*DIGOXIN-SPECIFIC ANTIBODIES**

By Vincent P. Butler, Jr.,† and James P. Chen

FIRST (COLUMBIA UNIVERSITY) MEDICAL DIVISION, BELLEVUE HOSPITAL, AND DEPARTMENT OF MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

Communicated by André Cournand, November 25, 1966

The problem of digitalis toxicity is one of considerable importance to clinicians and pharmacologists.[1] Not only is therapy far from satisfactory, but no convenient method is currently available for the specific determination of cardiac glycosides in plasma and tissue fluids.

Although antibodies specific for a variety of haptens have been obtained following the classic technique of Landsteiner,[2] no record has been found of the use of cardiac glycosides for such studies. Digitalis-specific antibodies could conceivably be an extremely useful tool (1) in the immunological assay of digitalis in plasma and tissue fluids, (2) in the therapy of severe digitalis toxicity, and (3) in the study of the mechanism of action of digitalis. This report describes the coupling of the cardiac glycoside, digoxin,[3] to bovine serum albumin and the use of the resulting conjugate as an antigen to induce the formation of antibodies which exhibit digoxin specificity.

Materials and Methods.—Reagents: Bovine serum albumin (BSA) was obtained as fraction V powder from Pentex, Inc. "Lanoxin"-brand digoxin and tritiated digoxin (Dig-H^3; 112.6 μc/mg; 1 mg/ml in 95% ethanol) were generously supplied by Burroughs Wellcome and Company. Cortisone-1,2-H^3 (5.83 mc/mg), hydrocortisone-1,2-H^3(50.2 mc/mg), dehydroepiandrosterone-7α-H^3 (5.62 mc/mg), and dehydroepiandrosterone-7α-H^3 sulfate, ammonium salt (4.23 mc/mg) were obtained from the New England Nuclear Corporation and were chromatographically purified by Dr. William Drucker and then dissolved in absolute methanol. Isotopic compounds were diluted with buffer for use in dialysis experiments.

Preparation of protein-digoxin conjugates: Digoxin (Dig) which consists of a steroidal aglycone linked to three digitoxose residues (see Fig. 2) was conjugated to BSA by a periodate oxidation method suggested by Dr. Bernard Erlanger, Columbia University, and based on a technique originally described by Erlanger and Beiser[4, 5] for other hapten-protein conjugates. One BSA-Dig conjugate was prepared as follows: To 218.8 mg (0.28 mmole) digoxin were added 30 ml 0.1 *M* sodium periodate, 40 ml absolute ethanol, and 10 ml dioxane. After 30 min at room temperature, 1.8 ml 1 *M* ethylene glycol was added and the entire reaction mixture was added to 280 mg BSA in 10 ml water which had been adjusted to pH 9.3 with 5% K_2CO_3. The mixture was stirred for 1 hr at room temperature with dropwise addition of 5% K_2CO_3 to maintain the pH in the 9.0–9.5 range. After 1 hr, 150 mg sodium borohydride was added and the reaction mixture was set aside for 24 hr at room temperature. Approximately 5.4 ml 1 *M* formic acid was added to lower the pH to 5.5; at about pH 5.8, considerable precipitation occurred. After 1 hr at room temperature, 1.5 ml 1 *M* NH_4OH was added to raise the pH to 8.5. Some cloudiness persisted and the mixture was dialyzed overnight against running tap water. The pH was lowered to 4.8 by the dropwise addition of 1.85 ml 0.1 *N* HCl with considerable precipitation of protein. After 4 hr at 4°C, the suspension was centrifuged 1 hr at 1800 rpm at 4°C. The precipitate was partially redissolved in 0.15 *M* $NaHCO_3$, dialyzed for 5 days against running tap water, lyophilized, and stored as a greenish-white powder. When examined spectrophotometrically in 83% H_2SO_4,[6] this BSA-Dig preparation had absorption maxima at 388 and 465 mμ, respectively, which were absent in unconjugated BSA. These absorption maxima appeared to be related to absorption maxima of digoxin at 388 and 480 mμ, respectively, under comparable conditions. On the assumption that the difference between the molar extinction coefficients of BSA and BSA-Dig at 465 mμ was due to the presence of digoxin in the conjugate, a rough estimate was made that 2.7 digoxin residues were conjugated to each mole of BSA; an estimate of 1.4 digoxin residues per mole of BSA was made using the extinction coefficients at 388 mμ. Hydrolysis of the conjugate and precise analytical studies will be required to obtain an exact value (conjugates recently prepared by a

