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Preparation and Characterization of Antibodies to Menadione†

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ABSTRACT: Antibodies specific for menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) were raised in rabbits following immunization with menadione–protein complexes wherein the protein was covalently bound to menadione at the three position *via* thioether linkage. The antibodies precipitated with menadione bound similarly to a different carrier protein and they were isolated from antimenadione sera with menadione-bearing immunoadsorbants. The binding of univalent menadione–haptens by purified antibodies was studied by equilibrium dialysis. The average intrinsic association constants for the binding of menadione–butyrate by antibodies produced early after immunization and those obtained after secondary immunization were $1.1 \pm 0.3 \times 10^5$ and $2.6 \pm 0.6 \times 10^6$ M^{-1}, respectively. Heterogeneity of hapten-binding sites appeared to be greater in the late antisera. The tryptophan fluorescence of antimenadione antibodies was quenched by bound menadione but the degree of quenching was less than has been observed for some antidinitrophenyl antibody–ligand systems.

Antibody-like activity against menadione or vitamin K_3 (2-methyl-1,4-naphthoquinone), the aromatic nucleus of the K vitamins, has been observed with several myeloma proteins and with rabbit and guinea pig antibodies raised against various nitrophenyl groups (Eisen *et al.,* 1970; Rosenstein and Richards, 1972; Michaelides and Eisen, 1974).

Accordingly, we wanted to determine whether antibodies could be raised against menadione by conventional immunization procedures and to evaluate the binding affinities of these antibodies. This paper describes the preparation of menadione–protein antigens and menadione–haptens, and some properties of antimenadione antibodies.

Materials and Methods

Conjugation of Menadione to Proteins. Menadione–proteins were prepared by coupling menadione to bovine γ-globulin, BγG,[1] or human serum albumin, HSA, *via* a thioether linkage wherein thiol groups previously bound covalently to the proteins reacted with menadione by nucleophilic substitution (Nickerson *et al.,* 1963).

Thiol groups were attached to the proteins with *N*-acetylhomocysteine thiolactone (Singer, 1964). In a representative preparation, 400 mg of protein in 6 ml of 0.02 M potassium carbonate (pH 10.7) was mixed with 500 mg of *N*-acetylhomocysteine thiolactone in 2 ml of water and held for 18–24 hr at 4°. The solution was then adjusted to pH 5.0 with 2.5 N HCl, chromatographed on Sephadex G-25 (1.5 cm × 30 cm) in water, and the thiol content of the eluted protein was measured (Ellman, 1959). The pooled fractions of thiolated protein were brought to pH 5 with acetate buffer (pH 5.0) (final concentration 0.02 M) and stirred with a saturating amount of menadione for 30–60 min in the dark at room temperature. After decanting from residual solid menadione, the solution was dialyzed exhaustively against phosphate–saline[1] to remove unreacted menadione.

Ultraviolet absorption spectra of menadione–proteins were obtained in a Cary 14 recording spectrophotometer. The number of menadione groups per protein molecule was calculated from the dry weight of the protein and from absorbancy at 335 nm, which was assumed to be due entirely to the menadione moiety with a molar extinction coefficient of 2200 (Rosenberg, 1945). (This value is the same for free and protein-bound menadione in phosphate–saline.) Dry weight was determined by exhaustive dialysis of the proteins against distilled water followed by heating under vacuum at 100° to constant weight.

The menadione content of the conjugated proteins was also determined by a modification of a method (Strauss *et al* 1963) wherein menadione undergoes base-catalyzed hydrolytic cleavage from the protein, yielding sulfhydryl protein and phthiocol. For this assay 2 ml of 0.2 N NaOH

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[1] Abbreviations: BγG, bovine γ-globulin; phosphate–saline, 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4); menadione–caproate, menadione–*N*-acetylhomocysteine thiolactone–ε-aminocaproate; menadione–butyrate, menadione–*N*-acetylhomocysteine thiolactone–γ-aminobutyrate; HSA, human serum albumin.

