PERSISTENCE OF HAPten-ANTIBODY COMPLEXES IN THE CIRCULATION OF IMMUNIZED ANIMALS AFTER A SINGLE INTRAVENOUS INJECTION OF HAPten*

BY DONALD H. SCHMIDT, BETTE M. KAUFMAN, AND VINCENT P. BUTLER, JR.‡

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032)

(Received for publication 2 October 1973)

When injected intravenously into immunized animals, proteins and other macro-molecular antigens form complexes with their corresponding antibodies (2, 3), and the resulting antigen-antibody complexes are rapidly removed from the circulation and deposited in various tissue sites (4). When injected into immunized animals, low molecular weight antigens or haptens are also thought to form complexes with their corresponding antibodies (5), but little is known about the fate of these presumed complexes.

We have previously reported that the intravenous administration of a hapten, digoxin, into rabbits whose sera contained digoxin-specific antibodies resulted in a prompt decrease in antidigoxin antibody titer which persisted for at least 1 wk (5). It was considered likely that the fall in antibody titer reflected the formation of digoxin-antibody complexes, but no evidence in support of this hypothesis was obtained and no information was available to determine whether such complexes, if present, persisted in the circulation of the rabbit for prolonged periods or were rapidly cleared from the circulation in the manner in which protein-antibody complexes are cleared.

We now present evidence that, when injected into immunized animals, a specific hapten, namely digoxin, indeed forms complexes with its corresponding antibodies and that these complexes persist in the circulation for longer than 1 yr after a single intravenous injection of that hapten.

Materials and Methods

Reagents.—BSA1 was obtained as Fraction V from Pentex Biochemical, Kankakee, Ill. Non-radioactive digoxin was supplied in crystalline form and in a 40% propylene glycol-10%

* These studies were supported by U.S. Public Health Service Grants HL-10608, HL-02001, HL-05741, and by grants-in-aid from the New York Heart Association and the American Heart Association (72-853). This work was presented in part to the Immunology Section of the Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N.J., April 30, 1972 (1).

† Recipient of an Irma T. Hirschl Career Scientist Award; formerly, recipient of Research Career Development Award (HL-11315) from the National Heart and Lung Institute.

1 Abbreviations used in this paper: anti-RGG, anti-rabbit γ-globulin; BSA, bovine serum albumin; and BSA-Dig, BSA-digoxin.
ethanol solution (0.25 mg/ml) through the courtesy of Dr. Stanley T. Bloomfield, Burroughs Wellcome & Co., Inc., Research Triangle Park, N.C. Two lots of randomly labeled digoxin-\(^{3}H\) were employed in this study: one lot (R907, 144 \(\mu\)Ci/mg in 95% ethanol, supplied by Dr. Bloomfield) was used in all animal studies; while the other lot (185-165, 15.3 mCi/mg in 90% ethanol, 10% benzene; New England Nuclear Corp., Boston, Mass.) was used in digoxin-binding antibody determinations. 3-\(\alpha\)-Sucinyl digoxigenin tyrosine\(^{125}\)I was obtained in 1% acetic acid in methanol from Schwarz/Mann Laboratories, Orangeburg, N.Y. \(^{125}\)I (carrier-free) and \(^{131}\)I (35-50 mCi/\(\mu\)g) were obtained as NaI in NaOH from the Cambridge Nuclear Corp., Billerica, Mass. All other reagents were commercial products of the highest degree of purity available.

**Immunological.**—Synthetic BSA-digoxin (BSA-Dig) conjugates were prepared by the periodate oxidation method (6, 7) as described in detail elsewhere (8). White New Zealand rabbits were immunized with BSA or with a BSA-Dig conjugate in complete Freund's adjuvant mixture, as previously described (5). The duration of immunization and the number of antigen injections are listed in Table I. The presence of digoxin-binding antibodies was determined in serum obtained during the 24 h period prior to the intravenous administration of digoxin, by the dextran-coated charcoal method (9, 10) as follows: to 1 ml portions of buffered albumin (5) were added 50 
\(\mu\)l of various dilutions of test serum followed by 50 
\(\mu\)l of digoxin-\(^{3}H\) (30 ng/ml; 0.5 \(\mu\)Ci/ml). 10 min later, 0.25 ml of dextran T-70-coated charcoal (2% dextran, 20% charcoal in Tris-buffered saline) was added. The separation and counting of protein-bound digoxin-SH were carried out as previously described (5). Digoxin-binding antibody titers, which are functions of both antibody affinity and concentration, are expressed as the dilutions of antiserum (calculated by extrapolation and rounded off to the nearest unit divisible by 100), 50 
\(\mu\)l of which was capable of binding 50% of the added digoxin-\(^{3}H\).

**Measurement of Complement Levels.**—Serum complement levels were determined using the method of Kent et al. (11).

**Rabbit \(\gamma\)-Globulin and Antidigoxin Antibody Purification and Radiiodination.**—\(\gamma\)-Globulin from the serum of nonimmunized rabbits was purified by DEAE-cellulose chromatography (12), as previously described (6, 13). Antidigoxin antibody was purified from rabbit antidigoxin serum by the method of Curd et al. (14). Both preparations contained no detectable proteins.

| Rabbit no. | Immunizing antigen | Duration of immunization | No. of antigen injections\(^a\) | Digoxin-binding antibody titer\(^b\) | Weight \(kg\) |
|-----------|---------------------|-------------------------|-----------------------------|---------------------------------|-------------|
| DC-20     | BSA-Dig             | 33                      | 13                          | 1:1,600                         | 3.93        |
| DC-23     | BSA-Dig             | 33                      | 12                          | 1:600                           | 4.00        |
| DC-24     | BSA-Dig             | 32                      | 12                          | 1:800                           | 4.29        |
| DC-25     | BSA-Dig             | 28                      | 11                          | 1:800                           | 3.83        |
| DC-26     | BSA-Dig             | 28                      | 11                          | 1:2,400                         | 3.65        |
| BSA-20    | BSA                 | 23                      | 10                          | 0\(\S\)                         | 5.93        |
| BSA-26    | BSA                 | 58                      | 20                          | 0\(\S\)                         | 4.60        |
| BSA-33    | BSA                 | 72                      | 21                          | 0\(\S\)                         | 4.58        |
| BSA-37    | BSA                 | 72                      | 21                          | 0\(\S\)                         | 4.72        |

\(^a\) No further antigen injections were given after the single intravenous dose of digoxin-\(^{3}H\) was administered.

