to elicit these antibodies have not been reported. The antibodies used in this RIA react with methaqualone and four of its metabolites. The use of this RIA in the detection of methaqualone in human urine (42) and serum (64) is being evaluated.

AA. Synthetic Steroids

1. Betamethasone. To elicit antibodies specific for betamethasone, a synthetic glucocorticoid, O-(carboxymethoxyl)hydroxylamine was added to form betamethasone-3-(O-carboxymethyl)oxime, which was conjugated to bovine serum albumin by the mixed anhydride method; on the basis of incorporation of ^{3}H-betamethasone into the conjugate, it was estimated that the molar ratio of betamethasone to albumin was 26.5:1. Rabbits immunized with betamethasone-albumin conjugates formed antibodies which bound ^{3}H-betamethasone, as determined by a dextran-coated charcoal separation method. Betamethasone was an effective inhibitor of this binding; dexamethasone and beclomethasone were weak inhibitors and negligible inhibition was caused by betamethasone-17-valerate, by other synthetic steroids and by four naturally occurring corticosteroids. Betamethasone antibodies have been used, together with ^{3}H-

betamethasone, in the development of a dextran-coated charcoal RIA method which has been employed in the determination of betamethasone concentrations in plasma specimens from a patient receiving this drug (350).

2. Betamethasone 17-benzoate. Betamethasone 17-benzoate-21-hemisuccinate was prepared and conjugated to bovine serum albumin by the mixed anhydride method; approximately 44 residues of betamethasone 17-benzoate were present in each molecule of conjugate, as estimated spectrophotometrically. Alternatively, betamethasone 17-benzoate-3-(O-carboxymethyl)oxime was prepared and conjugated to human serum albumin by the carbodiimide method, approximately 15 molecules of betamethasone 17-benzoate being coupled to each molecule of albumin, as estimated spectrophotometrically. Rabbits injected with either conjugate formed antibodies capable of binding ^{3}H-betamethasone 17-benzoate, as demonstrated by a dextran-coated charcoal separation system. Antibodies to the 21-hemisuccinate derivative exhibited greater specificity for betamethasone 17-benzoate; cross-reactivity with betamethasone, dexamethasone, cortisol and other endogenous steroids was minimal. These antibodies have been used together with ^{3}H-betamethasone 17-benzoate in the development of a sensitive and specific dextran-coated charcoal RIA method capable of detecting as little as 50 pg of betamethasone 17-benzoate in extracts of human and rabbit plasma (473). This RIA method has been used to measure plasma concentrations of betamethasone 17-benzoate after its topical application in creams and gels to the skin of rabbits and of man (472, 473).

3. Cyproterone acetate. To elicit antibodies capable of binding cyproterone acetate, the hemisuccinate derivative of 11α-hydroxycyproterone acetate was prepared and was conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride reaction; it was estimated that 18 moles of steroid were coupled to each mole of albumin, as determined by the decrease in free amino groups in albumin after the conjugation procedure. Rabbits immunized with these protein-steroid conjugates formed antibodies capable of binding 11α-hydroxycyproterone acetate-11-succinoyl-^{125}I-iodohistamine, as determined by the dextran-coated charcoal separation method. Cyproterone acetate and its 11α-succinoyl derivatives were effective inhibitors of this binding, as were steroids with identical A and B rings, while the other steroids tested were relatively ineffective inhibitors of the binding of ^{125}I-labeled tracer by antibody. The inhibitory capacity of cyproterone acetate enabled the development of a dextran-coated charcoal RIA procedure for the measurement of this drug in human serum (494a).

4. Dexamethasone. To elicit antibodies specific for the synthetic glucocorticoid, dexamethasone, dexamethasone-21-hemisuccinate (198, 301, 412) and dexamethasone-3-(O-carboxymethyl)oxime (461) have been synthesized and conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method (198, 412) and by carbodiimide condensation (301, 461); it was estimated by various methods that the individual dexamethasone-albumin conjugates contained between 5 and 28 molecules of dexamethasone per molecule of conjugate. Rabbits immunized with conjugates prepared by each of these methods formed antibodies which bound ^{3}H-dexamethasone, as determined by the ammonium sulfate precipitation (198, 412), dextran-coated charcoal separation (198, 301) and double antibody (461) methods. Dexamethasone is an effective inhibitor of this binding. There is some degree of cross-reactivity of dexamethasone antibodies with certain steroid hormones of man, notably testosterone and cortisol (301, 412, 461), but these antibodies have been used, together with ^{3}H-dexamethasone, in the development of double antibody (461), ammonium sulfate (412) and dextran-coated charcoal (206,

301) RIA procedures for the determination of plasma and urinary dexamethasone concentrations in man. Dexamethasone RIA methods have been used in studies of the pharmacokinetics (206, 301, 412, 461), bioavailability (196, 285) and percutaneous absorption (737) of dexamethasone in man.