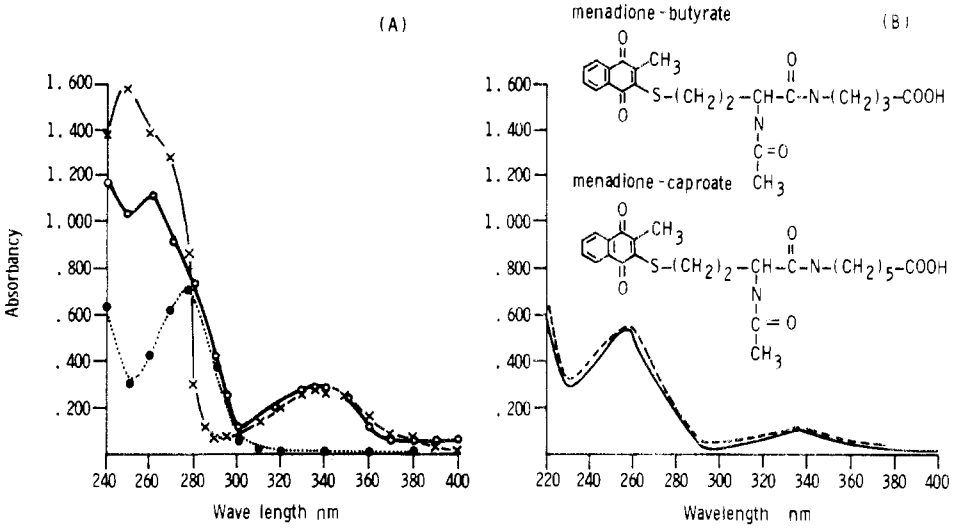

FIGURE 1: Ultraviolet absorption spectra of (A) menadione–HSA, 0.4 mg/ml, with 21 mol of menadione per 70,000 g of protein (O—O), unsubstituted menadione, 8×10^{-5} M (x—x), unsubstituted HSA, 1.08 mg/ml (●. . . .●), and of (B) menadione caproate 3.25×10^{-5} M (— —) and menadione–butyrate 3.25×10^{-5} M (- - -). All spectra were determined in phosphate–saline.

was added to 2 ml of menadione–protein; after 1 hr in the dark the phthiocol concentration was measured by absorbancy at 480 nm (ϵ 2500).

Preparation and Properties of Haptens. Menadione derivatives of ϵ-aminocaproate (menadione–caproate) and of γ-aminobutyrate (menadione–butyrate) (see Figure 1B) were prepared by stirring 2.5 g of the amino acid in 25 ml of 0.2 M potassium carbonate (pH 10.7) with 3.2 g of *N*-acetylhomocysteine thiolactone (previously dissolved in 20 ml of water) for 2 hr under N_2. The pH was then adjusted to 3.5–4.0 with 2.5 N HCl, 0.5–1.0 g of menadione was added, and stirring was continued for an additional 30 min. The mixture was filtered to remove unreacted menadione and made 0.15 M in salt with solid NaCl and the hapten was precipitated by acidification with concentrated HCl. The product was purified by repeated precipitation from phosphate–saline with concentrated HCl.

Purity was determined by thin-layer chromatography on silica gel G coated plates. Chromatograms were developed with ethyl acetate–2-propanol–water, 65:25:10 (v/v), and the menadione products were visualized by ultraviolet illumination and by exposure to iodine vapor. The R_F for menadione–caproate was 0.79 and for menadione–butyrate was 0.55.

Elemental analyses[2] confirmed expected compositions. Menadione–*N*-acetylhomocysteine thiolactone–ϵ-aminocaproate, Calcd: C, 59.8; H, 5.84; N, 6.06; S, 7.58; O, 20.8. Found: C, 60.14; H, 5.65; N, 6.12; S, 7.03; O, 20.76. Menadione–*N*-acetylhomocysteine thiolactone–γ-aminobutyrate, Calcd: C, 58.09; H, 5.5; N, 6.4; S, 8.0; O, 22.0. Found: C, 57.4; H, 5.2; N, 6.61; S, 7.64; O, 22.69.

The molar extinction coefficients in phosphate–saline were 25,000 at 256 nm and 2200 at 335 nm for each hapten (see Figure 1B). In establishing their extinction coefficients, the hapten concentrations were determined from the amino acid content as measured by automatic amino acid analyzer following hydrolysis in 6 N HCl at 100° under vacuum for 18 hr.

Menadione–caproate and menadione–butyrate are bright yellow granular compounds. They are light sensitive and unstable at pH values above 7.6.

