THE STRANGE CROSS-REACTION OF MENADIONE (VITAMIN K_3) AND 2,4-DINITROPHENYL LIGANDS WITH A MYELOMA PROTEIN AND SOME CONVENTIONAL ANTIBODIES*

By MARIA C. MICHAELIDES AND HERMAN N. EISEN‡

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110)

(Received for publication 30 May 1974)

The search for myeloma proteins with antihapten activity has shown that somewhere between roughly 1 and 10%, in different series, bind nitrophenyl ligands (1–4). Though the binding is generally exceedingly weak, the overall frequency is still surprisingly high (5). The reasons for the high frequency are not clear and a number of possibilities have been considered. One is that nitrophenyls are bound nonspecifically at hydrophobic sites on immunoglobulins (6). Another is that an unidentified, prevalent immunogen that happens to cross-react with nitrophenyls causes selective enrichment of the corresponding lymphocytes; neoplastic transformation of these cells could then result in myeloma tumors whose myeloma proteins bind various nitrophenyls (7).

To help evaluate these possibilities we have examined the binding of a variety of substances by protein 315, which has higher affinity for the 2,4-dinitrophenyl (DNP) group than any of the other myeloma proteins so far studied (5). The results show that (*a*) nonspecific binding does not account for protein 315's high affinity for DNP, and (*b*) a vitamin K-like molecule, 2-methyl-1,4-naphthoquinone, also called vitamin K_3 or menadione, is bound specifically and with considerable affinity at a site that probably overlaps the one that binds DNP ligands.

In order to extend the comparison of myeloma protein 315 with conventional antibodies, we also determined the temperature dependency of its binding reactions with ϵ-DNP-lysine and vitamin K_3, and we compared some of its binding properties with those of rabbit and guinea pig antibodies raised by ordinary immunization procedures against the 2,4-DNP, the 2,6-DNP, and the 2,4,6-(TNP) groups. All of the results emphasize the similarity between the ligand-binding properties of myeloma protein 315 and conventional antinitrophenyl antibodies, many of which also exhibit the "strange" (i.e., unexpected) cross-reaction with vitamin K_3. The recognition of this cross-reaction

* This work was supported in part by a research grant (AI-03231) and a training grant (T5I-AI-257) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by a contract with the Research and Development Command of the Army, recommended by the Commission on Immunization of the Armed Forces Epidemiological Board (USDA-49-193-MD-2330).

‡ Present address: Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

and some of the other results described here were previously summarized in a symposium on "Experimental Approaches to Homogeneous Antibody Populations" (5).

Materials and Methods

MOPC-315.—This tumor was generously provided by Dr. Michael Potter, National Institutes of Health, Bethesda, Md.; it was maintained by serial subcutaneous transplantation at about 3-wk intervals in BALB/c mice from Cumberland Farms, Tenn., for about 1 yr and then in BALBcAnN mice provided by Dr. Ralph J. Graff. Blood from tumor-bearing mice was drawn by cardiac puncture, and serum was stored at about −20°C.

Protein 315.—The protein was isolated from serum by two methods (2, 8). In both the protein was recovered in its 7S form (S uncorr. = 6.6), had the same affinity for ε-DNP-L-lysine as previously reported (2), and gave a single precipitin band in immunoelectrophoresis with rabbit antiserum prepared against serum from BALB/c mice.

Antibodies to Nitrophenyls.—Rabbit antibodies to the 2,4-DNP group were isolated (9) from antisera obtained several weeks after a booster injection of 2,4-DNP-hemocyanin, given 3 mo after an initial injection of the same antigen. Purified rabbit antibodies to the 2,4,6-TNP group and purified guinea pig antibodies to the 2,6-DNP group were generously provided by Dr. J. R. Little. The anti-TNP antibodies were obtained after a booster injection of the TNP-BγG given 1 after primary immunization with the same antigen (10). The anti-2,6-DNP antibodies were isolated as described (11).