\(^b\) See Materials and Methods for definition of digoxin-binding antibody titer.

\(\S\) No detectable binding at a dilution of 1:20.
other than γ-globulin as determined by cellulose acetate electrophoresis and immunoelectrophoresis. The rabbit γ-globulin was radioiodinated with 125I and the antidigoxin antibody was radiiodinated with 125I by the chloramine T method (15). The antidigoxin antibody retained its capacity to bind digoxin as determined by its capacity to bind 3-O-succinyl digoxigenin tyrosine-125I before and after purification and radioiodination. Radioiodinated γ-globulin and antidigoxin antibody were diluted in 0.15 M phosphate-buffered saline solution, pH 7.4 (16), and centrifuged at 40,000 rpm for 90 min (Spinco model L-2 ultracentrifuge, Spinco Division, Beckman Instrument Co., Palo Alto, Calif.) to remove aggregated material (17), prior to use in animal experiments.

Metabolic Studies.---

Digoxin: Digoxin-3H for injection was prepared as follows: to 1 ml of digoxin-3H (lot R907, 1 mg/ml in 95% ethanol) were added 4 ml of nonradioactive digoxin solution (0.25 mg/ml) and 20 ml 0.85% NaCl; 1 ml of the resulting solution contained 0.08 mg digoxin and 5.76 μCi. Unanesthetized rabbits whose weights ranged between 2.58 and 5.93 kg were given 5 ml of this digoxin solution per kilogram of body weight (0.4 mg digoxin per kilogram). Following the administration of digoxin the rabbits were placed in metabolic cages and fed a regular diet. Serum samples were obtained at 12 and 24 h and daily thereafter up to 10 days. Rabbits whose serum contained digoxin-specific antibodies were bled daily for 4 additional days and then bled every 3 days for 2 additional weeks, weekly for 1 mo, and then every 2 wk until the animals died or were sacrificed. Urine samples were collected daily for the first 10 days in all animals studied. The total volume of each sample was determined, following which a 10 ml portion was removed and frozen for subsequent analysis. Stool samples were also collected daily for 10 days. The daily weight was recorded and the specimens stored at 4°C until analyzed.

Digoxin concentration measured as total radioactivity was determined in serum, urine, and stool samples. The amount of radioactivity in various specimens was determined by counting in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Internal standards of digoxin-3H were added to each sample and the samples recounted to correct for quenching.

Serum and urine radioactivity were measured by the addition of 50 μl to 15 ml of a modified Bray's scintillation mixture (13), heating at 60°C for 10 min (10), and then counting. Fecal radioactivity was determined as follows: each 24-h collection of feces was added to 50-150 ml deionized water and homogenized in a model "45" homogenizer, (The Virtis Co., Inc., Gardiner, N.Y.). The homogenate was then lyophilized. The dry weight of the homogenate was determined. A 20-50 mg sample was then combusted (Packard Tri-Carb model 305 tissue oxidizer, Packard Instrument Co., Inc.) and transferred to 15 ml of a scintillation mixture containing 300 mg 1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene, 5 g 2,5-diphenyloxazole, 100 g naphthalene, 45 ml absolute methanol, 135 ml toluene, and 720 ml dioxane. The samples were then assayed for radioactivity in the liquid scintillation spectrometer, as described above.

The radioactivity in the samples analyzed was expressed in microcuries on the basis of observed counting efficiency (which varied from 23 to 31% in various samples), and then calculated as nanograms digoxin on the basis of the specific activity of the administered glycoside (72 μCi/mg). It was assumed that metabolic degradation of the digoxin-3H was minimal (18) and therefore no correction was made for the presence of radioactive metabolic degradation products in the specimens analyzed.

The radioactivity in the samples analyzed was expressed in microcuries on the basis of observed counting efficiency (which varied from 23 to 31% in various samples), and then calculated as nanograms digoxin on the basis of the specific activity of the administered glycoside (72 μCi/mg). It was assumed that metabolic degradation of the digoxin-3H was minimal (18) and therefore no correction was made for the presence of radioactive metabolic degradation products in the specimens analyzed.

The serum radioactivity was plotted on a logarithmic scale (ordinate) against time on a linear scale (abscissa) and followed until the curve became monoeponential (19). This final linear portion of the curve was used to compute the half time of the slowest component of the serum digoxin disappearance curve (20). It is thought that this slowest component is predominantly influenced by metabolism and excretion of digoxin (21). The data was then also analyzed by a digital computer technique for nonlinear regression analysis by which parameters were estimated by a least squares procedure (22). This allowed accurate compartmental analysis of the data by dividing the curves into exponential functions.
Radioiodinated γ-globulin and antibody: 131I-γ-globulin (5 mCi/kg body weight) and 125I-antidigoxin antibody (15 mCi/kg) were administered in 10 ml 0.15 M phosphate-buffered saline to three groups of animals: one group of nonimmunized animals received only 131I-γ-globulin and 125I-antidigoxin antibody; the second group of nonimmunized animals received, in addition, 0.25 mg digoxin and 8 ml of rabbit antidigoxin serum (digoxin-binding titer 1:2000); the third group of digoxin-immunized rabbits were given autologous 125I-antidigoxin antibody and 0.25 mg digoxin in addition to 131I-γ-globulin. The disappearance rates of radio-labeled γ-globulin and antidigoxin antibody were determined by measuring serum radioactivity in an Auto-Gamma spectrometer (Packard Instrument Co., Inc.) on specimens obtained at 1, 6, and 24 h, and daily thereafter for a period of 3 wk. Corrections were made for background, decay during counting, and channel crossover. This data was also analyzed by computer as described for digoxin, and the curves divided into exponential functions. These curves were all described by two exponentials. The t1/2 of the slower exponential was considered as the more important in assessing the metabolism of γ-globulin and of antibody.