5. Ethynylestradiol. The 6-(O-carboxymethyl)oxime derivative of 6-oxo-17α-ethynylestradiol, a synthetic estrogen, was prepared from 3-methoxy-6-oxoestrone and conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method to yield a conjugate which contained 42 steroid residues per mole of albumin, as estimated spectrophotometrically (540). By using another approach, ethynylestradiol-3-hemisuccinate was synthesized and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin, also by the mixed anhydride method, yielding a conjugate which contained 39 residues of steroid per molecule of albumin (702). Finally, an ethynylestradiol derivative substituted in position 7 with a 3-thiopropionic acid side chain was coupled *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride reaction, yielding a conjugate which contained 27 steroid residues per molecule of protein carrier (158). Rabbits immunized with each of these ethynylestradiol-albumin conjugates have formed antibodies capable of binding ^{3}H-ethynylestradiol, as demonstrated by the dextran-coated charcoal separation method (158, 702). Ethynylestradiol antibodies have exhibited excellent specificity; their cross-reactivity with naturally occurring estrogens and other synthetic estrogens has, in general, been minimal, thus enabling their use in the development of sensitive and specific dextran-coated charcoal RIA methods capable of detecting picogram quantities of ethynylestradiol in extracts of human plasma (158, 184, 702).

6. Fluoxymesterone. The 3-(O-carboxymethyl)oxime derivative of fluoxymesterone, a synthetic androgen, was synthesized and conjugated to the free amino groups of bovine serum albumin by the carbonyldiimidazole reaction, yielding a conjugate which contained approximately 38 moles of steroid per mole of albumin, as estimated spectrophotometrically. Rabbits immunized with fluoxymesterone-albumin conjugates formed antibodies capable of binding ^{3}H-fluoxymesterone, as determined by the double antibody method. Fluoxymesterone was an effective inhibitor of this binding, while androgens and other naturally occurring steroids were ineffective inhibitors, thus enabling the use of these antibodies in the development of a sensitive and specific double antibody RIA method, capable of detecting 25 pg of drug in 0.1 ml of unextracted human or animal serum (142).

7. Medroxyprogesterone acetate. Two methods have been utilized to conjugate medroxyprogesterone acetate to protein carriers. In the first of these, the 3-(O-carboxymethyl)oxime derivative of medroxyprogesterone acetate was prepared and conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method (161). In the other approach, the 11α-hydroxy derivative of medroxyprogesterone acetate was synthesized and allowed to react with succinic anhydride; the hemisuccinate ester of 11α-hydroxymedroxyprogesterone acetate was then conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin in the presence of carbonyldiimidazole. Approximately 9 moles of medroxyprogesterone acetate were conjugated to each mole of albumin by this method, as estimated spectrophotometrically (575). Rabbits and a goat immunized with these conjugates produced antibodies capable of binding ^{3}H-medroxyprogesterone acetate, as determined by the double antibody method. Medroxyprogesterone acetate was an effective inhibitor of this binding and, while certain analogs also inhibited binding, the inhibition produced by naturally occurring steroid hormones was negligible, thus permitting the development of

double antibody (161, 575) and dextran-coated charcoal (304) RIA methods for the measurement of serum concentrations of medroxyprogesterone acetate in experimental animals and man. These RIA methods have been used to study the pharmacokinetics of medroxyprogesterone acetate when administered orally, intramuscularly and *via* intravaginal rings (161, 304, 374, 575).

8. **Mestranol.** To elicit antibodies specific for mestranol, a synthetic estrogen, the 6-(O-carboxymethyl)oxime derivative of 6-oxomestranol was prepared and conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method, yielding a conjugate which contained approximately 32 molecules of steroid per molecule of albumin as estimated spectrophotometrically (540). Alternatively, a mestranol derivative containing a 3-thiopropionic acid side chain substituted in position 7 was synthesized and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the mixed anhydride method; this conjugate contained 29 steroid residues per molecule of albumin, as estimated spectrophotometrically (158). Rabbits immunized with either of these conjugates formed antibodies capable of binding ^{3}H-mestranol, as determined by the dextran-coated charcoal separation method. Mestranol was an effective inhibitor of this binding, while ethynylestradiol was a weak inhibitor, and there was minimal cross-reactivity with naturally occurring estrogens (158, 541), thus enabling the use of these antibodies in the development of a dextran-coated charcoal RIA method which has been used to study the pharmacokinetics of mestranol in women receiving this drug (184).