Reagents.—2-[*methyl*-^{14}C]-1,4-naphthoquinone (menadione, vitamin K_3) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. (spec act given as 9.7 mCi/mmol); m.p. 103°–104°C. It was indistinguishable from unlabeled menadione when used as ligand in fluorescence-quenching titrations (e.g., Fig. 2, below). Nonradioactive menadione was crystallized from methanol (m.p. 105°C); its molar extinction coefficient in 0.15 M NaCl − 0.01 K phosphate, pH 7.5 (phosphate-saline) was 20,000 at 252 nm (λmax).

2,4-Dinitronaphthol (m.p. 138°) had a molar extinction coefficient of 14,360 at 395 nm (λmax) in phosphate-saline (0.15 M NaCl − 0.01 K phosphate, pH 7.4). 1,4-Naphthoquinone, *p*-benzoquinone, and methyl *p*-benzoquinone were purified by sublimation. When necessary, other ligands were recrystallized until melting points agreed with literature values.

4-Mononitrophenyl (MNP)-aminocaproate, 2,6-DNP aminocaproate, and 2,4,6-TNP aminocaproate were all generously donated by Dr. J. R. Little (see ref. 11). ε-2,4-DNP-L-lysine (DNP-lysine) was obtained from Sigma Chemical Co., St. Louis, Mo. [^{3}H]-ε-2,4-DNP-L-lysine was prepared as described (2).

Equilibrium Dialysis.—The binding of various ligands was evaluated from their inhibition of the binding of [^{3}H]-ε-DNP-L-lysine by protein 315 in equilibrium dialysis. K_I, the intrinsic association constant for the inhibiting ligand, was determined from Karush's equation (12):

$$K_I = (r/r' - 1)(1 + Kc)/I \qquad \text{(eq. 1)}$$

where r and r' refer, respectively, to moles [^{3}H]-ε-DNP-L-lysine bound per mole protein in the absence (r) and presence (r') of the inhibitor, whose free (unbound) concentration is I; the free concentration of the reference ligand ([^{3}H]-ε-DNP-L-lysine) is c. I was taken as the total concentration of inhibitor, which was added in large molar excess over the protein (at least 1,000–10,000-fold), while c was measured by counting ^{3}H in the protein-free compartment. Based on equilibrium dialysis in the absence of the inhibitor, K, the intrinsic association constant for ε-DNP-L-lysine, was 1.2×10^7 M^{-1} (4°C), in agreement with a previous finding (2).

All equilibrium dialysis measurements were carried out at 4°C (unless specified otherwise) in small lucite chambers with 0.1 ml of protein solution (containing inhibitors as indicated) separated by a Visking membrane from 0.1 ml of buffer with [^{3}H]-ε-DNP-L-lysine (13). After

standing 40–48 h (without agitation), duplicate 25-μl portions from each compartment were counted in 10 ml of Bray's solution in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Protein concentration was measured by absorbancy at 278 nm, using the extinction coefficient (1.24 cm²/mg) and mol wt (150,000) determined previously (2, 14). Calibration curves relating r and c (see above) were constructed from equilibrium dialysis experiments with [^{3}H]-ε-DNP-L-lysine as the sole ligand; for a given value of c in the presence of an inhibitor the corresponding r' value was obtained for use in equation 1. The K_I value determined in this manner for menadione was confirmed by equilibrium dialysis with radiolabeled [^{14}C]menadione in the absence of ε-DNP-L-lysine (compare Fig. 1 and Table III).

Fluorescence Quenching.—Binding was also evaluated by the method of fluorescence quenching (15). The maximum quenching values (Qmax) used for calculation of intrinsic association

