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Characterization of Antibodies of High Affinity and Specificity for the Digitalis Glycoside Digoxin*

Thomas W. Smith, Vincent P. Butler, Jr., and Edgar Haber

ABSTRACT: Immunization of rabbits with a conjugate of the steroid glycoside digoxin coupled by a periodate oxidation method to amino groups of lysine in human serum albumin resulted in the production of high titer digoxin-specific antibodies with exceptionally high affinity and specificity late in the immune response. Studies of antisera from an animal heavily immunized over a period of 97 weeks showed 5.8 mg/ml of digoxin-specific antibody. The average intrinsic affinity constant (K_0) of this antiserum, determined by Sips analysis of equilibrium dialysis data, was 1.7×10^{10} M^{-1}; relative homogeneity was indicated by a heterogeneity index (a) of 0.92. The K_0 for digitoxin, which differs by only a single OH group at the C_{12} position, was 32-fold lower. A ninefold increase was found in K_0 for digoxin between the 6th and 32nd weeks of immunization in another animal serially studied. Hapten inhibition experiments with digitoxin deslanoside, digoxigenin, cholesterol, cortisol, dehydroepiandrosterone, 17β-estradiol, progesterone, and testosterone were carried out on a number of antisera utilizing a dextran-coated charcoal method for separation of bound and free ligand.

High specificity for antigenic determinants of the steroid nucleus of digoxin, tending to increase with time following immunization, was demonstrated. These antibodies may be employed in the measurement of 3×10^{-13} mole/ml concentrations of digoxin in physiologic fluids.

Previous studies have shown that antibodies specific for steroid haptens can be obtained by immunization of animals with antigens consisting of steroid molecules coupled to carrier proteins (Lieberman *et al.*, 1959; Zimmering *et al.*, 1967; Gross *et al.*, 1968). It has been suggested that such antibodies might be useful in the measurement of low concentrations of steroid compounds (Goodfriend and Sehon, 1961). This principle has been applied to the determination of 17β-estradiol and testosterone (Beiser and Erlanger, 1967) and the steroid glycosides digoxin (Butler and Chen, 1967) and digitoxin (Oliver *et al.*, 1968). The steroid hapten–antibody interaction has also been advanced as a possible model for hormone binding by target tissue receptors (Zimmering *et al.*, 1967).

Previous studies of average intrinsic affinity constants (K_0) for steroid-specific antibodies, obtained by immunization of unbred ewes with steroid–bovine serum albumin conjugates (Zimmering *et al.*, 1967), yielded values of the order of 10^5 M^{-1}. These appear too low to allow the practical use of such antibodies in radioimmunoassay systems where sensitivities of the order of 10^{-12} mole are required. The specificity of antibodies to steroid haptenic determinants has been evaluated by quantitative precipitin analysis, equilibrium dialysis, and hapten inhibition (Lieberman *et al.*, 1959; Zimmering *et al.*, 1967; Gross *et al.*, 1968). These studies have generally shown substantial cross-reactivity among related steroid compounds when tested against steroid-specific antisera.

Butler and Chen (1967) have reported a technique for coupling the steroid glycoside digoxin to ϵ-amino groups of

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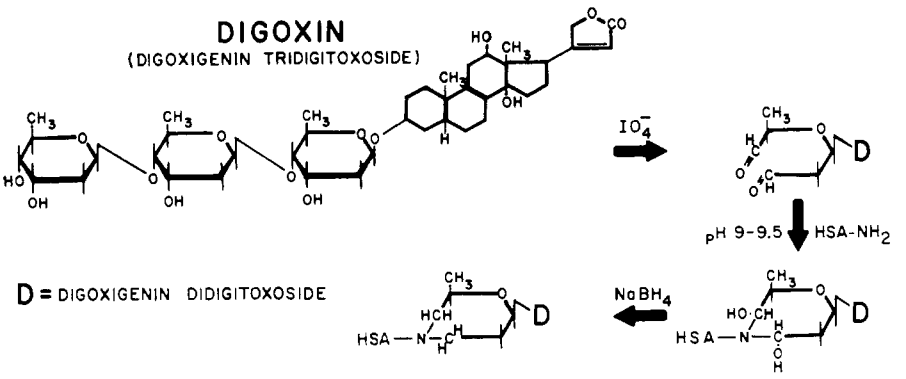


FIGURE 1: Proposed mechanism for conjugation of digoxin to human serum albumin, as modified from Butler and Chen (1967), HSA, human serum albumin molecule in which NH_2 is supplied by lysine and N-terminal residues.

lysine in a protein carrier, and have shown that the conjugate is immunogenic in rabbits. It is the purpose of this communication to describe in detail the characteristics of rabbit anti-digoxin antisera with very high affinity and specificity for the steroid moiety of digoxin.