slightly modified method have had extinction coefficients at these wavelengths consistent with the presence of five digoxin residues per mole of albumin, but these preparations have not yet been studied immunologically).

Immunological procedures: After control preimmunization sera had been obtained, rabbits were immunized by the injection of BSA-Dig, 1 mg/ml in complete Freund's adjuvant, three injections of 0.4 ml in the toe pads over a 4-week period. Most animals received a single booster injection intramuscularly. Most antisera reported herein represent a pooled series of terminal bleedings obtained from individual animals about 10 weeks later. Control antisera were obtained by immunizing in a similar manner with BSA, human serum albumin (HSA), human gamma globulin (HGG), whole human serum (WHS), hen's egg albumin (HEA), purin-6-oyl-BSA (Pur-BSA),[7] purin-6-oyl-HSA (Pur-HSA),[7] glucosyl-phenylazo-BSA (Glu-BSA),[8] and galactosyl-phenylazo-BSA (Gal-BSA).[8]

Equilibrium dialysis studies[9, 10] were carried out at 4°C in isotonic Tris-buffered saline, pH 7.5.[11] Antiserum, gamma globulin, or buffer was added to Visking dialysis tubing together with radioactive digoxin or steroid. The bag was tied and allowed to dialyze against an equal volume (5–10 ml) of buffer in a screw-capped test tube with frequent mixing (in recent experiments, a mechanical shaker has been used); after 5 days, 0.5–1.0-ml aliquots were removed for counting of radioactivity in 7.5–20 ml (usually 15 ml) of a scintillation mixture containing 6 gm 2,5-diphenyloxazole, 375 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, and 100 gm naphthalene in 1 liter dioxane. Measurements of radioactivity were carried out in a Packard Tri-Carb scintillation counter. Good agreement between final results was observed when the isotope was placed inside and outside the dialysis bag at the start of an experiment, but recoveries of added radioactivity were more satisfactory when isotope was added outside the dialysis bag. The efficiency of counting was excellent in buffer solution, γ-globulin solutions, and most control rabbit sera, but it was impaired in most anti-BSA-Dig sera, particularly in high concentration. Since no significant binding of radioactivity to dialysis tubing or glassware was observed, the amount of radioactivity inside any dialysis bag containing anti-BSA-Dig serum (or a 1:5 dilution of control rabbit serum) was calculated by subtracting the amount of radioactivity observed outside the dialysis bag from the total amount recovered in buffer control tubes. The amount of protein-bound radioactivity was calculated by subtracting the observed amount of free radioactivity outside the dialysis bag and the calculated amount inside from the total amount of radioactivity recovered. No significant volume change inside or outside dialysis tubing was noted during these experiments.

For inhibition studies, nonradioactive digoxin and other compounds were dissolved in 95% ethanol prior to dilution with buffered saline; the final concentrations of ethanol in the reaction mixture did not inhibit the binding of tritiated digoxin by anti-BSA-Dig sera.

γ-Globulin fractions of rabbit sera were prepared chromatographically by isolation of the first 280-mμ absorbing peak obtained from DEAE cellulose[12] in 0.01 *M* pH 7.5 phosphate containing 0.015 *M* saline.[13] In seven of nine preparations, a single γ-globulin arc was seen in immunoelectrophoresis with goat or sheep anti-whole rabbit serum; an additional β-globulin component was detected in the preparations from sera BSA-2 and DB-16X.