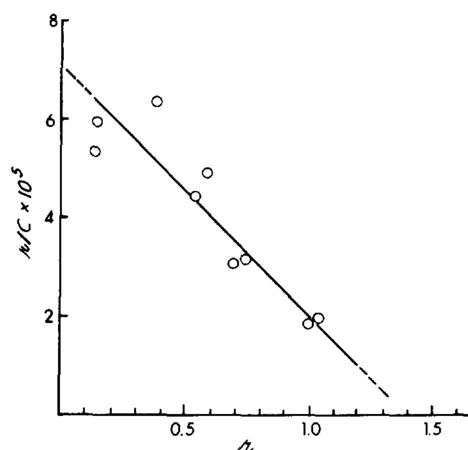

FIG. 1. Binding of [^{14}C]menadione by myeloma protein 315. Equilibrium dialysis was carried out with protein at 95 μg/ml. Each point is the average of duplicate determinations. Final concentrations of menadione ranged from 0.26 to 5.3×10^{-6} M/L. The volume on either side of the membrane was 0.1 ml. PBS Buffer; 4°C.

constants were: 69% for protein 315 (2), 75% for rabbit IgG anti-2,4-DNP (15), 55% for rabbit IgG anti-2,4,6-TNP (10), and 90% for guinea pig anti-2,6-DNP (11). Absorption spectra and difference absorption spectra were determined in a Cary spectrophotometer, model 14, as described (16).

RESULTS

The possibility that DNP ligands are bound nonspecifically at hydrophobic sites on protein 315 was tested with 1,8-anilinonaphthalene sulfonate (1,8-ANS; ref. 6). At 4×10^{-4} M, 1,8-ANS reduced the binding of [^{3}H]-ε-DNP-L-lysine (free concentration, 2×10^{-6} M) by less than 10% and the K_I for 1,8-ANS was $<2 \times 10^2$ M^{-1}. 1,8-ANS also had a negligible effect on the binding of ε-DNP-lysine by a conventional preparation of purified rabbit anti-DNP antibodies, whose average affinity for the DNP group was low (5.5×10^5 M^{-1} for ε-DNP-L-lysine and K_I for 1,8-ANS ca. 3×10^3 M^{-1}).

Further evidence for the specificity of the binding of ε-DNP-lysine by protein 315 was provided by competitive equilibrium dialysis with ε-pipsyl-L-lysine and ε-dansyl-L-lysine, whose K_I values (vs. [^{3}H]-ε-DNP-L-lysine) were ca. 5×10^3 and ca. 7×10^3 M^{-1}, respectively, or about 1,000-fold less than the affinity for ε-DNP-L-lysine.

A number of purines, pyrimidines, and nucleic acids were also tested for ability to inhibit the binding of ε-DNP-L-lysine, because of the suggestion by Schubert et al (3) that some myeloma proteins that appear to have antinucleic acid activity also react (though weakly, ref. 5) with DNP groups. The results are shown in Table I. The K_I values for 5-acetouracil caproate and 5-iodoorotic acid were $1–2 \times 10^4$ M^{-1}, or about 1,000-fold less than for ε-DNP-L-lysine. The other nucleotides were bound much less ($K_I < 10^4$ M^{-1}), and no inhibition was detected with several nucleic acid preparations.

The K_I values for some other substances are listed in Table II and III. The binding of a 2,4-dinitrophenylazo dye is in accord with the known affinity for DNP ligands. However, a number of structurally unrelated substances were also bound to a notable extent (tetrahydrofolate, riboflavin, flavin mononucleotide in Table II); of these, the most impressive binding was seen with menadione. The affinity of protein 315 for the latter was confirmed by equilibrium dialysis with [^{14}C]menadione in the absence of a competitive ligand: the intrinsic association constant was 5×10^5 M^{-1} (Fig. 1), in agreement with both the value obtained by fluorescence quenching (6×10^5 M^{-1}, Fig. 2) and the K_I value in Table III (6×10^4 M^{-1}).

The number of combining sites for menadione on protein 315 appeared to be about 1.4/150,000 daltons (Fig. 1), and the same number of sites for ε-DNP-L-lysine was found in a previous study (2). However, protein 315 has since been shown to have close to 2.0 sites/150,000 daltons for both menadione and ε-DNP-lysine, providing equilibrium dialysis is carried out at higher protein concentrations than were used in the experiment of Fig. 1 (0.05 mg/ml), or a protective protein, such as gelatin, is added (14).