9. **Methylprednisolone.** The 3-(O-carboxymethyl)oxime derivative of the synthetic glucocorticoid hormone, methylprednisolone, was prepared and conjugated to the free amino groups of bovine serum albumin by the carbonyldiimidazole reaction. Rabbits immunized with the methylprednisolone-albumin conjugate formed antibodies capable of binding ^{3}H-methylprednisolone, as determined by the double antibody method. Methylprednisolone and related synthetic steroids were effective inhibitors of this binding, while cortisol was a weak inhibitor and other naturally occurring steroid hormones were ineffective inhibitors at physiological concentrations. These antibodies have been used in the development of a sensitive and specific double antibody RIA method, which is capable of detecting femtogram quantities of methylprednisolone in unextracted serum and which has been used to study the pharmacokinetics of this drug in experimental animals and in man (144).

10. **Metyrapone.** The carboxymethyloxime derivative of metyrapone, a synthetic inhibitor of adrenal cortical 11β-hydroxylase, has been prepared and coupled to the free amino groups of bovine serum albumin by the carbodiimide method. The conjugate contained approximately 5 metyrapone residues per mole of albumin. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-metyrapone, as determined by the double antibody method. Metyrapone was an effective inhibitor of this binding. However, reduced metyrapone, an active metabolite, was also an effective inhibitor of this binding. Therefore, a column chromatography procedure must be performed on plasma extracts to separate metyrapone from reduced metyrapone; metyrapone and reduced metyrapone can then be measured individually by double antibody RIA procedures. This assay method has been used to study the conversion of metyrapone to reduced metyrapone in man (463); it has also been used to determine plasma levels of metyrapone and reduced metyrapone in patients with normal and abnormal clinical responses to metyrapone, administered for assessment of the integrity of the pituitary-adrenal axis (638).

11. **Nandrolone.** The 3-(O-carboxymethyl)oxime derivative of the anabolic steroid, nandrolone (19-nortestosterone),

has been synthesized and conjugated to bovine serum albumin by the carbodiimide method, yielding a conjugate which contained an average of 16 nandrolone residues per mole of albumin, as estimated spectrophotometrically. Sheep immunized with this conjugate formed antibodies capable of binding nandrolone-^{3}H, as determined by a dextran-coated charcoal separation method. Nandrolone was a very effective inhibitor of this binding, while testosterone was a moderately effective inhibitor. Other naturally occurring steroids exhibited little inhibitory effect thus permitting the use of nandrolone-specific antibodies together with nandrolone-^{3}H in the development of a dextran-coated charcoal RIA method which was used initially in the measurement of drug concentrations in animal urine (353a) and more recently has been used to measure nandrolone concentrations in the unextracted plasma of experimental animals (353b).

12. **Norethindrone (norethisterone).** The 3-(O-carboxymethyl)oxime derivative of the synthetic progestin, norethindrone, also known as norethisterone, has been synthesized and coupled to the free amino groups of bovine serum albumin, usually by the mixed anhydride method, yielding conjugates which contained from 21 to 25 moles of norethindrone per mole of albumin carrier, as estimated spectrophotometrically, (100, 499, 701, 703). Norethindrone-11α-hemisuccinate-bovine serum albumin (100) and norethindrone-17β-hemisuccinate-bovine serum albumin conjugates (704) also have been prepared. Rabbits and sheep immunized with these conjugates have formed antibodies capable of binding ^{3}H-norethindrone (480, 499, 701, 704) or an ^{125}I-labeled norethindrone derivative, such as norethindrone 3-(O-carboxymethyl)oxime-^{125}I-labeled histamine, norethindrone 11α-hemisuccinate-^{125}I-labeled histamine, norethindrone 17β-acetate 3-(O-carboxymethyl)oxime-^{125}I-labeled histamine (100, 101, 303, 480) or norethindrone 3-(O-carboxymethyl)oxime-^{125}I-labeled tyrosine methyl ester (703, 704), as demonstrated by the double antibody or dextran-coated charcoal separation methods. Nonradioactive norethindrone is an effective inhibitor of the binding of radiolabeled norethindrone and norethindrone derivatives by antibody; while other synthetic steroids cross-react significantly with many antinorethindrone sera, these antisera do not, in general, exhibit significant cross-reactivity with the naturally occurring steroids of man. Antinorethindrone sera have been employed, together with ^{3}H-norethindrone or with ^{125}I-labeled norethindrone derivatives, in the development of sensitive and specific dextran-coated charcoal RIA methods capable of detecting picogram quantities of the drug in extracts of human plasma (480, 499, 701, 703, 704). These RIA methods have been employed in studies of the pharmacokinetics of norethindrone in human subjects (480, 499, 703) and in rhesus monkeys (499).