Immune Complexes.—The dextran-coated charcoal and double antibody methods were used to document the presence of hapten-protein complexes in serum.

The dextran-coated charcoal method (5, 9, 10) was carried out as follows: to each of four tubes, 1.0 ml of a 1:20 dilution of the test serum in 0.35% buffered albumin (5) was delivered. To two tubes, 0.25 ml of dextran-coated charcoal (2% dextran T-70, 20% charcoal in Tris-buffered saline, pH 7.4) was added and to two control tubes was added 0.25 ml buffered albumin (5). The contents of the test tubes were mixed and then centrifuged at 2,000 rpm for 60 min at 4°C. The supernatant was then poured out into scintillation vials to which 15 ml of modified Bray's liquid scintillation counting solution (13) was added, and the vials were then counted as described above for serum samples. Protein-binding was expressed as the amount of digoxin (radioactivity) present in the supernatant of the tubes to which dextran-coated charcoal had been added, divided by the total amount of digoxin in the test sera, as determined in control tubes containing buffered albumin without charcoal.

The double antibody method (23) was utilized to determine whether or not the digoxin was immunoglobulin bound. Test serum (50 μl of a 1:4 dilution in buffered albumin) was added to duplicate to 1.0 ml of sheep anti-rabbit γ-globulin (anti-RGG), employed as whole serum or as a globulin fraction prepared by the sodium sulfate method (24). The tubes were placed in a 37°C water bath for 1 h, refrigerated at 4°C for 5 days, and then centrifuged for 1 h at 4°C and 2,000 rpm. The precipitates were washed three times with chilled 0.85% NaCl, digested in 0.5 ml Soluene (Packard Instrument Co., Inc.), transferred to scintillation vials with the aid of two 5 ml portions of Dimilume (Packard Instrument Co., Inc.) and then counted as described above for serum samples. Protein-binding was expressed as the amount of digoxin (radioactivity) present in the supernatant of the tubes to which dextran-coated charcoal had been added, divided by the total amount of digoxin in the test sera, as determined in control tubes containing buffered albumin without charcoal.

The double antibody method (23) was utilized to determine whether or not the digoxin was immunoglobulin bound. Test serum (50 μl of a 1:4 dilution in buffered albumin) was added in duplicate to 1.0 ml of sheep anti-rabbit γ-globulin (anti-RGG), employed as whole serum or as a globulin fraction prepared by the sodium sulfate method (24). The tubes were placed in a 37°C water bath for 1 h, refrigerated at 4°C for 5 days, and then centrifuged for 1 h at 4°C and 2,000 rpm. The precipitates were washed three times with chilled 0.85% NaCl, digested in 0.5 ml Soluene (Packard Instrument Co., Inc.), transferred to scintillation vials with the aid of two 5 ml portions of Dimilume (Packard Instrument Co., Inc.) and then counted as described above for serum samples. Protein-binding was expressed as the amount of digoxin (radioactivity) present in the supernatant of the tubes to which dextran-coated charcoal had been added, divided by the total amount of digoxin in the test sera, as determined in control tubes containing buffered albumin without charcoal.

The double antibody method (23) was utilized to determine whether or not the digoxin was immunoglobulin bound. Test serum (50 μl of a 1:4 dilution in buffered albumin) was added in duplicate to 1.0 ml of sheep anti-rabbit γ-globulin (anti-RGG), employed as whole serum or as a globulin fraction prepared by the sodium sulfate method (24). The tubes were placed in a 37°C water bath for 1 h, refrigerated at 4°C for 5 days, and then centrifuged for 1 h at 4°C and 2,000 rpm. The precipitates were washed three times with chilled 0.85% NaCl, digested in 0.5 ml Soluene (Packard Instrument Co., Inc.), transferred to scintillation vials with the aid of two 5 ml portions of Dimilume (Packard Instrument Co., Inc.) and then counted as described above for serum samples. Protein-binding was expressed as the amount of digoxin (radioactivity) present in the supernatant of the tubes to which dextran-coated charcoal had been added, divided by the total amount of digoxin in the test sera, as determined in control tubes containing buffered albumin without charcoal.

Identification of Digoxin.—To demonstrate that the tritium radioactivity in certain test sera reflected the persistence, at least in part, of digoxin-3H, 1.0 ml 25% ethanol was added to 1.0 ml serum; the solution then was passed over a 2 × 100 cm column of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, Calif.) suspended in 25% ethanol. Fractions containing tritium (early protein-containing fractions in the case of antidigoxin sera, or late protein-free fractions in the case of normal control sera) were pooled, lyophilized, and resuspended in 95% ethanol. Following centrifugation to remove ethanol-insoluble material (principally protein), the supernatant solution was concentrated by evaporation, resuspended in 95% ethanol, and again centrifuged to remove precipitated material. After a second evaporation step, 50 μl nonradio-
active digoxin (3 ng/ml in 95% ethanol) was added. A 10 μl sample was removed for counting, and the remaining 40 μl was subjected to thin layer chromatography on Silica Gel G (Analtech, Inc., Wilmington, Del.) as previously described (13). Test plates were scraped in 0.5-cm segments, and the fractions thus obtained were combusted, counted in a scintillation spectrometer, and a zonal profile drawn. Results were expressed as percentages of recovered radioactivity found in the digoxin peak on the thin layer chromatogram.

RESULTS

Disappearance of Serum Digoxin.—In Fig. 1 a representative computer-fitted serum digoxin-3H disappearance curve observed in one of nine nonimmunized rabbits is plotted together with a representative disappearance curve from one of four additional control rabbits immunized with BSA. The serum t1/2 in the nonimmunized rabbit illustrated in Fig. 1 was 2.8 days and the dominant t1/2 in the rabbit immunized with BSA was 8.6 days (Table II).