Experimental Procedures

Digoxin and digoxigenin were kindly supplied in crystalline form by Burroughs-Wellcome Co., Tuckahoe, N. Y. Thin-layer chromatography (silica gel G, using acetone–glacial acetic acid–cyclohexane, 49:2:49) showed no demonstrable contaminants. Crystalline digitoxin donated by Wyeth Laboratories, Radnor, Pa., and crystalline deslanoside, the gift of Sandoz Pharmaceuticals, Hanover, N. J., were also pure by thin-layer chromatography (solvent system as above).

Digoxin tritiated in the 12α position ([^{3}H]digoxin[1]) (specific activity 3.2 Ci/mmole) and randomly tritiated digitoxin (specific activity 0.72 Ci/mmole) were obtained from New England Nuclear Corp., Boston, Mass., and used without further modification. Radiochemical purity was greater than 97% by the supplier's radiochromatographic and reverse isotope dilution criteria, further supported by our finding of at least 98% binding to digoxin-specific antibody in the presence of antibody excess.

Cortisol, cholesterol, dehydroepiandrosterone, 17β-estradiol, progesterone, and testosterone were kindly supplied by Dr. Lewis Engel and were chromatographically pure.

Activated charcoal coated with dextran (mol wt 80,000) was prepared according to Herbert *et al.* (1965).

Human serum albumin, fraction V powder, was obtained from Pentex, Inc., Kankakee, Ill.

Preparation of Antigen. Synthetic human serum albumin–digoxin (HSA–digoxin) conjugates were prepared by a periodate oxidation method based on that previously described for protein–digoxin conjugates (Butler and Chen, 1967) (Figure 1). A representative protocol follows: to 436 mg of (0.56 mmole) digoxin suspended in 20 ml of absolute ethanol at room temperature, 20 ml of 0.1 M sodium metaperiodate was added dropwise with magnetic stirring; after 25 min, 0.6 ml of 1 M ethylene glycol was added. Five minutes later, the reaction mixture was added dropwise, with magnetic stirring, to 560 mg of human serum albumin in 20 ml of H_2O which had previously been adjusted to pH 9.5 with 0.4 ml of 5% K_2CO_3; the pH was maintained in the 9.0–9.5 range by the dropwise addition of 2.0 ml of 5% K_2CO_3. After 45 min, the pH was stable and 0.30 g of sodium borohydride, freshly dissolved in 20 ml of H_2O, was added. Three hours later, 7.6 ml of 1 M formic acid was added to lower the pH to 6.5. After 1 hr at pH 6.5, the pH was raised to 8.5 by the addition of 1.5 ml of 1 M NH_4OH. The entire reaction mixture was then dialyzed overnight against cold running tap water. The following day the pH was lowered from 6.85 to 4.5 by the addition of 2.8 ml of 0.1 N HCl. After 1 hr at room temperature and 4 hr at 4°, most of the protein had precipitated. The suspension was centrifuged 1 hr at 4° and 1000*g*, after which the supernatant was discarded. The precipitate was dissolved in 5 ml of 0.15 M $NaHCO_3$, dialyzed against cold running tap water for 4 days, and lyophilized. The yield of the final product was 461 mg. Its absorption spectrum in concentrated H_2SO_4 (Brown and Wright, 1960) was compared with that of HSA and digoxin in a Beckman Model 2400 DU spectrophotometer, using a 1-cm path length. The HSA–digoxin conjugate possessed absorption maxima at 388 and 466 mμ which appeared to correspond to absorption maxima for digoxin at 388 and 475 mμ under similar conditions. Assuming molecular weights of 67,000 for HSA and 72,500 for the conjugate, calculated molar extinction coefficients at 388 mμ were: HSA, 5900; HSA–digoxin, 173,000; and digoxin, 25,100. Using these values it was estimated that the HSA–digoxin conjugate prepared by the above method contained an average of 6.7 digoxin residues/molecule of HSA, in contrast to an average of 1.4 residues/molecule of albumin in a conjugate prepared by the method described previously (Butler and Chen, 1967).