13. **Norgestrel.** The 3-(O-carboxymethyl)oxime (101, 703), 11α-hemisuccinate (101) and 3-(O-carboxymethyl)oxime-ϵ-aminocaproic acid (639) derivatives of the synthetic progestin, norgestrel, have been prepared and coupled to the free amino groups of bovine serum albumin, usually by the mixed anhydride reaction. Rabbits immunized with these conjugates have formed antibodies capable of binding norgestrel 3-(O-carboxymethyl)oxime-^{125}I-labeled tyrosine methyl ester (703, 704) or norgestrel 3-(O-carboxymethyl)oxime-^{125}I-labeled histamine (101, 102, 639), as determined by the dextran-coated charcoal separation method. Norgestrel and certain of its metabolites were effective inhibitors of this binding but, in general, naturally occurring steroids in physiological concentrations exhibited little or no inhibition of binding (639, 703), thus enabling the development of sensitive and specific dextran-coated charcoal RIA methods for the determination of norgestrel concentrations in extracts of human plasma (639, 703, 704). These RIA methods have been used in studies of the pharmacokinetics of norgestrel in women after its oral (639, 703) and intravaginal (639) administration.

14. Prednisolone. The 21-hemisuccinate derivative of the synthetic glucocorticoid hormone, prednisolone, has been prepared and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method (143) or by the mixed anhydride method (589). Conjugates prepared by these methods contained between 6 and 20 prednisolone residues per molecule of albumin (462, 589). Rabbits immunized with prednisolone-21-hemisuccinate-albumin conjugates formed antibodies capable of binding ^{3}H-prednisolone, as determined by the double antibody (143, 462) and ammonium sulfate precipitation methods (589). These antibodies react primarily with prednisolone but, in most instances, cross-react significantly with 20β-hydroxyprenisolone, a metabolite, and with hydrocortisone. Antibodies to prednisolone-21-hemisuccinate-albumin conjugates have been used, together with ^{3}H-prednisolone, in the development of double antibody (143, 145, 462) and ammonium sulfate (589) RIA methods for the measurement of prednisolone concentrations in the serum of experimental animals and of man. These RIA methods have been employed in studies of the pharmacokinetics and bioavailability of prednisolone in man (143, 462, 647a, 660a) and in experimental animals (145). Since the antibodies employed do not cross-react extensively with prednisone, these RIA methods have also been used to detect the appearance of prednisolone in the serum of prednisone-treated humans or experimental animals, reflecting the *in vivo* conversion of prednisone to prednisolone (143, 145, 462, 589).

A prednisolone-3-(O-carboxymethyl)-oxime-bovine serum albumin conjugate has also been prepared. Antibodies to this conjugate react more avidly with hydrocortisone than they react with prednisolone, thus permitting their use, together with ^{3}H-hydrocortisone, in the development of a sensitive and specific double antibody RIA method for the measurement of hydrocortisone concentrations in extracts of human plasma (141).

15. Prednisone. The 21-hemisuccinate derivative of the synthetic glucocorticoid hormone, prednisone, was prepared and conjugated *via* its carboxyl group to the free amino groups of bovine serum albumin by the carbodiimide method (140). A conjugate prepared by this method contained 18 moles of prednisone per mole of albumin carrier (462). Rabbits immunized with prednisone-21-hemisuccinate-albumin conjugates formed antibodies capable of binding ^{3}H-prednisone, as determined by the ammonium sulfate precipitation (140) or double antibody (462) methods. Nonradioactive prednisone was an effective inhibitor of this binding; cortisone also inhibited the binding of ^{3}H-prednisone by antiprednisone sera, but prednisolone was a weak inhibitor and endogenous steroids other than cortisone exhibited no significant inhibition (140, 462, 589). Antiprednisone sera have been used, together with ^{3}H-prednisone, in the development of sensitive and specific ammonium sulfate (140, 145, 589) and double antibody (462) RIA methods for the determination of prednisone concentrations in the serum of experimental animals and man. These assay procedures are usually performed on serum specimens which have been diluted sufficiently to minimize interference with cortisone and are capable of detecting as little as 6 pg of prednisone in 0.1 ml of unextracted diluted serum. Prednisone RIA procedures, used in conjunction with prednisolone RIA methods, are useful in determining the interconversion rates of these two compounds in experimental animals and in man (140, 145, 462, 589).