In the nine nonimmunized rabbits (Table II), the mean 12-h serum digoxin concentration was 92 ± 6 ng/ml and the serum t1/2 was 3.4 ± 0.7 days. The mean 12-h serum digoxin concentration in the four rabbits immunized with BSA (Table II) was somewhat higher, 126 ng/ml, and the dominant t1/2 somewhat longer, 9.4 days, than the values observed in nonimmunized animals.

In Fig. 2, the serum disappearance curve of digoxin-3H in each of five individual digoxin-immunized rabbits is illustrated. The sera from all five rabbits contained antidigoxin antibodies in high titer (Table I). 12-h serum digoxin concentrations were almost 100 times greater than those observed in control

---

**Fig. 1.** Representative computer-fitted disappearance curves of serum digoxin-3H in two control animals: D8, a nonimmunized rabbit (○); and BSA 37, which had been immunized with unconjugated BSA (●).
TABLE II

Serum Concentrations and Serum Disappearance Rates of Digoxin-3H

| Rabbit no. | 12-hr serum concentration (ng/ml) | Half-time of exponential (days)* | Length of study (days) |
|------------|----------------------------------|---------------------------------|-----------------------|
| 1st        | 2nd                             | 3rd                             |                       |
| Nonimmunized |                                 |                                 |                       |
| D-1        | 94                              | 3.38                            | 10                    |
| D-3        | 89                              | 4.54                            | 10                    |
| D-4        | 60                              | 2.64                            | 10                    |
| D-5        | 94                              | 2.69                            | 10                    |
| D-6        | 114                             | 3.88                            | 10                    |
| D-7        | 92                              | 4.11                            | 28                    |
| D-8        | 71                              | 2.82                            | 16                    |
| D-9        | 97                              | 3.33                            | 29                    |
| D-10       | 115                             | 2.98                            | 29                    |
| Mean (± SD)| 92 (± 6)                        | 3.37 (± 0.67)                   | 17                    |

Immunized with BSA

- BSA-20: 118 ng/ml, t1/2 0.51 days, 9.39 days, 32 days
- BSA-26: 111 ng/ml, t1/2 0.54 days, 10.09 days, 15 days
- BSA-33: 144 ng/ml, t1/2 0.53 days, 9.47 days, 34 days
- BSA-37: 131 ng/ml, t1/2 0.50 days, 8.63 days, 41 days
- Mean: 126 ng/ml, t1/2 0.52 days, 9.40 days, 31 days

Immunized with BSA-Dig

- DC-20: 9,600 ng/ml, t1/2 0.77 days, 8.19 days, 113.7 days, 330 days
- DC-23: 5,390 ng/ml, t1/2 0.45 days, 6.12 days, 50.5 days, 410 days
- DC-24: 7,800 ng/ml, t1/2 0.5‡ days, 6.4‡ days, 56.9 days, 425 days
- DC-25: 9,500 ng/ml, t1/2 0.6‡ days, 7.5‡ days, - days, 21 days
- DC-26: 9,040 ng/ml, t1/2 0.5‡ days, 7.3‡ days, 67.5 days, 450 days
- Mean: 8,300 ng/ml, t1/2 0.56 days, 7.10 days, 72.2 days, 327 days

* Serum digoxin-3H disappearance was described by a single exponential (compartment) in nonimmunized rabbits, two exponentials in rabbits immunized with BSA, and three exponentials in rabbits immunized with BSA-Dig. A t1/2 was determined for each exponential, although the t1/2 of the final, slowest exponential was considered to be dominant in each group of animals.

‡ Exponential could not be derived from computer analysis; value was obtained graphically.

rabbits and varied between 5,400 and 9,600 ng/ml. In these rabbits the dominant serum half time of digoxin varied from 51 to 114 days.

In Fig. 3, a representative computer-fitted disappearance curve of radioactive digoxin in a normal nonimmunized rabbit is plotted as a function of time in weeks instead of days and compared with a representative computer-fitted disappearance curve of digoxin-3H in a digoxin-immunized rabbit. The rapid disappearance of digoxin in the nonimmunized animal is in marked contrast with the serum digoxin disappearance curve in the rabbit which had been in-
munized with a BSA-Dig conjugate. In five digoxin-immunized rabbits, the serum digoxin disappearance curves were described by three exponentials (compartments). The mean $t_{1/2}$ of the first exponential was 0.56 days, that of the second 7.1 days, and that of the third and dominant exponential was 72 days, which was 21 times longer than the single $t_{1/2}$ of 3.4 days observed in the nonimmunized rabbits (Table II). The mean 12-h serum digoxin concentration in digoxin-immunized rabbits (Table II) was 8,300 ng/ml as compared with 92 ng/ml observed in the control group. At 1 yr, the mean serum digoxin concentration in the digoxin-immunized group had fallen to 85 ng/ml (Fig. 2), a value comparable with the 12-h serum digoxin concentration in nonimmunized animals (Table II).

Excretion of Digoxin.—The per cent of the administered dose of labeled digoxin which was excreted by the rabbits in 10 days is shown in Fig. 4. In nine nonimmunized rabbits, 77% of the total administered dose was excreted in 10 days. In the four rabbits immunized with BSA in whom digoxin-3H had a slightly longer half-life than in the nonimmunized rabbits (Fig. 1), a total of 57% was excreted. In contrast, the five antidigoxin rabbits excreted a mean total of 8% of the administered dose, 6% in the feces and 2% in the urine. This diminished excretion was in keeping with the prolonged serum half-life of the hapten.

Immune Complexes.—To determine whether the persistence of tritiated digoxin in the circulation reflected its presence in complex with antibody in the
serum of digoxin-immunized animals, the binding of radioactivity in the sera of nonimmunized control rabbits and the rabbits immunized with BSA-digoxin was determined (Table III). In the nonimmunized control rabbits bled at 15 and 30 min, a mean of 9% of the labeled digoxin was protein bound as determined by the dextran-coated charcoal method and 2% was γ-globulin-bound as determined by the double antibody method. In contrast, in the rabbits immunized with BSA-Dig, in serum obtained between 12 h and 11 mo after the infusion of digoxin, a mean of 95% of the total serum digoxin was protein-bound as determined by the dextran-coated charcoal method and 95% was bound to immunoglobulin as measured by the double antibody method.