^{14}C-labeled menadione–butyrate was prepared with 4-amino-*n*-butyric acid-^{14}C (U) 204 Ci/mol (Amersham/Searle) using the method described for the unlabeled hapten but with 1000-fold less of each reagent: the total reaction volume was 0.75 ml and included 2.5 mg of unlabeled γ-aminobutyrate. The specific activity of the purified product was 2.25×10^4 cpm/nmol.

Preparation of Immunoadsorbants (Menadione–Sepharose.) In order to attach menadione to a solid support, lysine was first coupled *via* its α-amino group to activated Sepharose, homocysteinyl groups were then added to the ϵ-amino group of the attached residues, and, finally, menadione was attached to the free sulfhydryl group of the homocysteinyl moiety. In a representative preparation, 25 g of Sepharose 4B-200 was activated with cyanogen bromide (Axen *et al.*, 1967) and stirred with *N*-ϵ-*t*-Boc-L-lysine (6 mg/g of Sepharose) in 0.1 M $NaHCO_3$ at 4° overnight. The *t*-Boc moiety was then removed by stirring the modified Sepharose for 5 hr in 1.0 M HCl in glacial acetic acid. After thorough washing with water, *N*-α-lysine–Sepharose was suspended in 0.2 M potassium carbonate (pH 10.7) and stirred with *N*-acetylhomocysteine thiolactone (10 mg/g of Sepharose) for 2–4 hr at 4°. The washed Sepharose was finally stirred overnight with menadione, 1 mg/g of Sepharose, in 0.1 M acetate (pH 5.0) at room temperature. The resin was filtered, washed extensively on the filter with water, and then washed exhaustively by stirring with frequent changes of water for several days to remove traces of unbound menadione.

Menadione–Sepharose was pale yellow; on prolonged exposure to light it became brown and lost ability to bind antibodies to menadione. Although more menadione residues per gram of Sepharose could be attached than in the adsorbants used in these experiments (initial ratio: 1 mg of menadione/g of Sepharose), we found that with a higher menadione content it was difficult to elute the bound antibodies; and the resulting yields were low.

Preparation of Antibodies to Menadione. New Zealand white rabbits were injected in their foot pads with 1.0 mg of menadione–BγG containing 25–30 mol of menadione/150,000 g of protein, in Freund's complete adjuvant. Serum

[2] Elemental analyses were done by Gailbraith Laboratories, Knoxville, Tenn.

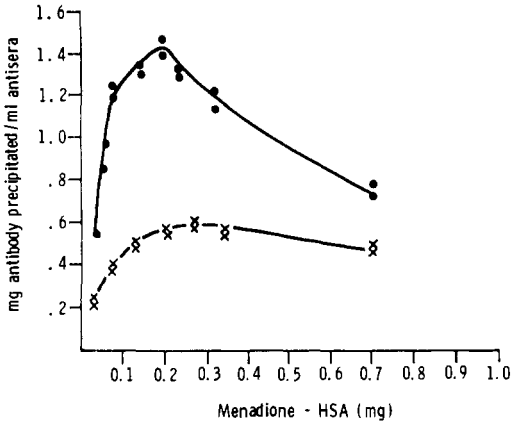

FIGURE 2: Precipitation of early (x) and late (●) antimenadione antibodies with menadione-HSA; 0.5 ml of antiserum was used per tube and the washed precipitates were dissolved in 1.0 ml of 0.5% sodium lauryl sulfate prior to ultraviolet absorbancy measurements. Correction for menadione-HSA contribution to absorbancy at 278 nm of dissolved precipitates was based on the ratio of absorbancy at 278 and 335 nm of menadione-HSA itself and absorbancy at 335 nm of the dissolved precipitates. [$(A_{278}/A_{335,\ \text{menadione-HSA}}) \times A_{335,\ \text{precipitate}} = A_{278}$ in the precipitate due to menadione-HSA.] This correction was subtracted from the total absorbancy at 278 nm, giving absorbancy due only to antibody.

was harvested by cardiac puncture just before immunization, and at 2 weeks, and again at 2 months after immunization. Two months after the initial injection the animals were injected again with 1.0 mg of menadione-protein in Freund's complete adjuvant and bled 10 days later. Sera were stored at −20°.

Purification of Antimenadione Antibodies. In a typical preparation 25 ml of antiserum was passed twice over about 10 ml of packed menadione-Sepharose.