16. Triamcinolone acetonide. Triamcinolone acetonide-21-hemisuccinate has been conjugated to bovine serum albumin by the mixed anhydride reaction; the resulting conjugate contained 17 moles of steroid per mole of albumin. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-triamcinolone acetonide, as determined by the dextran-coated charcoal separation method. Triamcinolone and triamcinolone acetonide were effective inhibitors of this binding

while various naturally occurring steroid hormones, including cortisol, progesterone, testosterone, dehydroepiandrosterone and aldosterone did not exhibit significant inhibitory effects, thus enabling the use of triamcinolone acetonide antibodies together with radiolabeled drug in the development of a dextran-coated charcoal RIA method capable of detecting as little as 200 pg of the nonradioactive drug in plasma samples. This RIA method has been used in studies of the pharmacokinetics of triamcinolone acetonide in rats and in the measurement of plasma levels of the drug in psoriatic patients being treated with topical application of triamcinolone acetonide cream (534a).

17. Trienbolone acetate. The 3-(O-carboxymethyl)oxime derivative of trienbolone acetate, a potent synthetic anabolic steroid, has been synthesized, as has the 17-hemisuccinate derivative of its major metabolite, trienbolone. These steroid derivatives have been conjugated to the free amino groups of bovine serum albumin by the mixed anhydride method. Rabbits immunized with the trienbolone acetate conjugate formed antibodies which bound ^{3}H-trienbolone acetate, as determined by the dextran-coated charcoal separation method; this binding was inhibited by nonradioactive trienbolone acetate, but was not significantly inhibited by trienbolone or by other synthetic and endogenous steroids. Rabbits immunized with the trienbolone-17-hemisuccinate-albumin conjugate formed antibodies which bound ^{3}H-trienbolone, as determined by the dextran-coated charcoal separation method; this binding was effectively inhibited both by trienbolone and by trienbolone acetate, but was not significantly inhibited by endogenous steroids. Antibodies to trienbolone acetate and to trienbolone have been used, together with the corresponding tritiated steroids, in the development of sensitive and specific dextran-coated charcoal RIA methods capable of detecting less than 100 pg of the corresponding unlabeled steroids in extracts of bovine plasma and tissues (308).

BB. Toxins

1. Amanitin. Amanitins are the principal toxins of the toadstools *Amanita phalloides* and *Amanita verna;* their molecular weights are in the 900 range. To elicit antibodies, β-amanitin was conjugated to rabbit serum albumin by the carbodiimide method, yielding a conjugate with a molar ratio of amanitin to albumin of 1:4; rats immunized with these amanitin-albumin conjugates formed antibodies capable of binding a tritiated amanitin derivative, as determined by the ammonium sulfate precipitation method (226). Alternatively, the N-hydroxysuccinimide ester of β-amanitin was prepared and coupled to the free amino groups of bovine serum albumin; the amanitin-albumin conjugate was then cross-linked *via* its remaining free amino groups to the amino groups of poly(L-lysine) by the glutaraldehyde method; rabbits immunized with the amanitin-albumin-polylysine conjugate formed antibodies which bound a tritiated amanitin derivative, as determined by the dextran-coated charcoal separation method (219). The binding of the tritiated amanitin derivative, ^{3}H-O-methyl-demethyl-γ-amanitin was inhibited by α-, β and γ-amanitins (219, 226), thus enabling the development of an ammonium sulfate RIA method capable of detecting as little as 500 pg of amanitins in 1 ml of serum; this RIA method has been used to study the clearance of α-amanitin from the blood of poisoned mice (226).

2. Arsenicals. The diazonium derivative of *p*-aminophenylarsenic acid has been conjugated to protein carriers, and rabbits immunized with these conjugates have formed antibodies specific for azophenylarsonate (521, 614). These antibodies appeared to confer some protective effect against the toxic effects of an arsenical compound in mice (614), but the use of antibodies to arsenicals in immunoassay procedures has not been reported.

3. Carcinogens. The isocyanate derivatives of numerous carcinogenic substances have been conjugated to the free amino groups of protein carriers *via* car-

bamido linkages; these substances have included anthracene, 1,2,5,6-dibenzanthracene (165), 1,2-benzanthracene (168), 3,4-benzpyrene, 10-methyl-1,2-benzathracene, β-anthracene (169, 170), 2-acetylaminofluorene, 4-dimethylaminostilbene, 2'-methyl-4-dimethylaminostilbene (174, 524) and 4-dimethylaminoazobenzene (20, 21). Carcinogenic compounds such as β-naphthylamine (383), 2-aminofluorene (376) or analogs such as azobenzene and *p*-dimethylaminoazobenzene (105) have been diazotized and then conjugated to protein carriers. The O-carboxymethyloxime derivatives of aflatoxin B_1 and of a structural analog have been conjugated to the amino groups of poly(L-lysine) by the carbodiimide method, and the resulting conjugate was electrostatically complexed with succinylated bovine serum albumin before immunization (408). A hemisuccinyl derivative of 2-acetylaminofluorene has been synthesized and conjugated *via* its carboxyl group to the free amino groups of protein carriers (119). Another carcinogen, 1,2-naphthoquinone has been conjugated directly to protein carriers without chemical manipulation, by an unspecified mechanism (509). Finally, a hemisuccinyl derivative of 4-aminobiphenyl has been synthesized and conjugated to bovine thyroglobulin (345). Rabbits immunized with these carcinogen-protein conjugates have formed antibodies with specificity for the introduced carcinogenic groups (20, 21, 105, 119, 164, 166, 167, 171–173, 345, 376, 383, 408, 509).