Identification of Digoxin.—In serum obtained from four digoxin-immunized rabbits 4–9 mo after the administration of digoxin-3H, 66% of the recovered radioactivity migrated together with digoxin on thin layer chromatography. In serum obtained from a nonimmunized rabbit 30 min after administration of digoxin-3H, 89% of the recovered radioactivity migrated with digoxin on similar thin layer chromatographic analysis.

Serum Complement.—Sera from three rabbits immunized with BSA-Dig,
PERSISTENCE OF HAPTO-ANTIBODY COMPLEXES

Fig. 4. Percent of administered digoxin-3H excreted in urine and feces in 10 days in three groups of rabbits: nonimmunized, those immunized with BSA, and those immunized with BSA-digoxin.

TABLE III

| Rabbit no. | Time of bleeding | Serum digoxin-3H ng/ml | Dextran-coated charcoal method | Double antibody method* |
|------------|------------------|------------------------|--------------------------------|-------------------------|
|            |                  |                        | %                             | %                       |
| NI-5       | 15 min           | 755                    | 6                             | 1                       |
|            | 30 min           | 589                    | 6                             | 1                       |
| NI-6       | 15 min           | 526                    | 11                            | 2                       |
|            | 30 min           | 357                    | 11                            | 2                       |
| Immunized with BSA-Digoxin |                   |                        |                               |                         |
| DC-20      | 12 h             | 10,339                 | 97                            | 100                     |
|            | 1 mo             | 3,072                  | 96                            | 88                      |
|            | 10 mo            | 698                    | 94                            | 87                      |
| DC-23      | 72 h             | 3,913                  | 88                            | 91                      |
|            | 1 mo             | 1,968                  | 95                            | 97                      |
|            | 9 mo             | 53                     | 100                           | 87                      |
| DC-24      | 48 h             | 5,413                  | 98                            | 99                      |
|            | 2 mo             | 3,082                  | 92                            | 95                      |
|            | 11 mo            | 238                    | 100                           | 89                      |
| DC-25      | 48 h             | 8,329                  | 100                           | 99                      |
|            | 10 days          | 4,733                  | 95                            | 98                      |
| DC-26      | 12 h             | 9,446                  | 94                            | 99                      |
|            | 1 mo             | 3,538                  | 89                            | 99                      |
|            | 9 mo             | 205                    | 92                            | 98                      |

* Test performed on 1:20 dilution of serum.
† Test performed on 1:4 dilution of serum.
obtained 13–14 mo after giving digoxin, contained 60–100 50%-complement units per milliliter, values which were similar to those determined in sera obtained from normal control rabbits and to values reported by other investigators in normal rabbit sera, using a similar method (25).

Disappearance of γ-Globulin and Digoxin-Specific Antibodies.—To determine whether the prolongation of half-life of digoxin in the serum of digoxin-immunized rabbits was accompanied by a prolongation of the half-life of antidigoxin antibody, the disappearance of 131I-γ-globulin and 125I-antidigoxin antibody from the circulation of three groups of rabbits was studied. Computer-fitted curves from one of the rabbits in each group are shown in Fig. 5, and serum disappearance times from all animals studied are listed in Table IV.

In four nonimmunized rabbits given only 131I-γ-globulin and 125I-antidigoxin

![Fig. 5. Representative computer-fitted disappearance curves of serum 125I-antidigoxin antibody (○) and pooled 131I-γ-globulin (○). (A) Rabbit AB-9, a nonimmunized animal, received antidigoxin antibody and γ-globulin without digoxin. (B) Rabbit AB-5, also nonimmunized, received 0.25 mg digoxin together with antidigoxin antibody and γ-globulin. (C) Rabbit DC-37, which had been immunized with BSA-digoxin, received 0.25 mg digoxin together with γ-globulin and autologous antidigoxin antibody.](image-url)
PERSISTENCE OF HAPTEN-ANTIBODY COMPLEXES

TABLE IV

Serum Disappearance Rates of $^{131}$I-Rabbit $\gamma$-Globulin and $^{125}$I-Antidigoxin Antibody

| Rabbit no. | Injectate | Half time of exponential* | Half time of exponential | Half time of exponential | Half time of exponential |
|-----------|-----------|---------------------------|-------------------------|-------------------------|-------------------------|
|           |           | 1st exponential | 2nd exponential | 1st exponential | 2nd exponential |
| AB-2      | $\gamma$-Globulin and antidigoxin antibody | 0.50 | 9.78 | 0.56 | 8.52 |
| AB-6      | $\gamma$-Globulin and antidigoxin antibody | 0.51 | 6.58 | 0.49 | 7.09 |
| AB-9      | $\gamma$-Globulin and antidigoxin antibody | 0.37 | 4.31 | 0.40 | 3.76 |
| AB-10     | $\gamma$-Globulin and antidigoxin antibody | 0.16 | 3.81 | 0.30 | 3.53 |
| Mean      | $\gamma$-Globulin and antidigoxin antibody | 0.39 | 6.12 | 0.44 | 5.73 |

| AB-4      | $\gamma$-Globulin, antidigoxin antibody | 0.47 | 4.29 | 0.40 | 3.93 |
| AB-5      | $\gamma$-Globulin, antidigoxin antibody | 0.37 | 6.30 | 0.28 | 4.82 |
| AB-7      | $\gamma$-Globulin, antidigoxin antibody | 0.38 | 4.17 | 0.21 | 3.65 |
| AB-8      | $\gamma$-Globulin, antidigoxin antibody | 0.57 | 4.02 | —    | —    |
| Mean      | $\gamma$-Globulin, antidigoxin antibody | 0.45 | 4.70 | 0.30 | 4.13 |

| AB-11     | $\gamma$-Globulin, autologous antidigoxin antibody | 0.37 | 3.49 | 0.41 | 3.36 |
| AB-12     | $\gamma$-Globulin, autologous antidigoxin antibody | 0.52 | 4.43 | 0.42 | 3.91 |
| DC-37     | $\gamma$-Globulin, autologous antidigoxin antibody | 0.52 | 5.00 | 0.39 | 3.68 |
| Mean      | $\gamma$-Globulin, autologous antidigoxin antibody | 0.46 | 4.30 | 0.41 | 3.65 |

* The disappearance of radioactivity for both $^{131}$I-$\gamma$-globulin and $^{125}$I-antidigoxin antibody was described as a two-exponential function. The $t_{1/2}$ of the longer exponential was considered to be dominant.