After thorough washing with phosphate-saline, the antibodies were eluted with 0.01 M menadione-caproate in phosphate-saline. The effluent was run directly over an anion exchange column (Dowex 1-X8, H^+ ion form, Calbiochem) to remove menadione-caproate. The concentration of protein in the effluent from the second column was monitored spectrophotometrically at 278 nm. The activity of the purified antibodies was tested by quantitative precipitin analysis, by equilibrium dialyses, and by fluorescence quenching.

Precipitin Analyses. Antisera or purified antibodies were incubated with menadione-HSA at 37° for 1 hr, and at 4° for 18–24 hr. Precipitates were collected by centrifugation, washed three times with phosphate-saline at 4° and dissolved in 1.0 ml of 0.5% sodium lauryl sulfate, and absorbance was measured at 335 and 278 nm. The antibody content was determined by subtracting from the total absorbance at 278 nm the contribution due to menadione-HSA. This contribution was assessed as indicated in the legend of Figure 2. The extinction coefficient for rabbit antibody was taken at 14 (ϵ_{278}(1%). (The absorbancies of menadione-HSA and of rabbit immunoglobulin G were the same in 0.5% lauryl sulfate as in phosphate-saline.) Some precipitates were hydrolyzed in 0.1 N NaOH to cleave menadione from the antigen or hapten, and the resulting phthiocol was removed by extraction with chloroform or dialysis against phosphate-saline. Protein then could be determined spectrophotometrically at 278 nm.

Equilibrium Dialyses. These experiments (Karush and Karush, 1971) were carried out with 100-μl Plexiglass dialyses chambers (Gateway Immunosera, Cahokia, Ill.); 100 μl of antibody solution was dialyzed against an equal volume of different concentrations of radioactive haptens for 48 hr at 4°. Two 25 μl aliquots were withdrawn from each side of the chamber and radioactivity was determined by liquid scintillation counting in Bray's solution (Bray, 1960).

Fluorescence Quenching. Titrations measuring quenching of protein fluorescence by hapten (Eisen and McGuigan, 1971) were carried out at 4° in a temperature controlled cell in an Aminco Bowman spectrofluorometer.

Other Methods and Reagents. Antisera to *p*-azobenzene arsonate, produced by immunizing rabbits with azobenzene arsonate-BγG, was generously provided by E. S. Simms and W. Grey. Rabbit anti-HSA sera was obtained from Gateway Immunosera (Cahokia, Ill.). ε-Aminocaproic acid, γ-aminobutyric acid, and *N*-acetylhomocysteine thiolactone were obtained from Mann Research Laboratories. Bovine γ globulin, human serum albumin, *N*-ε-*t*-Boc-L-lysine, and menadione were obtained from the Sigma Chemical Company, St. Louis, Mo. Menadione was recrystallized three times from methanol before use. Menadione-proteins, menadione-haptens, and menadione-Sepharose were kept at pH 7.6 or lower to prevent spontaneous base-catalyzed hydrolysis to phthiocol and sulfhydryl compounds. They were also protected from light to minimize what is assumed to be light-catalyzed free radical degeneration.

Results

Characterization of Menadione-Proteins and Haptens. Menadione proteins were a bright clear yellow and were stable for several months if kept frozen (*ca.* −20°) and protected from light. Breakdown was evidenced by a gold and eventually brown discoloration.

Menadione-HSA and menadione-BγG were each soluble up to at least 25 mg/ml in low ionic strength buffer (0.02 M acetate, pH 5.0); but when dialyzed against phosphate-saline, menadione-HSA remained in solution while menadione-BγG formed curd-like precipitates, even at protein concentrations as low as 1.0 mg/ml. However, when incorporated into complete Freund's adjuvant, the precipitated menadione-BγG was an effective immunogen (see below).