Experimental and Results.—Anti-BSA-Dig sera contained more antibody which reacted with BSA-Dig than reacted with unconjugated BSA, and digoxin was capable of partial inhibition of the precipitation reaction between BSA-Dig and anti-BSA-Dig serum.[14] These observations were consistent with the presence of antidigoxin antibodies in anti-BSA-Dig serum but further studies were required to establish their presence definitively.

Direct evidence for the presence of digoxin-specific antibodies in anti-BSA-Dig sera was obtained in equilibrium dialysis experiments employing Dig-H^3. In these experiments, antibodies to BSA and other nondigoxin determinants would not be expected to exert any action. Unlike digitoxin[1, 15, 16] and steroid hormones,[17] little digoxin was bound by normal rabbit serum. In contrast, 12 of 14 anti-BSA-Dig sera in 1:5 dilution bound more than 98 per cent of the added Dig-H^3 (Table 1). Digoxin-binding capacity could not be demonstrated in rabbit antisera to other

TABLE 1

BINDING OF TRITIATED DIGOXIN BY RABBIT SERUM

Rabbit no.	Preimmunization: Final Observed Distribution of Radioactivity (cts/5 min/ml) Inside	Outside	Per cent added radioactivity outside	Per cent bound by serum	After Immunization with BSA-Dig: Final Observed Distribution of Radioactivity (cts/5 min/ml) Inside	Outside	Per cent added radioactivity outside	Per cent bound by serum
DB-1	41,526	41,779	51.0	0.0	63,861	643	0.8	98.4
DB-2	20,529*	39,006	47.6	4.8	46,010	161	0.2	99.6
DB-3	39,207	39,880	48.7	2.6	33,209	77	0.1	99.8
DB-4	40,809	38,106	46.5	7.0	36,552	115	0.1	99.8
DB-6	37,273	40,430	49.3	1.4	57,920	53	0.1	99.8
DB-8	41,342	37,811	46.1	7.8	68,307	48	0.1	99.8
DB-9	41,877	39,686	48.4	3.2	68,095	232	0.3	99.4
DB-10					62,891	12,169	14.8	70.4
DB-11	42,172	39,833	48.6	2.8	64,339	217	0.3	99.4
DB-12					55,097	107	0.1	99.8
DB-13					66,029	198	0.2	99.6
DB-14	39,601	39,251	47.9	4.2	63,208	99	0.1	99.8
DB-15	38,760	41,765	51.0	0.0	51,070	146	0.2	99.6
DB-16	41,029	39,878	48.7	2.6	46,932	34,729	42.4	15.2
Buffer controls	41,641	39,270	47.9	(4.2)	41,738	41,275	50.4	(0.0)

One ml Dig-H^3 (0.45 μc/ml) and 2 ml serum, diluted with buffer to final volume of 10 ml. were placed in dialysis tubing and dialyzed against equal volume of buffer at 4°C. Equal concentrations of radioactivity were found inside and outside dialysis tubing in buffer control in which Dig-H^3 had initially been placed outside tubing.
* Preimmunization serum from rabbit DB-2 was severely hemolyzed.

antigens (Table 2). The digoxin-binding capacity of anti-BSA-Dig serum DB-9 was not removed by prior absorption with BSA but was substantially diminished after absorption with BSA-Dig.[14] Chromatographically separated γ-globulin from anti-BSA-Dig sera possessed strong Dig-H^3 binding capacity, while γ-globulins from normal rabbit sera and from rabbit antisera to other antigens lacked this capacity (Tables 3 and 4).