† Exponential could not be obtained from computer analysis; value was obtained graphically.

antibody without digoxin, the serum half times were 6.1 and 5.7 days, respectively. In four nonimmunized rabbits given $^{131}$I-$\gamma$-globulin, $^{125}$I-antidigoxin antibody, and 0.25 mg digoxin, the serum half times of $\gamma$-globulin and antibody were 4.7 and 4.1 days, respectively. In three rabbits whose sera contained digoxin-specific antibodies given an injection containing $^{131}$I-$\gamma$-globulin, 0.25 mg digoxin, and autologous radioiodinated purified antibody, the serum half-times of $\gamma$-globulin and of antidigoxin antibody were 4.3 and 3.7 days.

DISCUSSION

When a foreign serum protein of high molecular weight is injected into the circulation of an animal whose serum contains antibodies specific for that protein, the injected protein forms complexes with the specific antibody and is removed from the circulation of that animal more rapidly than it is cleared in the absence of antibody (2). When injected into the circulation of animals whose sera contain hapten-specific antibodies, hapten-protein conjugates are rapidly cleared in a similar manner (26).

In contrast, relatively little is known about the fate of low molecular weight
protein antigens or of unconjugated haptens in the circulation in the presence of antibody. Berson et al. showed that the biologic half-life of insulin-$^{131}$I in the circulation of humans with antibodies to insulin was approximately five times longer than in the circulation of individuals without such antibodies (27). Wheeler, Kagan, and Glick have made similar observations in rabbits immunized against the nonapeptide hormone oxytocin (28). Rothenberg et al., employing folic acid as a hapten, have immunized rabbits with protein-folic acid conjugates and have reported that the plasma clearance of $^{3}$H-folic acid was altered in immunized animals in comparison with nonimmunized rabbits. The plasma concentrations of folic acid were 3.5 times greater at 1 min and plasma clearance was threefold slower in the immunized rabbits as compared with the control animals (29).

The current study demonstrates that there is a very striking alteration in the clearance of the hapten, digoxin, from the circulation of animals immunized with a digoxin-BSA conjugate in comparison with the clearance of digoxin from the circulation of nonimmunized animals or of animals immunized with unconjugated BSA. Following a single intravenous injection of 0.4 mg digoxin per kilogram body weight, the serum digoxin concentration 12 h later was 8,300 ng/ml in digoxin-immunized animals, in contrast with concentrations of 92 ng/ml and 126 ng/ml in nonimmunized animals and BSA-immunized animals, respectively. Despite the high serum levels of digoxin, the excretion of digoxin was markedly diminished in the digoxin-immunized rabbits in contrast with control animals. The serum half-life of digoxin was markedly prolonged in the digoxin-immunized group in which the $t_{\frac{1}{2}}$ was 72 days, in comparison with the values of 3.4 days and 9.4 days in the nonimmunized rabbits and BSA-immunized rabbits, respectively. Most striking of all was the observation that the digoxin persisted in the circulation of digoxin-immunized rabbits for as long as 14 mo after a single intravenous injection of the hapten and without any subsequent injections of the immunogenic digoxin-protein conjugate. As late as 1 yr after this single digoxin injection, the mean serum digoxin concentration in the digoxin-immunized animals was 85 ng/ml, a value comparable with the mean 12-h value in nonimmunized rabbits.

Evidence that these alterations in digoxin metabolism in digoxin-immunized rabbits are due to the presence of circulating hapten-antibody complexes was obtained. The majority of the serum radioactivity in digoxin-immunized animals was immunoglobulin bound whereas in nonimmunized rabbits only 2% of the serum radioactivity was so bound. Most of the serum radioactivity was indeed intact hapten, as evidenced by the fact that the serum radioactivity from four rabbits obtained 4–9 mo after the infusion of digoxin contained at least 66% digoxin-$^{3}$H as determined by thin layer chromatographic analysis.

On the basis of these observations, we have concluded that hapten-antibody complexes may persist in the circulation of immunized animals for periods greater than 1 yr after a single intravenous injection of hapten. It is presumed
that the binding of digoxin to antibody markedly inhibits its renal and fecal excretion and its metabolic degradation. Less clear, however, is the basis for its prolonged persistence in the serum in complex with rabbit γ-globulin, the half-life of which has been estimated to be 5–8.6 days (30). Since complexing with hapten may confer some protection to antibody against degradation by proteolytic enzymes in vitro (31, 32), experiments were carried out to determine whether digoxin might inhibit the metabolic degradation of antidigoxin antibody in vivo. In these experiments, the half-life of 125I-antidigoxin antibody in the presence of digoxin (4.1 days) did not differ significantly from its half-life in the absence of digoxin (5.7 days) nor from the half-life of pooled normal γ-globulin labeled with 125I (5.1 days). Thus, in the current study no evidence was obtained in support of the hypothesis that the hapten, digoxin, prolongs the half-life of antidigoxin antibody in vivo. Our data seems most consistent with the hypothesis that, as digoxin is released from the antibody molecule in the normal process of immunoglobulin catabolism, the released molecule is immediately or soon thereafter bound by another preformed or newly formed antidigoxin antibody molecule. It is possible, of course, that digitalis-binding sites, which are believed to be on the membranes of all mammalian cells (33), may serve as a temporary reservoir for digoxin during the postulated transfer from one antibody molecule to another.