The ultraviolet absorption spectrum of menadione-HSA is given in Figure 1A. The absorbance maximum at 335 nm corresponds to that of free menadione and of the menadione-caproate and menadione-butyrate haptens (Figure 1B; other properties of the haptens are given under Materials and Methods). Because of prominent menadione absorbancy at 280 nm, spectrophotometric measurement of the protein moiety in a menadione-protein conjugate presents difficulties. In these conjugates, accordingly, protein was measured either by dry weight or from the absorbancy at 278 nm of the thiolated protein before addition of menadione: in the latter case the assumption was made that no protein was lost in reaction with menadione or in the subsequent dialysis to purify the conjugate. Menadione residues of the conjugate were measured by absorbancy at 335 nm, using the molar extinction coefficient of menadione haptens (2200 at 335 nm), or by measurement of phthiocol released by alkaline hydrolysis (see Materials and Methods section).

Table I compares the results of several procedures for estimating moles menadione residues per mole of protein. In general, the several approaches gave concordant results, and the least cumbersome method was used most frequently (*i.e.*, absorbancy of thiolated protein before addition of menadione to determine protein concentration and absorbancy of the final product at 335 nm to measure the con-

TABLE I: Comparison of Different Approaches to Measurement of the Number of Menadione Groups in Menadione–Proteins.[a]

Method of Determining		Mol of Menadione/mol of Protein[b]	
Protein	Menadione	Menadione–BγG	Menadione–HSA
Dry weight	A_{335}	25.0	22.0
Dry weight	Phthiocol	26.0	23.3
A_{278} after SH addition	A_{335}	28.0	18.4
A_{278} after SH addition	Phthiocol	27.0	24.4

[a] Protein in menadione–BγG and in menadione–HSA was measured by dry weight or by ultraviolet absorbancy after thiolation (before addition of menadione); menadione residues were measured by absorbancy (335 nm) or by phthiocol release following alkaline hydrolysis. [b] Each value represents an average of three determinations on three different samples.

centration of the menadione residues). With menadione–BγG, which had only limited solubility in phosphate–saline, the spectrophotometric measurements were made with the protein in 0.02 M acetate (pH 5.0).

The optimal sulfhydyl content for reaction with menadione was 50–60 SH groups per molecule of BγG and 40–50 SH groups per molecule of HSA. The maximal level of conjugation was 25–35 groups of menadione/molecule of BγG and 19–24 groups menadione per molecule of HSA.

Characterization of Antisera to Menadione. Forty-four of forty-six rabbit sera harvested 2 weeks after injecting menadione–BγG had antimenadione activity: by precipitin reaction with menadione–HSA, they contained 0.40–0.65 mg of antibody/ml of antiserum. Two months later, the antisera had 0.25–0.53 mg antibody/ml. At that time 18 animals were injected a second time with menadione–BγG and bled 10 days later: by precipitin reaction with menadione–HSA these antisera had 1.0–1.8 mg of antimenadione antibody/ml. Sera obtained before immunization from six rabbits failed to precipitate with menadione–HSA.

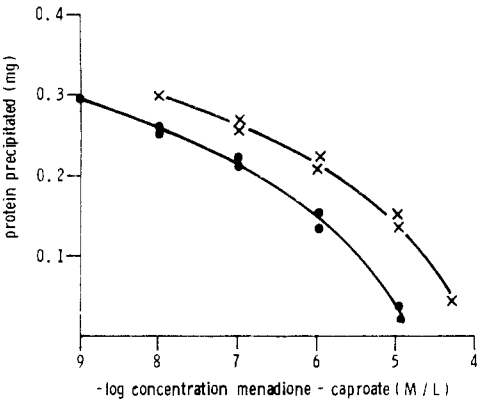

FIGURE 3: Hapten inhibition of precipitation of early (x) and late (●) antimenadione antibodies by menadione–HSA. Precipitates were formed at equivalence with the appropriate antisera. (In absence of inhibiting hapten, precipitates contained 0.34 mg of antibody, plus 0.16 mg of antigen for early antisera and 0.048 mg of antigen for late antisera.) The final volume of all tubes was 0.5 ml. Diluent was phosphate–saline.

Figure 2 shows precipitin reactions with menadione–HSA and pooled sera from the bleeding 2 weeks after the first injection (early sera) and pooled sera from bleedings taken 10 days after the second injection (late sera): at equivalence the early pool had 0.58 mg of antibody/ml and the late pool had 1.44 mg of antibody/ml. Early and late pools of sera from other rabbits subjected to the same immunization schedule had 0.48–0.62 and 1.2–1.7 mg of antibody/ml, respectively. At equivalence (based on supernatant tests) the mole ratio of antibody/antigen was 1.0–1.5 for the early pools and 3.1–4.5 for the late pools.