Nonradioactive digoxin was, as expected, a very potent inhibitor of the binding of Dig-H^3 by anti-BSA-Dig sera. In Figure 1 is plotted the inhibition observed

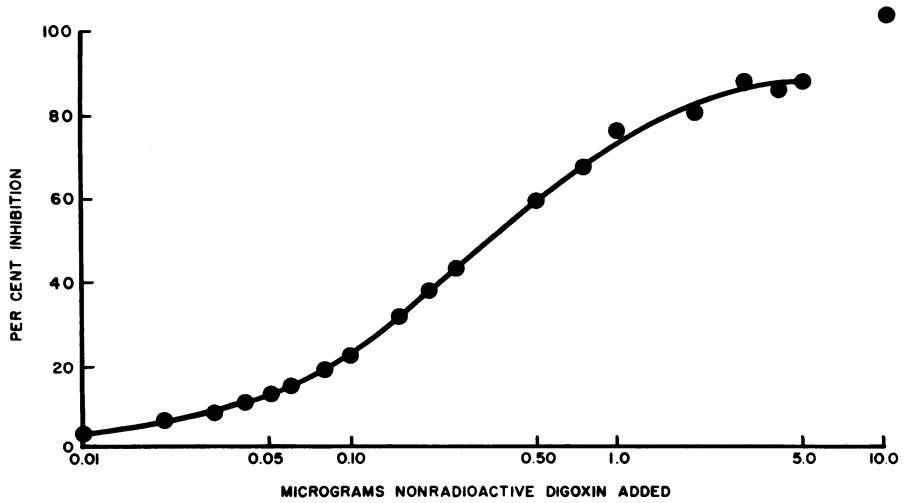

FIG. 1.—Inhibition by nonradioactive digoxin of binding of 0.03 μc Dig-H^3 by 10 ml anti-BSA-Dig serum DB-6 (1:640 dilution). The 3067 cts/10 min/0.5 ml outside the dialysis bag in the buffer control was used as a theoretical 100% inhibition value; 541 cts/10 min/0.5 ml outside the bag in the presence of antibody and in the absence of nonradioactive digoxin as an inhibitor was used as a 0% inhibition value.

TABLE 2

PERCENTAGE OF 0.03 μC DIG-H³ BOUND BY 10 ML OF 1:25 DILUTIONS OF VARIOUS RABBIT ANTISERA

Serum	Per cent bound	Serum	Per cent bound	Serum	Per cent bound
Anti-BSA-3	0.8	Anti-HGG-4	3.0	Anti-Pur-BSA-E22-29	0.0
Anti-HSA-1	1.8	Anti-HEA-1	1.4	Anti-Pur-HSA-E22-42	1.6
Anti-HSA-3	0.4	Anti-HEA-2,3	3.0	Anti-Glu-BSA, anti-Gal-BSA-E289	0.2
Anti-WHS-5	1.4				
Anti-BSA-Dig-DB-8	98.2			Anti-BSA-Dig-DB-9	98.4

TABLE 3

BINDING OF TRITIATED DIGOXIN BY RABBIT γ-GLOBULIN

γ-Globulin		Per cent radioactivity recovered	Cts/10 min/0.5 ml		Recovered Radioactivity		
Rabbit serum	Conc. (μg N/ml)		Inside	Outside	Percentage Inside	Outside	Per cent protein-bound
DB-9 Anti-BSA-Dig	25	100.1	7222	85	98.8	1.2	97.6
BSA-2 Anti-BSA	37	101.2	3768	3623	51.0	49.0	2.0
DB-16X Preimmune	31	98.4	3681	3504	51.2	48.8	2.4
Buffer control		97.6	3555	3573	49.9	50.1	
DB-14 Anti-BSA-Dig	33	98.8	7193	52	99.3	0.7	98.6
E22-42 Anti-Pur-HSA	46	100.1	3677	3662	50.1	49.9	0.2
AN-14X Nonimmune	30	97.1	3608	3510	50.7	49.3	1.4
Buffer control		98.3	3664	3540	50.9	49.1	

Chromatographically separated γ-globulin was dialyzed against equal volume of buffer containing Dig-H³ (0.003 μc/ml). Comparable values were obtained when Dig-H³ was added to dialysis bag together with γ-globulin and dialyzed against buffer (Table 4), but recovery of radioactivity was less satisfactory.