Immunological Procedures. For immunization, an emulsion was prepared containing equal volumes of HSA–digoxin (2 mg/ml) in 0.85% NaCl or 0.15 M phosphate-buffered saline (pH 7.4) and complete Freund's adjuvant mixture containing approximately 1 mg/ml of killed tubercle bacilli.

Rabbits were immunized by the injection of 0.1–0.2 ml of antigen preparation into each of four individual toe pads once weekly for 3 weeks followed by repeated intramuscular injections of 0.4 ml, usually at 1- or 2-week intervals except in the case of rabbit 700, which was boosted only by repeat toe pad injections at 10, 15, and 30 weeks. Antisera were obtained by serial bleedings of the animals at the times indicated in Table I. Individual bleedings were studied except in the case of rabbit 20, in which four consecutive bleedings

[1] Abbreviations used are: [^{3}H]digoxin, 12α-tritiated digoxin; HSA, human serum albumin; HSA–digoxin, human serum albumin–digoxin conjugate.

were pooled. Electrophoresis of eight antisera from rabbit 46 obtained over a 2-year period showed a diffuse rise in the γ-globulin concentration, plateauing 14 weeks following initial immunization.

Equilibrium Dialysis. Dialyses were carried out in plastic chambers (Technilab Instruments, Los Angeles, Calif.) with 1-ml volumes on either side of a sheet of washed dialysis tubing (average pore diameter 48 Å; Arthur H. Thomas Co., Philadelphia, Pa.). From 0.025 to 2.5 μl of whole antiserum in 1 ml of buffer [0.15 M NaCl–0.01 M K_2HPO_4, pH 7.4, containing 1.0 mg/ml of crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill.) to inhibit nonspecific adherence of antibody to surfaces] was placed on one side of the membrane and varying amounts of tritiated digoxin or digitoxin in the same volume of the buffer on the other. Gravimetric measurements indicated that 96.5–98% of the volumes added was recovered at the end of each run. The recovery of added radioactivity was similarly between 96 and 98% in each case. The chambers were rotated at 6 rpm for 18 hr at 25° and equilibrium across the dialysis membrane was assured by counting the radioactivity in control chambers to which no specific antibody had been added. Corrections were made for the small differences in volume on either side of the membrane at the end of the period of dialysis. No nonspecific binding was found when normal rabbit serum was substituted for antiserum.

At the completion of dialysis, bound and free hapten concentrations were measured by liquid scintillation counting in the medium described by Bray (1960), employing an Ansitron liquid scintillation counter. Samples were then recounted following addition of a tritium internal standard to determine counting efficiency.

Antibody concentration was determined by extrapolation to infinite free hapten concentration (Eisen, 1964) using a reciprocal bound *vs.* reciprocal free plot (Nisonoff and Pressman, 1958). Least-squares linear regression analysis of values at each ligand concentration was carried out with the aid of an SDS-940 computer.

The average intrinsic association constants were calculated with the aid of the same computer, from equilibrium dialysis data utilizing the modified Sips equation (Karush, 1956; Nisonoff and Pressman, 1958; Eisen, 1964) $\log r/n - r = a \log C - a \log K_0$, where r equals moles of hapten bound per mole of antibody at free hapten concentration C; n is the number of moles of hapten bound at antibody binding site saturation, and a is an index of heterogeneity describing the distribution of association constants about the average intrinsic association constant, K_0.

Sips plots were subjected to least-squares analysis and correlation coefficients for the lines obtained were 0.97 or better in each case.

Hapten Inhibition Studies. [^{3}H]Digoxin (3.94×10^{-12} mole) was placed in 1 ml of the buffer at 25° in 12 × 75 mm test tubes. Various compounds to be tested for their ability to inhibit antibody binding of [^{3}H]digoxin were added and thoroughly mixed. An amount of antiserum which would bind 45–50% of the tritiated digoxin in the absence of interfering substances was then added and the mixture was allowed to equilibrate for 12 hr, after which no further changes were noted. At the completion of this incubation, bound and free [^{3}H]digoxin were separated by selective adsorption of the free [^{3}H]digoxin by 5-min contact with dextran-coated charcoal.