The use of carcinogen specific antibodies to detect carcinogens in tissues and biological fluids has not been extensive to date. Antibodies to 4-dimethylaminoazobenzene have been used to localize, by the immunofluorescent method, protein-bound carcinogen in rat liver and to demonstrate, by precipitation reactions, the presence of the 4-dimethylaminoazobenzene prosthetic group in rat liver antigens during the early stages of 4-dimethylaminoazobenzene carcinogenesis (20, 21). Fluorescein-conjugated antibodies specific for the 2-azofluorenyl group have been used to investigate the behavior and localization of hepatocellular components capable of binding 2-acetylaminofluorene in rat livers during chemical carcinogenesis; these experiments provided evidence that carcinogen-binding components are present in high concentrations at the cell boundary and in the perinuclear zone, and that these components are partially deleted in cells of hyperplastic nodules, which appear at the later stages of carcinogenesis and are almost completely deleted in carcinomatous cells (652). More recently, similar immunofluorescent techniques have been used to study the distribution of carcinogen-binding cells (105) and of carcinogen prosthetic groups (106) in the livers of rats fed large doses of *p*-dimethylaminoazobenzene or 3'-methyl-*p*-dimethylaminoazobenzene.

Antibodies to aflatoxin B_1 or its nonmutagenic analog, 5,7-dimethoxycyclopentenon(2,3-c)coumarin, have been shown by the double antibody method to be capable of binding ^{3}H-aflatoxin B_1. Aflatoxin B_1 inhibits this binding more effectively than other aflatoxins, thus enabling the development of sensitive and specific double antibody RIA methods for the detection of aflatoxin B_1 in serum and urine and in crude extracts of foodstuffs in which aflatoxin B_1 contamination is sometimes encountered. As little as 60 pg of the aflatoxin can be detected in body fluids, and as little as 1 μg/kg can be detected in foodstuffs (408). Antibodies to 2-acetylaminofluorene have been used, together with an ^{125}I-tyramine derivative, in the development of an RIA method capable of detecting picomole quantities of this carcinogen (119).

4. Genistein. Genistein, 5,7,4'-trihydroxyisoflavone, and several closely related isoflavones, which occur as glycosides in many forage legumes, exert an estrogen-like effect in animals and have been isolated as the free aglycones from the plasma of sheep grazing in clover pastures. Excessive ingestion of these phytoestrogens has been implicated as a possible cause of serious infertility in grazing sheep and cattle. Genistein-2-carboxylic

acid was prepared and, using the carbodiimide method, was conjugated *via* its carboxyl group to the free amino groups of a synthetic copolymer of tyrosine, glutamic acid and lysine. The resulting conjugate contained 4.3% genistein, as estimated spectrophotometrically. Rabbits immunized with the genistein-polypeptide conjugate formed antibodies which bound the ^{125}I-labeled genistein-polypeptide conjugate, as determined by the ammonium sulfate precipitation method. Genistein and a closely related estrogenic isoflavone, biochanin-A, inhibited this binding, while estradiol-17β was not an effective inhibitor, which suggests that antibodies to isoflavones might be used in the development of an RIA method for the detection of phytoestrogens in the plasma of grazing sheep and cattle (32).

5. Insecticides. DDA [2,2-bis(*p*-chlorophenyl)acetic acid], a DDT analog in which the trichloroethane moiety is replaced by an acetic acid residue, was reacted with thionyl chloride to form the acid chloride which, in turn, was conjugated directly to the amino groups of bovine fibrinogen (272). Alternatively, the anhydride of DDA was synthesized and added to bovine serum albumin, resulting in the coupling of approximately 25 DDA residues to the free amino groups of each albumin molecule (117). Rabbits immunized with DDA-protein conjugates formed antibodies which caused hemagglutination of erythrocytes coated with DDA-protein conjugates. DDA or DDA derivatives inhibited these passive hemagglutination reactions, but the use of antibodies to DDA in immunoassay systems has not been reported (117, 272).