TABLE 4

PER CENT BINDING OF TRITIATED STEROIDS BY RABBIT γ-GLOBULIN

γ-Globulin			H³-Digoxin (0.003 μc/ml)		H³-Cortisone (0.006 μc/ml)		H³-Hydrocortisone (0.006 μc/ml)		H³-Dehydroepiandrosterone (0.005 μc/ml)		H³-Dehydroepiandrosterone sulfate (0.007 μc/ml)	
Rabbit serum		μg N/ml	O	I	O	I	O	I	O	I	O	I
DB-8	Anti-BSA-Dig	31	99.0	99.4	58.8	57.4	44.2	46.6	22.2	23.6	19.6	19.0
DB-9	Anti-BSA-Dig	25	97.6	N.D.	34.0	33.2	32.2	23.4	72.4	69.2	75.2	N.D.
DB-14	Anti-BSA-Dig	33	98.6	98.4	25.0	23.6	7.0	6.6	10.0	7.6	6.4	6.2
BSA-2	Anti-BSA	37	2.0	0.6	1.4	0.0	0.6	2.0	0.0	0.0	0.0	1.0
E22-42	Anti-Pur-HSA	46	0.2	2.4	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0
TCP-23	Anti-Pur-BSA	31	3.2	8.2	1.8	1.0	1.6	2.6	1.4	1.4	1.6	1.6
DB-16X	Pre-immune	31	2.4	3.0	0.0	0.0	0.0	1.6	1.8	0.2	0.0	0.0
AN-8X	Non-immune	30	2.2	0.0	0.0	3.0	0.6	1.6	4.0	0.0	0.0	2.0
AN-14X	Non-immune	30	1.4	3.0	0.2	2.8	3.2	0.0	2.2	0.0	1.6	0.0

Values recorded are percentages of recovered radioactivity bound to protein when tritiated steroid, in the concentrations noted, was added outside (O) or inside (I) a dialysis bag containing chromatographically separated γ-globulin. N.D. = Not done.

TABLE 5

PER CENT INHIBITION BY VARIOUS STEROIDS OF THE BINDING OF TRITIATED DIGOXIN (0.006 μC/ML) BY A 1:640 DILUTION OF ANTI-BSA-DIG SERUM DB-6

Inhibitor	Mol wt[18]	0.31 μg	0.6 μg	1.25 μg	2.50 μg	5.0 μg	10 μg
Digoxin	(780.9)	43.9	51.9	55.3	81.2	84.6	94.9
Digitoxin	(764.9)	29.1	28.5	26.1	37.7	37.8	55.4
Oubain octahydrate	(728.8)	11.1	5.7	14.6	13.6	20.1	12.1
Corticosterone-21-acetate	(388.5)				7.0	7.0	14.1
Cortisone-21-acetate	(402.5)				1.9	4.9	1.5
Cholesterol	(386.6)				2.1	0.4	5.5

when increasing amounts of nonradioactive digoxin were added to a constant amount of anti-BSA-Dig serum and tritiated digoxin.

The inhibitory capacity of digoxin was compared with that of digitoxin which lacks the C_{12} hydroxyl group of digoxin,[18] but is otherwise chemically identical. Digitoxin has some inhibitory capacity, but, on a molar basis, digoxin is about ten times as effective in producing 50 per cent inhibition of the reaction between Dig-H^3 and anti-BSA-Dig serum DB-6 (Table 5). Under similar conditions, oubain, a more distantly related cardiac glycoside, exerted slight inhibition. Cortisone, corticosterone, and cholesterol also appeared to possess minimal inhibitory activity (Table 5), but it was not possible to be certain that this inhibition was significant. The cross-reactivity of antidigoxin antibodies was conclusively demonstrated by showing that γ-globulin from anti-BSA-Dig sera, but not from various control sera, possessed a significant capacity to bind tritiated cortisone, hydrocortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate (Table 4).