TABLE I: Summary of Immunization Data.[a]

Rabbit Number	Antiserum Designation	Weeks of Immunization	Bleeding Number	Number of Injections of Antigen
20	20/31–38	31–38	5–8 (pool)	26–30
39	39/22	22	4	15
46	46/3	3	1	3
	46/17	17	2	11
	46/97	97	16	63
700	700/6	6	2	4
	700/17	17	4	6
	700/32	32	6	7

[a] Individual antisera are referred to by rabbit number followed by time of bleeding in weeks following initial immunization (*e.g.*, 700/6 refers to rabbit number 700, 6 weeks following first antigen dose).

Following centrifugation, the supernatant containing antibody-bound [^{3}H]digoxin was added to 15 ml of Bray's solution and counted. Internal [^{3}H]digoxin standards were then added for quenching correction and the vials were recounted. All samples were run in duplicate, and replicate determinations gave a standard deviation of ±3%. In the absence of specific antiserum the charcoal adsorbed more than 99% of the tracer counts added. When excess antibody was present greater than 98% of [^{3}H]digoxin remained in the supernatant following contact with coated charcoal.

Results

Equilibrium Dialysis Studies. ANTIBODY CONCENTRATION. A typical plot of reciprocal bound *vs.* reciprocal free hapten concentration for antiserum 46/97 is shown in Figure 2. All such plots were linear over ranges of binding site saturations from less than 15 to 90% or greater and showed no tendency to deviate from linearity at low bound to free ratios. This allows reliable extrapolation to full antibody saturation. Digoxins-pecific antibody concentrations determined by this technique, assuming a molecular weight of 150,000 and two binding sites per antibody molecule, are included in Table II. Values varied from 5.8 mg/ml in a late antiserum from the heavily immunized rabbit 46 to 0.11 mg/ml in an early antiserum from rabbit 700.

SIPS ANALYSIS. Table II shows the average intrinsic association constants (K_0) and the heterogeneity indices (a) for antisera on which equilibrium dialysis data were obtained. The values given are means of duplicate determinations. Variation in duplicate K_0 values was from 2 to 14%, and for a values from 3 to 10%.

A typical Sips plot for antiserum 46/97 is shown in Figure 3. The high affinity ($K_0 = 1.7 \times 10^{10}$) of the antibody is accompanied by relative homogeneity of binding constants, as indicated by the average a value of 0.92. The closely related compound digitoxin (Figure 4) which differs only in the absence of the OH group at the C_{12} position, exhibits an

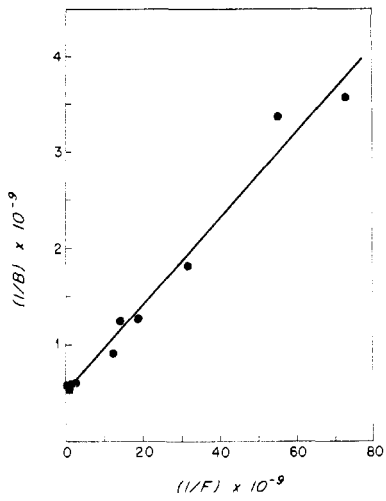


FIGURE 2: Plot of the reciprocals of bound (B) and free (F) molar hapten concentrations as determined by equilibrium dialysis of tritiated digoxin with 0.025 μl of antiserum 46/97. The line, determined by least-squares regression analysis, has a correlation coefficient of 0.99 and the experimental points shown extend over a range from 14 to 90% saturation of antibody combining sites.