Fifty per cent inhibition of the early and late pools was obtained with menadione–caproate at 1×10^{-5} and 1×10^{-6} M, respectively (Figure 3); with a control precipitin reaction of HSA with rabbit anti-HSA no inhibition was observed with menadione–caproate at 5×10^{-3} M.

Purified Antimenadione Antibodies. Antimenadione antibodies were specifically bound on menadione–Sepharose and could be specifically eluted from this adsorbant by 0.01 M menadione–caproate in phosphate–saline, with an overall yield of 70–80% (Table II). The resulting purified anti-

TABLE II: Specific Retention and Release of Antimenadione Antibodies from Menadione–Sepharose.

Serum Added[a]	Total Antibody Added[b] (mg)	Protein Eluted with Menadione–Caproate (mg)	Protein Eluted with Guanidine-HCl (mg)	Yield[c] Amount Eluted with Menadione–Caproate) (%)
Antimenadione (early pool)	140	114	18	82
Antimenadione (late pool)	140	102	33	73
Antiazobenzenearsonate (pool)	140	10.5	7.6	8

[a] Antimenadione antisera (early pool, 240 ml, 0.58 mg of antihapten antibody/ml), antimenadione (late pool, 87 ml, 1.6 mg of antihapten antibody/ml), and antiazobenzenearsonate (pool, 108 ml, 1.29 mg of antihapten antibody/ml) were cycled twice over 75 ml of menadione–Sepharose. The Sepharose was washed thoroughly with phosphate–saline and eluted with 100–125 ml of 0.1 M menadione–caproate in phosphate–saline. The eluate was freed of hapten by ion exchange chromatography (Dowex 1-X8 H^+ ion form). The menadione–Sepharose was then washed free of hapten with phosphate–saline and residual protein was eluted with 7 M guanidine-HCl. Protein in both eluates was measured by absorbancy at 278 nm. [b] Antibody content of the sera was based on quantitative precipitin analyses. [c] Yield is the amount of protein eluted with menadione–caproate/total antibody added, × 100.

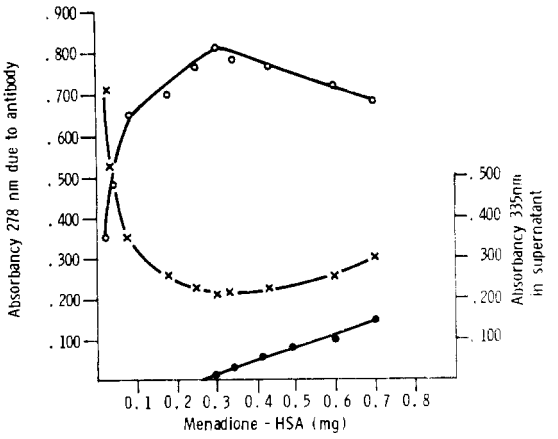

FIGURE 4: Precipitation of purified antimenadione antibodies with menadione-HSA. Each tube contained 0.72 mg of purified antimenadione antibody (A_{278} = 1.0) in a final volume of 1.0 ml. Absorbance at 278 nm of the washed dissolved precipitates (O) and of the supernatants (x), corrected for menadione-HSA content (see legend to Figure 2), represents precipitated and unprecipitated antibody, respectively. Absorbance of the supernatant at 335 nm (●) represents unprecipitated menadione-HSA.

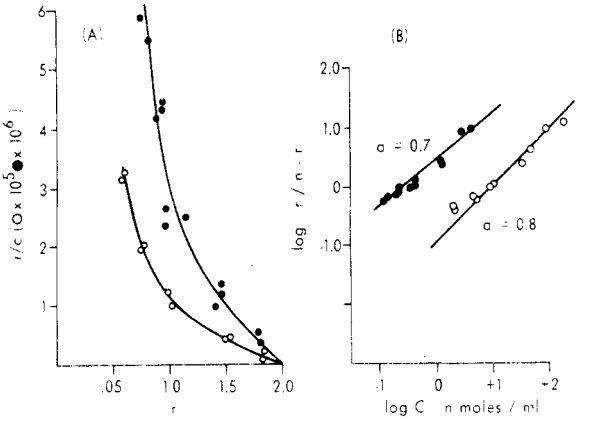

FIGURE 5: Equilibrium dialysis with [^{14}C]menadione-butyrate and purified antimenadione antibodies from early (O) and late (●) antisera. Antibody concentrations were 1.0-1.2 mg/ml for early antibodies and 0.25-0.35 mg/ml for late antibodies. The solvent was phosphate-saline; temperature, 4°. (A) Data plotted according to $r/c = nK - rK$; (B) data plotted according to $\log [r/(n - r)] = a \log c + a \log K_0$ (Karush and Karush, 1971).