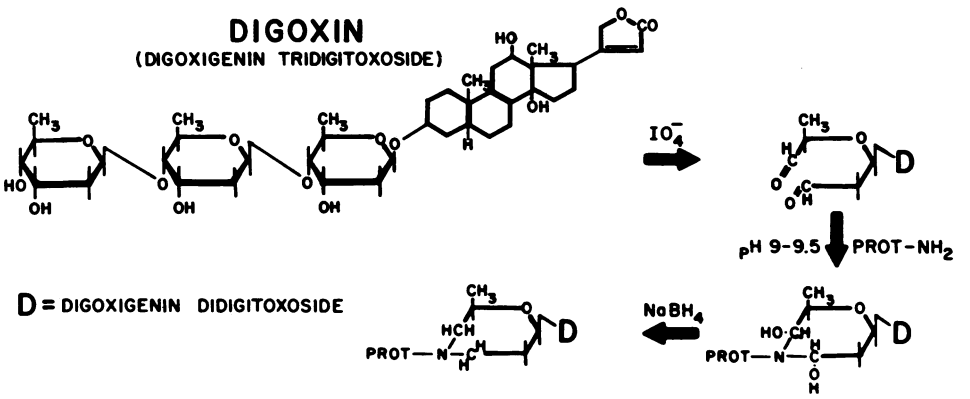

FIG. 2.—Proposed method of conjugation of digoxin to protein based on studies of Khym,[5] Erlanger and Beiser,[4] and Brown and Read[19] (*PROT* = protein backbone in which $-NH_2$ is supplied by lysine residues).

Discussion.—The digoxin-containing antigen used in these studies was effective in eliciting digoxin-specific antibodies but the nature of the binding of digoxin to protein has not yet been chemically defined. The terminal digitoxose of digoxin (Fig. 2) contains two vicinal hydroxyl groups which, if oxidized by periodate, would theoretically be capable of reacting with free amino groups of protein. The possible reaction mechanism shown in Figure 2 is slightly modified[19] from that proposed by Erlanger and Beiser[4] for the conjugation of periodate-sensitive ribose derivatives to protein. In the case of digoxin, however, the lactone ring of the aglycone conceivably could react also with free amino groups under the alkaline conditions employed; this and other possible reaction mechanisms cannot be ruled out until hydrolysis of the digoxin-protein conjugate and chemical identification of the hydrolytic products have been performed.

Definitive evidence for the presence of digoxin-specific antibodies in most anti-BSA-Dig sera was obtained in equilibrium dialysis experiments. It is clear from the data presented in Table 1 that anti-BSA-Dig sera possess a potent digoxin-

binding capacity; this capacity was not present in preimmunization sera and, as seen in Table 3, it was a property of the γ-globulin fraction of anti-BSA-Dig sera. It was not a nonspecific result of immunization because antisera to BSA or other antigens and γ-globulin fractions derived from these antisera lacked this digoxin-binding capacity (Tables 2–4). The digoxin-specificity of these antisera was further confirmed by the demonstrations that digoxin is a potent inhibitor of the binding of tritiated digoxin by anti-BSA-Dig serum (Fig. 1) and that, on a molar basis, digoxin is a far more effective inhibitor of this binding than the other cardiac glycosides and steroids studied (Table 5).

Like antibodies to steroid haptens,[20–23] digoxin-specific antibodies cross-react with other steroids. The cross-reactivity with digitoxin was anticipated in view of its close chemical similarity to digoxin, and the cross-reaction was readily apparent in inhibition experiments (Table 5). The cross-reactivity with steroids was not conclusively demonstrated in these experiments but was apparent (Table 4) when the binding of tritiated steroid hormones to relatively large amounts of γ-globulin from anti-BSA-Dig sera was directly demonstrated.

This communication contains the first known description of the experimental production of antibodies with specificity for cardiac glycosides. Recently, the undiluted sera of two digitoxin-treated patients (one with digitoxin-induced thrombocytopenic purpura, the other with increased dosage requirements) were found to contain 10–18 per cent of a given amount of added digitoxin-H^3 in electrophoretically separated γ-globulin fractions, whereas normal sera contained 4–7 per cent in their γ-globulins after similar starch gel electrophoresis.[24] This digitoxin-binding capacity, while clinically important, is too weak to be of practical value in the studies for which the antibodies described in the current report were prepared.