The half-ester of the organophosphate insecticide, Malathion, was mixed with thionyl chloride to form an acid chloride derivative, which was conjugated directly to the amino groups of protein carriers (272). Alternatively, the anhydride of a Malathion metabolite, O,O-dimethyl S-(1,2-bis-carboxyethyl)-phosphorodithioate, was synthesized and conjugated directly to the free amino groups of bovine serum albumin, yielding a conjugate which contained 12 residues of the Malathion metabolite per molecule of albumin, as estimated by the phosphorus content of the conjugate (117). Rabbits immunized with the conjugates formed antibodies which, after absorption with the protein carrier, agglutinated erythrocytes coated with the corresponding hapten-protein conjugate. Malathion and a Malathion derivative were capable of inhibiting these passive hemagglutination reactions, but the use of antibodies to Malathion derivatives in immunoassay systems has not been reported (117, 272).

To elicit antibodies capable of detecting the structurally related organochlorine insecticides, aldrin and dieldrin, 6,7-dihydro-6-carboxyaldrin was synthesized and activated by the addition of N-hydroxysuccinimide and N,N′-dicyclohexylcarbodiimide; the activated N-hydroxysuccinimide ester was then conjugated directly to human serum albumin. Rabbits immunized with this hapten-protein conjugate formed antibodies capable of binding an ^{125}I-labeled tyramine derivative of 6,7-dihydro-6-carboxyaldrin, as determined by the double antibody method. Dieldrin, aldrin and structurally related compounds were effective inhibitors of this binding, while other polychlorinated insecticides inhibit too weakly to interfere with the assay of dieldrin or aldrin. This specificity has enabled the development of a double antibody RIA method capable of detecting as little as 150 pg of dieldrin or 700 pg of aldrin (407).

6. Ochratoxin A. Ochratoxin A, a dihydro-isocoumarin derivative linked through its 7-carboxyl group to L-β-phenylalanine, is a nephrotoxic metabolite produced by several fungal species. Ochratoxin A was conjugated *via* the carboxyl group in its phenylalanine moiety to the free amino groups of bovine γ-globulin by the carbodiimide method. Rabbits immunized with this conjugate formed antibodies capable of binding an ^{125}I-labeled ovalbumin-

ochratoxin A conjugate, as determined by the double antibody method. This binding could readily be inhibited by as little as 2 ng of ochratoxin A, thus suggesting that the antibodies may be useful in the development of an RIA method for the detection and measurement of ochratoxin A in biological fluids (1).

7. Paralytic shellfish poison. Paralytic shellfish poison (PSP) is a potent neurotoxin which is produced by *Gonylaulax catanella* and related dinoflagellates and which is concentrated, without ill effects, in clams and mussels which use these dinoflagellates as a food source. However, human ingestion of PSP may result in paralysis and, occasionally, death; toxic manifestations usually occur in man only after shellfish have been exposed to unusually large numbers of these dinoflagellates (a phenomenon associated with the discoloration of sea water, which has been referred to as the "red tide"). The chemical structure of PSP is not known, but it has the empirical formula $C_{10}H_{17}N_7O_4$-2HCl (474, 590). With formaldehyde as a coupling agent, PSP has been conjugated to bovine serum albumin. Rabbits immunized with PSP-albumin conjugates have formed antibodies which agglutinated erythrocytes coated with various PSP-protein conjugates and which protected mice against the toxic effects of a lethal dose of PSP (346). By using inhibition of passive hemagglutination of PSP-coated erythrocytes or inhibition of flocculation of PSP-coated bentonite particles, it is possible to detect PSP in contaminated shellfish (347).

8. Paraquat. A preliminary note has appeared, describing the development of antibodies specific for the herbicide, paraquat, and the use of these antibodies in the development of an RIA method for the detection of paraquat in human plasma (419a). This RIA has been used to document decreases in blood paraquat concentrations during therapeutic plasmapheresis in a patient who had swallowed a large amount of this herbicide (183a).

9. Strychnine. An amino derivative of mononitrostrychnine was prepared and diazotized; the diazonium derivative was coupled to protein carriers. Rabbits immunized with monoaminostrychnine-hemocyanin conjugates produced antibodies which formed precipitates with monoaminostrychnine-casein conjugates. Strychnine and certain of its derivatives inhibited this precipitation reaction but the use of antibodies to strychnine in an immunoassay procedure has not been reported (312).