A finding of uncertain significance in this study was the prolongation of the mean serum disappearance time of digoxin-3H in BSA-immunized animals (t1/2 = 9.4 days) in comparison with nonimmunized animals (t1/2 = 3.4 days). Associated with the slowed disappearance of digoxin-3H from the sera of BSA-immunized animals was a 10-day digoxin-3H excretion of 57% of the administered dose, in comparison with 77% digoxin-3H excretion in nonimmunized rabbits. No digoxin-binding capacity has been detected in the sera of BSA-immunized rabbits (Table I) and the basis for the altered metabolism of digoxin-3H in these animals is not known. It is, however, of interest to note that, by computer analysis of the digoxin-3H disappearance curves in digoxin-immunized rabbits (Table II), a component with a t1/2 of 7.1 days was noted (similar to the dominant exponential, t1/2 = 9.4 days, in the BSA-immunized rabbits); it is conceivable that this early component in the digoxin-immunized animals is a reflection of an as yet undefined process which may occur as a nonspecific result of the immunization procedure employed. In this connection, it is of interest to note that we have previously reported that rabbits which have been injected with Freund's complete adjuvant mixture, with or without protein antigens not containing digoxin, frequently tolerate digoxin doses which are usually lethal in nonimmunized animals (5).

The unexpectedly long persistence of digoxin-antibody complexes in the circulation of the rabbits in this study raises the possibility that drugs and other low molecular weight determinants may persist in complex with antibody for prolonged periods in the circulation of man and experimental animals.
Such complexes could be of clinical importance in certain instances of delayed and prolonged drug allergies and of some idiosyncratic drug reactions (34). It is well recognized that experimental animals immunized with protein antigens exhibit a greater increase in serum γ-globulin concentration than can be accounted for on the basis of the appearance of antibody capable of precipitating with the immunizing antigen (35). In this connection, the present study raises the possibility that some or all of these so-called “nonspecific” immunologic responses to protein antigens may reflect the presence in serum of specific antibodies, the antigen-binding sites of which are occupied by low molecular weight determinants (perhaps degradation products of the protein antigen) and which therefore are not specifically precipitable by the corresponding protein antigen when conventional precipitin techniques are employed (36).

Finally, the persistence of antibody-hapten complexes in the circulation suggests that these complexes may not be deposited in tissues. Since haptens and other low molecular weight determinants are capable, by virtue of their ability to react with antibody-combining sites, of inhibiting or reversing the precipitation of certain antigen-antibody complexes in vitro, it seems reasonable to speculate that haptens and certain low molecular weight determinants (e.g., enzymatic digests of protein or nucleic acid antigens) may prevent or reverse the formation and tissue deposition of immune complexes caused by the interaction between antibodies and macromolecular antigens in experimental animals and man (36). In this connection, it is known that, in rabbits with immune complex glomerulonephritis due to BSA-anti-BSA complexes, the intravenous administration of sufficient BSA to produce a state of extreme antigen excess promotes dissolution of renal antigen-antibody complexes (37, 38). However, the use of a protein antigen may result in increased antibody formation; thus, its beneficial effect may be evanescent and may be followed by a greater degree of antigen-antibody complex deposition than had been present initially. In contrast, hapten and low molecular weight determinant groupings have the advantage of being nonimmunogenic and, moreover, the unexpectedly long persistence of hapten-antibody complexes in the circulation of the animals in the current study suggests that their postulated effect on immune complex deposits may be a very protracted one.

**SUMMARY**

To study the fate of a low molecular weight antigen (hapten) in the circulation of animals whose sera contain antibodies specific for that low molecular weight antigen, a single injection of digoxin-3H (0.4 mg/kg) was administered intravenously to 18 rabbits. Thirteen animals (nine nonimmunized and four immunized with bovine serum albumin) served as control animals. In five rabbits which had been immunized with a digoxin-bovine serum albumin conjugate and whose sera contained digoxin-specific antibodies, the mean 12-h serum digoxin concentration was 8,300 ng/ml (control: 92 ng/ml) and the mean
PERSISTENCE OF HAPten-ANTIBody COMplexes

serum concentration 12 mo after the single injection of digoxin-3H was 85 ng/ml. In digoxin-immunized rabbits, less than 10% of the digoxin-3H was excreted in the first 10 days (control: 77% recovered in urine and feces) and the mean biological half-life of digoxin, as calculated from serum digoxin-3H disappearance curves, was 72 days (control: 3.4 days). In sera of digoxin-immunized rabbits, more than 90% of the circulating digoxin-3H was immunoglobulin bound, as determined by the double-antibody and dextran-coated charcoal methods.

The serum disappearance rate of 125I-antidigoxin antibodies was similar in nonimmunized and in immunized animals and in the presence or absence of digoxin.

It is concluded that the biological half-life of a hapten may be markedly prolonged when the hapten is bound to specific antibody. The persistence of antibody-hapten complexes in the circulation suggests that these complexes may not be deposited in tissues and raises the possibility that low molecular weight determinants may be capable of preventing or reversing the deposition of immune complexes, containing macromolecular antigens, in the tissues of experimental animals and man.

The authors would like to thank Mrs. Eileen Pilliner, Mrs. B. Denise Raynor, and Mr. Robert Sciacca for their superb technical assistance. We are grateful to Dr. Sam M. Beiser, Department of Microbiology, Columbia University, for the gift of a globulin fraction prepared from sheep antirabbit γ-globulin serum.

BIBLIOGRAPHY

1. Schmidt, D. H., B. M. Kaufman, and V. P. Butler, Jr. 1972. Persistence of hapten-antibody complexes in the circulation of immunized animals after a single intravenous injection of hapten. J. Clin. Inves. 51:86a. (Abstr.)
2. Dixon, F. J., and D. W. Talmage. 1951. Catabolism of 118I labelled bovine gamma globulin in immune and non-immune rabbits. Proc. Soc. Exp. Biol. Med. 78:123.
3. Dixon, F. J., and P. H. Maurer. 1953. Fate of antibody following in vivo combination with specific antigen. Proc. Soc. Exp. Biol. Med. 84:442.
4. Cochrane, C. G. 1971. Mechanisms involved in the deposition of immune complexes in tissues. J. Exp. Med. 134:75s.
5. Schmidt, D. H., and V. P. Butler, Jr. 1971. Immunological protection against digoxin toxicity. J. Clin. Invest. 50:866.
6. Butler, V. P., Jr., and J. P. Chen. 1967. Digoxin-specific antibodies. Proc. Natl. Acad. Sci. U. S. A. 57:71.
7. Erlanger, B. F., and S. M. Beiser. 1964. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc. Natl. Acad. Sci. U. S. A. 52:68.
8. Smith, T. W., V. P. Butler, Jr., and E. Haber. 1970. Characterization of antibodies of high affinity and specificity for the digitalis glycoside digoxin. Biochemistry. 9:331.
9. Herbert, V., K.-S. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay of insulin. J. Clin. Endocrinol. Metab. 25:1375.
10. Smith, T. W., V. P. Butler, Jr., and E. Haber. 1969. Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. *N. Engl. J. Med.* 281:1212.