It is hoped that the ability of digoxin to inhibit the binding of tritiated digoxin (Fig. 1) by anti-BSA-Dig may prove useful in the development of an immunological assay for digoxin in plasma and other biological fluids. Current evidence, based on a biological assay, suggests that digoxin levels in the plasma of fully digitalized patients are less than 0.005 μg/ml.[25] This concentration is obviously less than that detectable by the immunological assay used in the current study. It should be possible, however, to extract the digoxin from 10–50 ml of plasma with chloroform[26] or some other suitable solvent prior to dissolving it in a small volume of ethanol: aqueous buffer medium suitable for use in an immunological assay. It also may be necessary to use another assay system such as chromatoelectrophoresis,[27] which, if more sensitive, would also provide the additional desirable characteristic of rapidity of analysis.

The extent to which the cross-reaction of anti-Dig antibodies with plasma steroids may interfere with an immunological assay for digoxin remains to be determined. Such interference, should it be significant, could be minimized by employing an extraction method which effectively separates glycosides from plasma steroids. Alternatively, it might be possible to absorb cross-reacting antibodies with steroid protein conjugates[20–23] or to inhibit the cross-reacting antibodies by performing all determinations in a medium containing an excess of steroids added *in vitro*.

Antibodies to steroid hormone haptens interfere with the physiological action of the corresponding steroid hormones,[28, 29] and thus it is reasonable to assume that digoxin-specific antibodies may interfere with the pharmacological effects of digoxin.

Experiments have been instituted to determine whether or not anti-BSA-Dig antibodies will prevent or ameliorate the electrocardiographic manifestations of digitalis toxicity in intact animals. The potential clinical value of digoxin-binding antibodies in patients with severe digoxin overdosage, particularly of the accidental variety,[30] is obvious.

Digoxin-specific antibodies may also be useful in the study of the mechanism of action of digoxin on the myocardium and on renal tubular epithelium. Several workers have demonstrated tritiated digoxin in the A-band of cardiac myofibrils by electron microscopy,[31-33] but physiological studies of cation flux[34] suggest that a more important site of action may be at the membrane level. The rapid disappearance of digoxin action following addition of antibody to a medium containing muscle cells might lend support to the hypothesis that the observed action of digoxin at the cell membrane level is more important pharmacologically than its apparent anatomical localization in the myofibril. Such studies have been initiated recently.

Summary.—Rabbits immunized with a synthetic protein-digoxin conjugate formed antibodies capable of specific binding of tritiated digoxin. Nonradioactive digoxin inhibited the binding of tritiated digoxin by antidigoxin antibody, and this inhibitory capacity of nonradioactive digoxin may prove useful in the development of an immunological assay for digoxin in plasma and biological fluids. The possible value of digoxin-specific antibodies in the treatment of experimental digoxin toxicity and in the study of mechanism of digoxin action has been discussed.

We are indebted to Dr. B. F. Erlanger, Columbia University, who suggested the conjugation method; to Dr. S. Bloomfield, Burroughs Wellcome and Company, who generously provided the nonradioactive and tritiated digoxin; and to Dr. W. Drucker, New York University School of Medicine, who purified the other tritiated steroids. Drs. C. Ragan, A. M. Briscoe, and S. M. Beiser gave helpful suggestions, and Miss Diane Lepore and Miss Ann Wargo provided superb technical assistance.

* Supported by USPHS grants AM-07183 and HE-10608 and gifts from the Strick Foundation and the Vermont Heart Association.

† Senior investigator, the Arthritis Foundation.

[1] Moe, G. K., and A. E. Farah, in *The Pharmacological Basis of Therapeutics*, ed. L. S. Goodman and A. Gilman (New York: Macmillan Co., 1965) 3rd ed., p. 665.