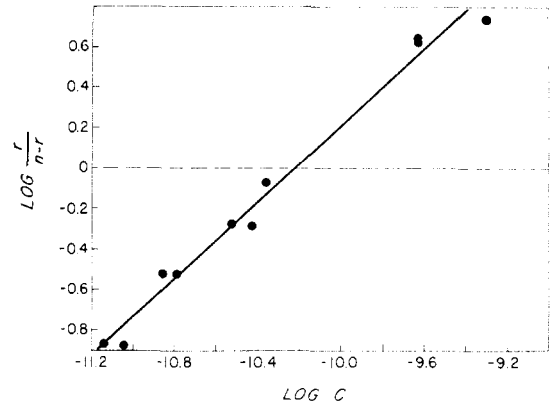


FIGURE 3: Example of Sips plot of equilibrium dialysis data for the homologous hapten digoxin with antiserum 46/97. Range of antibody saturation 12 to 85%; correlation coefficient 0.99. K_0 = 1.67 × 10^{10} M^{-1}; a = 0.95.

association constant, which although high, is 32-fold lower than that for digoxin (Table II). This indicates the remarkable specificity of the antibody. A ninefold rise in the K_0 value was observed between relatively early (6 weeks) and late (32 weeks) antisera from rabbit 700, concomitant with increasing titer and increasing heterogeneity as indicated by the decrease in the a value from 0.99 to 0.60.

Hapten Inhibition Studies. Antibody specificity was further defined by examining inhibition of binding of homologous hapten by various steroids or steroid glycosides. Figure 5 compares the ability of the steroid glycosides digoxin, digitoxin, and deslanoside (Figure 4) to displace [^{3}H]digoxin from the antibody binding sites of antiserum 46/97. It is apparent that digitoxin is a relatively poor competitor despite its close structural similarity, as might be predicted from the 32-fold lower K_0 value previously noted. Deslanoside, on the other hand, with an identical steroid nucleus but with an additional glucose moiety coupled to the terminal digitoxose residue, displays marked similarity to the binding characteristics of the homologous ligand digoxin. Table III summarizes data for all antisera studied, comparing the relative ability of digitoxin and deslanoside to compete with digoxin for antibody binding sites. Of particular interest is the rise in steroid nucleus specificity with time observed for rabbit 700, coincident with increasing antibody titer and increasing K_0. As the antiserum is better able to discriminate between the differing steroid portions of digoxin and digitoxin, there is an apparent decrease in discrimination between digoxin and deslanoside.

A number of steroid compounds were tested with this system as shown for antiserum 46/97 in Figure 6. Digoxigenin

TABLE II: Equilibrium Dialysis Data.[a]

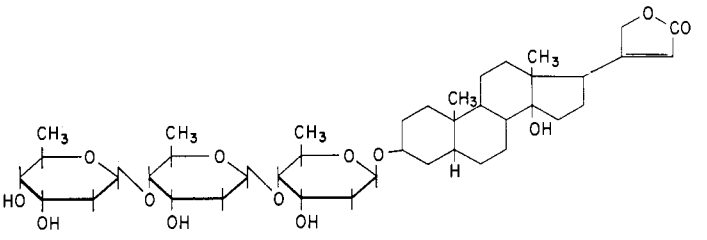
Antiserum	Hapten	Antibody Concn (mg/ml of Antiserum)	K_0	a
46/97	Digoxin	5.8	1.7 × 10^{10}	0.92
46/97	Digitoxin		5.3 × 10^{8}	0.83
700/6	Digoxin	0.11	1.2 × 10^{9}	0.99
700/32	Digoxin	1.5	1.1 × 10^{10}	0.60

[a] Summary of values derived from Sips analysis. K_0 values are averages of duplicate determinations which varied 2–14%; a values varied from 3 to 10%. Duplicate antibody concentrations agreed within 10%.

TABLE III: Antibody Titers and Hapten Inhibition Data for Steroid Glycosides.[a]

Antiserum	Titer	50% Inhibition DTX-DOX	50% Inhibition DES-DOX
20/31–38	38,000	19	1.3
39/22	63,000	18	1.1
46/17	29,000	35	1.3
46/97	40,000	41	1.1
700/6	420	14	1.7
700/17	2,600	13	1.1
700/32	15,000	37	1.2

[a] Titer, a function of both antibody concentration and affinity, is expressed as the final antiserum dilution at which 50% of 3.94 × 10^{-12} mole of tritiated digoxin are bound (conditions as described under hapten inhibition studies). The earliest antiserum studied, from rabbit 46 at 3 weeks, undiluted bound 35% of this quantity of hapten. Ratios listed are concentrations of digitoxin (DTX) or deslanoside (DES) relative to digoxin (DOX) required to decrease antibody binding of tritiated digoxin by 50%. Higher ratios therefore represent greater ability of the antiserum to distinguish between digoxin and digitoxin or deslanoside.