10. Tartrazine. Tartrazine, a synthetic organic dye used as a color additive in foods and drugs, has been conjugated to bovine serum albumin by two methods: a) by means of the bifunctional diazonium reagent, bis-diazotized benzidine, yielding a conjugate containing approximately 5 moles of tartrazine per mole of albumin, as estimated spectrophotometrically; and, b) by the carbodiimide method, utilizing the formation of a peptide bond between the carboxyl group of the pyrazolone ring of tartrazine and the free amino groups of albumin and yielding conjugates containing up to 8 residues of tartrazine per molecule of albumin (348). Rabbits immunized with either of these conjugates form antibodies which are capable of binding an ^{125}I-labeled tartrazine-rabbit serum albumin conjugate, as demonstrated by a solid-phase technique, employing antibody-coated tubes. Tartrazine is capable of inhibiting this binding, but the use of anti-tartrazine antibodies in the development of a tartrazine immunoassay has not been reported (349).

CC. Vitamins

1. Pantothenic acid. Pantothenic acid has been conjugated to bovine serum albumin through a bromoacetyl derivative, with the resulting conjugate containing 24 to 28 pantothenic acid residues per molecule of protein. Rabbits immunized with this conjugate formed antibodies capable of binding ^{3}H-pantothenic acid (726). These antibodies have been used, together with ^{3}H-pantothenic acid, in the develop-

ment of an ammonium sulfate RIA method for the measurement of blood pantothenic acid concentrations in human subjects. Results obtained by this RIA method exhibited a highly significant correlation with results obtained by a microbiological assay method (726a).

2. **Vitamin A.** Vitamin A acid (retinoic acid) was conjugated *via* its carboxyl group to the free amino groups of human serum albumin by the mixed anhydride method. It was estimated that between 28 and 37 molecules of retinoic acid had been incorporated into each molecule of conjugate, as determined spectrophotometrically and by the extent of the decrease in the number of free amino groups in albumin after the conjugation procedure. Rabbits immunized with retinoic acid-albumin conjugates formed antibodies capable of binding ^{3}H-retinol, as demonstrated by the dextran-coated charcoal method. Retinol, retinoic acid and certain related compounds are effective inhibitors of this binding, but technical problems have prevented the development of a practical RIA method to date (151).

3. **Vitamin B_{12}.** Two approaches have been used successfully to elicit antibodies capable of binding vitamin B_{12}. Firstly, a carboxypropyl derivative of vitamin B_{12} was prepared and conjugated *via* its carboxyl group to the free amino groups of poly(L-lysine) by the carbodiimide method; an electrostatic complex formed between the carboxypropyl-B_{12}-poly(L-lysine) conjugate and succinylated hemocyanin was used as the immunogen (243). Secondly, the carboxyamide groups in vitamin B_{12} were converted to carboxylic groups, which were coupled to the amino groups of bovine serum albumin by the carbodiimide method; this B_{12}-albumin immunogen contained about 1 molecule of vitamin B_{12} per 4 molecules of protein, as estimated spectrophotometrically (671). Rabbits and a guinea pig immunized with these antigens formed antibodies capable of binding ^{57}Co-cyanocobalamin, as demonstrated by membrane filtration (243) and dextran-coated charcoal (671) separation methods. Vitamin B_{12} is an effective inhibitor of this binding (243), thus enabling the use of anti-B_{12} antibodies in the development of a solid-phase antibody RIA method for the measurement of vitamin B_{12} in human serum (670).

4. **Vitamin D.** The 3-hemisuccinate of cholecalciferol (vitamin D_3) was synthesized and conjugated to bovine serum albumin by the mixed anhydride method, approximately 10 to 15 molecules of hapten being coupled to each molecule of albumin carrier. The poor solubility of vitamin D_3 in aqueous systems interfered with vitamin D_3-binding studies, but it was demonstrated that rabbits immunized with the cholecalciferol-albumin conjugate formed antibodies capable of binding ^{3}H-25-hydroxycholecalciferol, as demonstrated by the dextran-coated charcoal separation method. The ability of 25-hydroxycholecalciferol to inhibit this binding formed the basis for the development of a dextran-coated charcoal RIA method capable of detecting as little as 0.1 ng of 25-hydroxycholecalciferol in serum extracts (241).

5. **Vitamin K.** Menadione (vitamin K_3) was coupled to the thiol groups of bovine γ-globulin which had been thiolated with N-acetylhomocysteine thiolactone; approximately 25 to 35 menadione residues were conjugated to each molecule of γ-globulin, as estimated both spectrophotometrically and by chemical analysis of an alkaline hydrolysate of the conjugate. Rabbits immunized with menadione-bovine-γ-globulin conjugates formed antibodies which bound ^{14}C-menadione-butyrate as shown by equilibrium dialysis. The use of these antibodies in menadione immunoassays has not yet been reported (352, 353).

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