[2] Landsteiner, K., *The Specificity of Serological Reactions* (Cambridge, Mass.: Harvard University Press, 1945).

[3] Smith, S., *J. Chem. Soc.*, 508 (1930).

[4] Erlanger, B. F., and S. M. Beiser, these PROCEEDINGS, **52**, 68 (1964).

[5] Khym, J. X., *Biochemistry*, **2**, 344 (1963).

[6] Brown, B. T., and S. E. Wright, *J. Am. Pharm. Assn., Sci. Ed.*, **49**, 777 (1960).

[7] Butler, V. P., S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, these PROCEEDINGS, **48**, 1597 (1962).

[8] Mage, M., E. W. Bassett, S. W. Tanenbaum, and S. M. Beiser, *J. Immunol.*, **90**, 318 (1963).

[9] Kabat, E. A., *Kabat and Mayer's Experimental Immunochemistry* (Springfield, Ill.: Charles C Thomas, 1961).

[10] Eisen, H. N., *Methods Med. Res.*, **10**, 106 (1964).

[11] Butler, V. P., S. W. Tanenbaum, and S. M. Beiser, *J. Exptl. Med.*, **121**, 19 (1965).

[12] Tomasi, T., and H. G. Kunkel, *Methods Med. Res.*, **10**, 80 (1964).

[13] Stanworth, D. R., *Nature*, **188**, 156 (1960).

[14] Butler, V. P., to be published.

[15] Fawaz, G., and A. Farah, *J. Pharmacol. Exptl. Therap.*, **80**, 193 (1944).

[16] Lukas, D. S., and R. E. Peterson, *J. Clin. Invest.*, **45**, 782 (1966).

[17] Slaunwhite, W. R., H. Rosenthal, and A. A. Sandberg, *Arch. Biochem. Biophys.*, **100**, 486 (1963).
[18] The Merck Index of Chemicals and Drugs, 7th ed., Merck & Co., Rahway, N. J. (1960).
[19] Brown, D. M., and A. P. Read, *J. Chem. Soc.*, 5072 (1965).
[20] Beiser, S. M., B. F. Erlanger, F. J. Agate, and S. Lieberman, *Science*, **129**, 564 (1959).
[21] Lieberman, S., B. F. Erlanger, S. M. Beiser, and F. J. Agate, *Recent Progr. Hormone Res.*, **15**, 165 (1959).
[22] Goodfriend, L., and A. H. Sehon, *Can. J. Biochem. Physiol.*, **39**, 941 (1961).
[23] Klopstock, A., M. Pinto, and A. Rimon, *J. Immunol.*, **92**, 515 (1964).
[24] Young, R. C., R. L. Nachman, and H. I. Horowitz, *Am. J. Med.*, **41**, 605 (1966).
[25] Lowenstein, J. M., *Circulation*, **31**, 228 (1965).
[26] Jelliffe, R. W., *J. Lab. Clin. Med.*, **67**, 694 (1966).
[27] Yalow, R. S., and S. A. Berson, *Meth. Biochem. Anal.*, **12**, 69 (1964).
[28] Goodfriend, L., and A. H. Sehon, *op. cit.*, p. 961.
[29] Neri, R. O., S. Tolksdorf, S. M. Beiser, B. F. Erlanger, F. J. Agate, and S. Lieberman, *Endocrinology*, **74**, 593 (1964).
[30] Fowler, R. S., L. Rathi, and J. D. Keith, *J. Pediat.*, **64**, 188 (1964).
[31] Smith, J. R., and H. A. Fozzard, *Nature*, **197**, 562 (1963).
[32] Tubbs, F. E., L. Crevasse, and M. W. Wheat, *Circulation Res.*, **14**, 236 (1964).
[33] Conrad, L. L., and D. J. Baxter, *J. Pharmacol. Exptl. Therap.*, **145**, 210 (1964).
[34] Glynn, I. M., *Pharmacol. Rev.*, **16**, 381 (1964).