bodies were 81% precipitable by menadione-HSA at equivalence (Figure 4). At approach to equivalence the supernatants had no detectable free antigen or antibody (tested in different aliquots by adding fresh antiserum or menadione-HSA, respectively).

Equilibrium Dialysis. As is shown in Figure 5, equilibrium dialysis demonstrated that the purified antibodies had 2 sites/150,000 daltons for [^{14}C]menadione-butyrate. The Scatchard plots (Figure 5A) were nonlinear, indicating that the antibodies were heterogeneous with respect to affinity for this hapten. The average intrinsic association constant, K_0, was 1.1 ± 0.3 × 10^5 M^{-1} for antibodies isolated from the early sera and 2.6 ± 0.6 × 10^6 M^{-1} for antibodies from the late sera.

In accord with nonlinearity in the Scatchard plots, the heterogeneity indices (Sips, 1948), *a*, in Figure 5B, were clearly less than 1.0 (0.8 for the early antibodies and 0.7 for the late antibodies).

Fluorescence Quenching. The binding of menadione-butyrate and menadione-caproate quenched the tryptophan fluorescence of the antimenadione antibodies. When maximum quenching (Q_{max}) value is assumed to be 40%, the titration curves of Figure 6 correspond to average intrinsic association constants for the early and late antibodies of 1 × 10^5 and 2-3 × 10^6 M^{-1}, respectively (*i.e.*, the same as those measured by equilibrium dialyses). The Sips heterogeneity indices for the fluorescence quenching titrations are also in agreement with the equilibrium dialysis experiment: *a* was 0.8-0.9 for the early antibodies and 0.7-0.8 for the late antibodies.

Discussion

Our initial attempt to prepare menadione-proteins *via* amino group substitution on menadione were unsuccessful. However, the addition of sulfhydryl compounds to menadione (Fieser and Turner, 1947) and the elucidation of the reaction as one of nucleophilic substitution (Nickerson *et al.*, 1963) suggested to us that menadione could be conjugated to proteins or other compounds as long as they carried a reactive thiol. Because thiol groups evidently have a lower energy of activation than amino groups, the thiols are capable of carrying out nucleophilic attack on menadione under the mild conditions needed to maintain the integrity of proteins.

In compounds obtained by thiol substitution, the menadione moiety is linked by a thioether bond at the 3 position on the quinoid ring (see Figure 1B). The most prevelant or oxidized form of the quinone is preserved as evidenced by the yellow color of the menadione derivatives. Since these compounds are susceptible to base-catalyzed hydrolysis, yielding phthiocol and sulfhydryls, they have to be maintained at pH 7.6 or lower. Exposure to light yielded brown, often gummy, degradation products. The K vitamins and their analogs are degraded by light-induced free radical