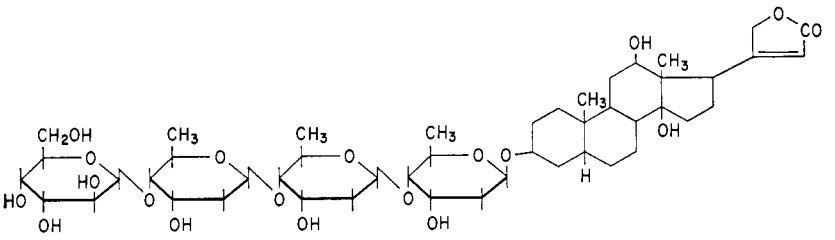


FIGURE 4: Structural formulas of steroid glycosides closely related to digoxin. Digitoxin differs only in the absence of the C-12 OH group, while deslanoside has an additional glucose residue coupled to the terminal digitoxose.

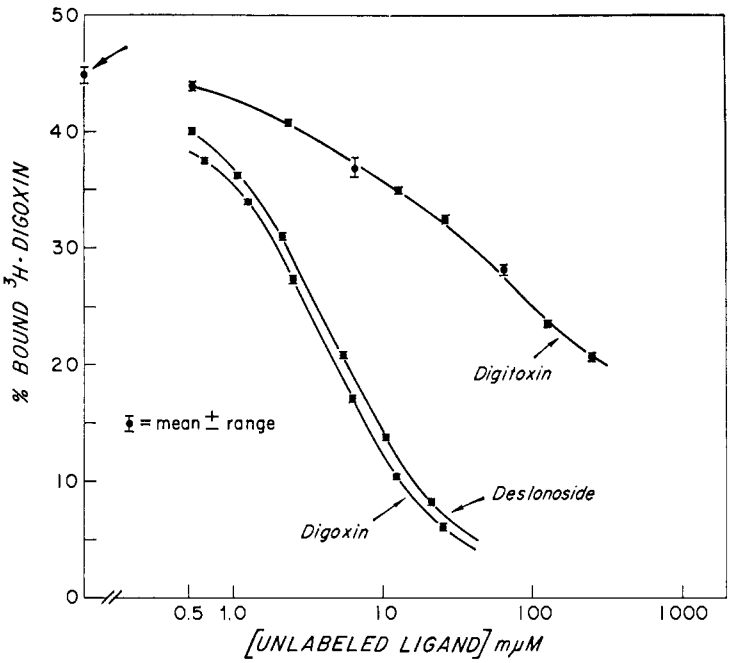


FIGURE 5: Hapten inhibition curves for antiserum 46/97 plotting on semilogarithmic scale the extent to which the steroid glycosides digoxin, deslanoside, and digitoxin displace tritiated digoxin from the antibody combining site. The control value with no unlabeled ligand added is indicated by the arrow on the ordinate.

the steroid nucleus of digoxin without the three digitoxose sugars coupled at C-3, rather closely parallels the ability of digoxin to compete with [^{3}H]digoxin for antibody binding sites. Cholesterol, cortisol, dehydroepiandrosterone, and 17β-estradiol show virtually no ability to displace [^{3}H]digoxin from the binding site, even when present in 10,000-fold molar excess. Progesterone and testosterone produce detectable decreases in [^{3}H]digoxin binding, but only when present in concentrations more than 1000-fold greater than that of the [^{3}H]digoxin. Table IV summarizes further hapten inhibition data for all antisera indicating the extent to which the steroid compounds cortisol, progesterone, and testosterone compete for antibody binding sites when present in 10,000-fold molar excess. It can be seen by comparison with Table III that greater specificity in terms of the ability of a given antiserum to distinguish between digoxin and digitoxin is closely correlated with greater ability to distinguish digoxin from cortisol, testosterone, and progesterone.