11. Kent, J. F., S. C. Bukantz, and C. R. Rein. 1946. Studies in complement fixation. I. Spectrophotometric titration of complement; construction of graphs for direct determination of the 50% hemolytic unit. *J. Immunol.* 53:37.

12. Tomasi, T., and H. G. Kunkel. 1964. Isolation of 7S and 19S gamma globulins. *Methods Med. Res.* 10:80.

13. Watson, J. F., and V. P. Butler, Jr. 1972. Biologic activity of digoxin-specific antiserum. *J. Clin. Invest.* 51:638.

14. Curd, J., T. W. Smith, J.-C. Jaton, and E. Haber. 1971. The isolation of digoxin-specific antibody and its use in reversing the effects of digoxin. *Proc. Natl. Acad. Sci. U. S. A.* 68:2401.

15. Hunter, W. M. 1967. The preparation of radioiodinated proteins of high activity, their reaction with antibody in vitro: the radioimmunoassay. In *Handbook of Experimental Immunology.* D. M. Weir, editor, F. A. Davis Co., Philadelphia, Pa. 608.

16. Butler, V. P., Jr., and J. H. Vaughan. 1964. Hemagglutination by rheumatoid factor of cells coated with animal gamma globulins. *Proc. Soc. Exp. Biol. Med.* 116:585.

17. Morse, J. H., and C. L. Christian. 1964. Immunological studies of the 11S protein component of the human complement system. *J. Exp. Med.* 119:195.

18. Doherty, J. E., W. H. Hall, M. L. Murphy, and O. W. Beard. 1971. New information regarding digitalis metabolism. *Chest.* 59:433.

19. Matthews, C. M. E. 1957. The theory of tracer experiments with 131I-labelled plasma proteins. *Phys. Med. Biol.* 2:36.

20. Wells, J. V., and H. H. Fudenberg. 1971. Metabolism of radio-iodinated IgG in patients with abnormal serum IgG levels. I. Hypergammaglobulinaemia. *Clin. Exp. Immunol.* 9:761.

21. Doherty, J. E., W. H. Perkins, and G. K. Mitchell. 1961. Tritiated digoxin studies in human subjects. *Arch. Intern. Med.* 108:531.

22. Dell, R. B., R. Sciacca, K. Lieberman, D. B. Case, and P. J. Cannon. 1973. A weighted least-squares technique for the analysis of kinetic data and its application to the study of renal 133Xenon washout in dogs and man. *Circ. Res.* 32:71.

23. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats. *Diabetes.* 12:115.

24. Kekwick, R. A. 1940. The serum proteins in multiple myelomatisis. *Biochem. J.* 34:1248.

25. Pincus, T., R. Haberkern, and C. L. Christian. 1968. Experimental chronic glomerulitis. *J. Exp. Med.* 127:819.

26. Lightfoot, R. W., R. E. Drusin, and C. L. Christian. 1970. Properties of soluble immune complexes. *J. Immunol.* 105:1493.

27. Berson, S. A., R. S. Yalow, A. Bauman, M. A. Rothschild, and K. Newerly. 1956. Insulin-I131 metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. Clin. Invest.* 35:170.
28. Wheeler, M., A. Kagan, and S. M. Glick. 1966. Radioimmunoassay of oxytocin. *Clin. Res.* 14:479. (Abstr.)
29. Rothenberg, S. P., F. Gizis, and B. Kamen. 1969. In vitro and in vivo effects of folate binding antibodies. *Clin. Res.* 17:341. (Abstr.)
30. Waldmann, T. A., and W. Strober. 1969. Metabolism of immunoglobulins. *Prog. Allergy.* 13:1.
31. Grossberg, A. L., G. Markus, and D. Pressman. 1965. Change in antibody conformation induced by hapten. *Proc. Natl. Acad. Sci. U. S. A.* 54:942.
32. Cathou, R. E., and T. C. Werner. 1970. Hapten stabilization of antibody conformation. *Biochemistry.* 9:3149.
33. Marks, B. H. 1972. Factors that affect the accumulation of digitalis glycosides by the heart. In *Basic and Clinical Pharmacology of Digitalis.* B. H. Marks and A. M. Weissler, editors. Charles C Thomas, Pub. Springfield, Ill. 69.
34. Goodfriend, T. L., M. E. Webster, and J. S. McGuire. 1970. Complex effects of antibodies to polypeptide hormones. *J. Clin. Endocrinol. Metab.* 30:565.
35. De Vos-Cloetens, C., V. Minsart-Baleriaux, and G. Urbain-Vansanten. 1971. Possible relationships between antibodies and non-specific immunoglobulins simultaneously induced after antigenic stimulation. *Immunology* 20:955.
36. Campbell, D. H. 1970. On the significance of inhibiting haptens. *Ann. N. Y. Acad. Sci.* 169:105.
37. Valdes, A. J., L. B. Senterfit, A. D. Pollack, and F. G. Germuth, Jr. 1969. The effect of antigen excess on chronic immune complex glomerulonephritis. *Johns Hopkins Med. J.* 124:49.
38. Wilson, C. B., and F. J. Dixon. 1971. Quantitation of acute and chronic serum sickness in the rabbit. *J. Exp. Med.* 134:7s.