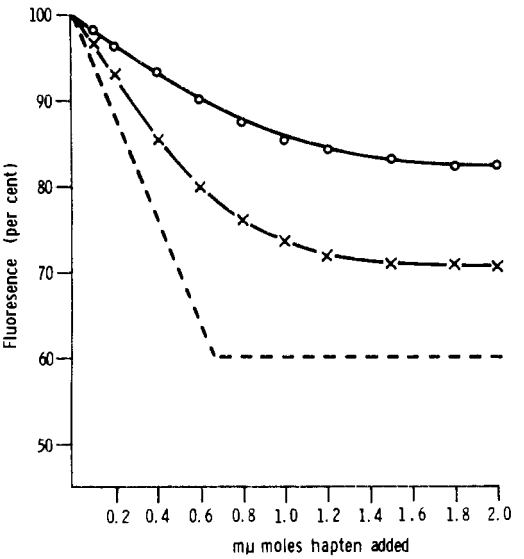

FIGURE 6: Fluorescence quenching of purified antimenadione antibodies from early (O) and late (x) antisera. Protein (1 ml, 0.33 nmol) was titrated with menadione-butyrate at 1 × 10^{-5} M, at 4°. Values are corrected for background fluorescence and volume changes. The Q_{max} value of 40% was selected so that the fluorescence quenching titrations gave the same intrinsic association constants as equilibrium dialyses carried out with the same antibody and hapten preparations. A Q_{max} value of 35% was observed in a separate titration with these antibodies and menadione-butyrate at 5 × 10^{-4} M; in the latter titration, corrections for attenuation of fluorescence by the high ligand concentration were based on parallel titrations with nonspecific immunoglobulins from unimmunized rabbits (Eisen and McGuigan, 1971).

generation (MacCorquodale *et al.*, 1939) and this is probably the mechanism by which these menadione derivatives are degraded. When these compounds were kept in the dark, and exposed to room light for only short intervals, they remained stable for many months.

An extensive repertoire of low molecular weight aromatic compounds (haptens) conjugated to carrier proteins or polypeptides can elicit the formation of antibodies to the haptenic groups. It is thus not surprising that menadione–proteins stimulate the production of antimenadione antibodies. Although menadione represents a portion of certain molecules (vitamin K and its analogs) that are normally present in mammals, it is well established that rabbit antibodies can be raised to some other substances that also exist in normal mammalian tissues, *e.g.*, thyroxin, pyridoxal, folic acid (Clutton *et al.*, 1938; Ungar-Waron and Sela, 1966; Rubenstein and Little, 1970).

The antibodies to menadione resemble those to other haptenic groups; *i.e.*, they engage in conventional precipitin reactions, these reactions can be inhibited specifically by univalent haptens (one hapten moiety per molecule), antihapten antibodies can be isolated in high yield with the aid of specific adsorbants, and the purified antibodies have two hapten binding sites per 150,000 daltons. As with most other antihaptens, antimenadione antibodies are also heterogeneous with respect to affinity. However, their heterogeneity indices (*a* values in Figure 5) are close to 1.0 suggesting that they are considerably less heterogeneous than the antibodies raised in rabbits by similar immunization procedures against many other haptens. It is possible that the natural prevalence of menadione-like molecules in these animals brings about tolerance of many potentially responsive lymphocytes, leaving only a restricted number of menadione-sensitive clones of lymphocytes to respond to immunization.

In accord with the overlap between the absorption spectrum of menadione (Figure 1B) and the fluorescence emission spectrum of antibodies (Velick *et al.*, 1960), the antibody fluorescence was quenched in the menadione–antimenadione complex. About 40% of the fluorescence (290 nm excitation; 350 nm emission) appears to be quenched when the ligand binding sites are saturated (Q_{max} = 40%). A similar value has been observed with rabbit antibodies to the 2,6-dinitrophenyl group (Q_{max} = 45–50%; Little *et al.*, 1969), but higher values obtain with rabbit antibodies, to the 2,4,6-trinitrophenyl group (Q_{max} = 50–60%; Little and Eisen, 1966), and especially to the 2,4-dinitrophenyl group (Q_{max} = 70–80%; Eisen and Siskind, 1964). The differences probably relate to varying distributions and orientations of tryptophan residues in rabbit immunoglobulin molecules of different specificities.

Our primary objective in this work was to determine whether antibodies could be raised against menadione and, if so, to establish some of the general characteristics of these antibodies. The results show that rabbits can make substantial amounts of antimenadione antibodies. Except for their possibly limited heterogeneity, these antibodies have the general properties of those raised to nitrophenyls and to many other aromatic haptenic groups.

The binding of menadione by some anti-Dnp immunoglobulins has been regarded as supporting evidence for the view that combining sites of antibodies have multiple binding functions (Eisen *et al.*, 1967; Richards and Konigsberg, 1973), largely on the assumption that Dnp and menadione are as structurally dissimilar as any aromatic haptens chosen at random. As is shown elsewhere, however, structural similarity between Dnp and the benzenoid moiety of menadione cannot be dismissed (Michaelides and Eisen, 1974); and in a separate report we shall show that some antimenadione immunoglobulins bind Dnp ligands, just as some anti-Dnp molecules bind menadione (M. F. M. Johnston and H. N. Eisen, in preparation).

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