Discussion

Careful studies of the immune response to several antigenic determinants have confirmed the tendency of antibody affinity to rise progressively with time following immunization (Eisen, 1966; Siskind and Benacerraf, 1969; Steiner and Eisen, 1967), although this is not invariably the case (Haber *et al.*, 1967; Wu and Rockey, 1969). Increasing cross-reactivity has generally accompanied the rise in affinity (Eisen and Siskind,

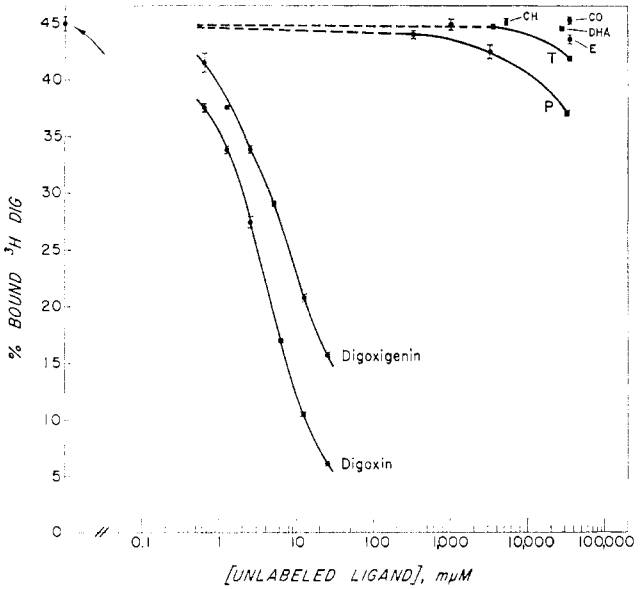


FIGURE 6: Hapten inhibition curves (antiserum 46/97) for the steroid compounds digoxigenin, cholesterol (CH), cortisol (CO), dehydroepiandrosterone (DHA), 17β-estradiol (E), testosterone (T), and progesterone (P), compared with the homologous hapten digoxin. The value for cholesterol represents the concentration at saturation of the aqueous buffer. The arrow on the ordinate denotes binding in the absence of unlabeled ligand. Individual values plotted are means, with ranges of duplicate determinations as shown.

TABLE IV: Hapten Inhibition Data for Steroid Compounds.[a]

Antiserum	Cortisol (%)	Testosterone (%)	Progesterone (%)
20/31–38	95	84	58
39/22	90	75	53
46/17	100	95	78
46/97	100	94	83
700/6	100	80	65
700/17	100	75	63
700/32	98	95	85

[a] Percentages shown represent the proportion of a tracer quantity (3.97×10^{-12} mole) of tritiated digoxin bound by each antiserum in the presence of a 10,000-fold molar excess of cortisol, testosterone, or progesterone, compared with the amount bound in the absence of any competing ligand. Higher percentages therefore denote greater antibody specificity for the homologous hapten digoxin.

1964), however, and it is therefore of particular interest that the late high-affinity antisera reported here retained remarkable specificity for the structure of the steroid nucleus of digoxin. There is a close similarity in hapten inhibition data for deslanoside and digoxigenin compared with the homologous hapten digoxin. This occurs despite the addition of a glucose moiety in deslanoside and the deletion of three sugar residues in the aglycone, indicating that the major determinants of binding specificity reside in the steroid portion of the molecule. This is amply confirmed by the marked loss of binding affinity when the C-12 OH group of the steroid nucleus is absent as in digitoxin despite the identity of the sugars coupled at the C-3 position. The anti-digitoxin antibody studied by Oliver *et al.* (1968) was found to bind digoxin only one-tenth as well as digitoxin, again implying as important role of the C-12 substituent in determining antibody–hapten binding. These findings are in accord with previous observations (Lieberman *et al.*, 1959) that the most important structural determinants of antibody specificity to steroid haptens tend to be those most distal to the site of coupling of steroid to carrier protein. In the present study, the additional distance between steroid nucleus and protein carrier interposed by three sugar residues may enhance the specificity of the immune response. An analogy may be found in the work of Sela *et al.* (1962) showing that in the case of polypeptide antigens accessibility of a particular antigenic grouping within the overall protein structure is a primary determinant of the antibody response.

The presence of the three digitoxose residues between the steroid nucleus and the carrier protein of the antigen may also influence the affinity of antibodies produced in the present experiments. Additional factors probably bear on the difference between the late K_0 values of the order of 10^{10} M^{-1} reported here and the much lower values previously obtained for antibodies to steroid haptens. Zimmering *et al.* (1967) studied antibodies elicited in ewes by a variety of steroid haptens coupled to bovine serum albumin. Bleedings similar in time to those in the present report were studied and doses of immunogen initially administered seem comparable, so that differing maturation of the immune response is not an obvious explanation. Differences in experimental animal species and preparation of antibody may bear on the discrepancy noted. In addition, differing technique for K_0 determination is pertinent since spectrophotometric methods used by Zimmering *et al.* do not permit determination of K_0 at levels much greater than 10^6 M^{-1} (Eisen, 1964). Conversely, our technique might well fail to detect a smaller population of antibodies with affinity in the 10^4 to 10^6 M^{-1} range, thus decreasing apparent antibody heterogeneity.

From the data presented, it is evident that antibodies of very high affinity and specificity for steroid haptenic determinants can be elicited in rabbits. The characteristics of these antibodies compare favorably with those of antibodies currently in use in radioimmunoassays for angiotensin II (Vallotton *et al.*, 1967) and a number of other protein and polypeptide hormones (Margoulies, 1968). It is therefore not surprising that they serve well in a sensitive and specific radioimmunoassay system (Smith *et al.*, 1969) for serum or urine levels of digoxin as low as 3×10^{-13} mole/ml. Of particular interest is the fact that the specificity of interaction allows determinations to be carried out in whole serum, eliminating the need for cumbersome extraction procedures. The possibility of extension of this general methodology to measurement of minute quantities of other important circulating steroid compounds is apparent.

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The Degradation of *trans*-Ferulic Acid by *Pseudomonas acidovorans**

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ABSTRACT: Washed cell suspensions of *Pseudomonas acidovorans*, grown with *trans*-ferulic acid as sole carbon source, oxidized vanillic acid, vanillin, protocatechuic acid, caffeic acid, and *cis*-ferulic acid at similar rates to *trans*-ferulic acid. Vanillic acid and vanillin were extracted from culture filtrates of *trans*-ferulic acid grown cells. Cell extracts were shown to convert [2-^{14}C]*trans*-ferulic acid into [2-^{14}C]acetate and vanillic acid only in the presence of nicotinamide–adenine dinucleotide.

Oxidized nicotinamide–adenine dinucleotide was required for the conversion of vanillin into vanillic acid *via* an oxidoreductase. Vanillic acid was oxidized to protocatechuic acid and formate by a monooxygenase requiring oxidized nicotinamide–adenine dinucleotide, reduced glutathione (GSH), ferrous ions, and formaldehyde. This mixture provided the reduced nicotinamide–adenine dinucleotide generating system required for mixed-function oxidation. Enzymes which convert formaldehyde into formate, requiring oxidized nicotinamide–adenine dinucleotide and reduced glutathione, and formaldehyde into methanol, requiring reduced nicotinamide–adenine dinucleotide, were separable by fractionation with ammonium sulfate. A reaction sequence for the complete degradation of *trans*-ferulic acid in which 3-methoxy-4-hydroxyphenyl-β-hydroxypropionic acid is an intermediate is proposed.

The microbial degradation of aromatic acids which contain the phenylpropane- (C_6–C_3) type structure may occur by (1) dihydroxylation of the benzene nucleus followed by ring fission leaving the side chain intact (Coulson and Evans, 1959; Dagley *et al.*, 1963, 1965; Blakely and Simpson, 1964; Seidman *et al.*, 1969); or (2) shortening of the side chain by a two-carbon fragment before ring fission (Webley *et al.*, 1962; Henderson, 1955; Cartwright and Smith, 1967).

trans-Ferulic acid is regarded as one of the simplest model compounds found in lignin (Siegel, 1954; Ishikawa and Takaichi, 1955). For example, α-conidendrin is believed to be synthesized by the condensation of dehydrogenated *trans*-ferulic acid with a quinone methide radical produced by dehydrogenation of a second molecule of coniferyl alcohol (Freudenberg and Geiger, 1963). When *Pseudomonas fluorescens* was grown with *trans*-ferulic acid as sole source of carbon, cell suspensions oxidized vanillin, vanillic acid, and protocatechuic acid in addition to the growth substrate (Cartwright and Smith,

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