In view of the structural difference between the DNP group and menadione (see Fig. 5, below) a study was made of the binding of this naphthoquinone by antibodies to diverse nitrophenyls. As is shown in Fig. 2, rabbit anti-2,4-DNP rabbit anti-2,4,6-TNP, and guinea pig anti-2,6-DNP also had substantial affinity for menadione. For purposes of comparison with menadione, the binding of 2,4-dinitronaphthol by these antibodies and by protein 315 is also shown in Fig. 2. From the summary given in Table IV it is clear that 315 bound menadione better than 2,4-dinitronaphthol, whereas the rabbit and guinea pig antinitrophenyls bound dinitronaphthol more strongly than menadione. As expected, the conventional antibodies were heterogeneous with respect to affinity (a values much less than 1.0), whereas the combining sites of protein 315 appeared homogeneous (a values were close to 1.0).

The specificity of protein 315 for menadione was indicated by the sharp

TABLE I

Binding of Some Purines, Pyrimidines, and Nucleic Acids by Protein 315

Inhibitors	Total (free) inhibitor concentration (I)	I/c*	Inhibition‡	K_I
	$M/L(\times 10^{-4})$		%	$L/M(\times 10^{-4})$
Purines				
Adenine	10.0	9,280	50	0.27
Adenosine	2.0	1,570	30	0.53
Adenylic acid	2.0	1,580	41	0.85
Deoxyadenylic acid	2.0	1,690	36	0.66
Guanosine				0.21
Guanylic acid	2.0	1,780	23	0.34
Deoxyguanylic acid	2.0	1,650	25	0.39
Pyrimidines				
5-Acetouracil caproic acid	1.0	830	46	2.44
5-Acetouracil	1.0	1,180	10	0.26
Thymine	10.0	9,860	27	0.09
Thymidine	1.0	1,120	11	0.30
Deoxythymidylic acid	2.0	1,530	27	0.46
Uracil	10.0	12,500	26	0.08
Uridine	10.0	14,000	18	0.05
Uridylic acid	2.0	1,830	25	0.38
5-Hydroxyuridine	10.0	13,000	21	0.06
5-Nitrouracil	10.0	9,770	25	0.06
Isoorotic	5.0	5,840	18	0.10
5-Iodoorotic acid	1.0	1,160	10	1.05
Cytidine	2.0	1,830	10	0.12
Cytosine	2.0	1,830	12	0.15
Cytidylic acid	2.0	1,890	22	0.31
Deoxycytidylic acid	2.0	1,530	28	0.48
Carboxymethylcytosine	10.0	10,370	18	0.06
Nucleic Acids				
BALB/c mouse spleen DNA	50§	1,300‖	N¶	<0.02
Salmon sperm DNA	50§	1,300‖	N	<0.02
Yeast RNA	1.0	570	N	<0.02

* Ratio of inhibitor concentration (I) to concentration of free [^{3}H]-ε-DNP-L-lysine (c).

‡ Inhibition of [^{3}H]-ε-DNP-L-lysine binding relative to its binding in absence of inhibitor (see eq. 1).

§ μg/ml.

‖ Corresponds to about 1.6×10^{-4} M/L per nucleotide (avg. mol wt = 300) or about 1×10^{-5} M if 10 nucleotides are assumed to represent one antigenic determinant, i.e., at 100–1,000-fold molar excess over DNP-L-lysine.

¶ None detectable (< 10%).

reduction in affinity for some analogues (see Fig. 5). For example, phthiocol was hardly bound to a detectable extent, and the K_I value for 1,4-napthoquinone was about one-tenth that of menadione (Table III).

Subsets of conventional anti-2,4-DNP antibodies were previously found to

TABLE II

Binding of Diverse Other Compounds by Protein 315

Inhibitors	Total (free) inhibitor concentration (I)	I/c*	Inhibition‡	K_I
	$M/L(\times 10^{-4})$		%	$L/M(\times 10^{-4})$
2,4-Dinitrophenylazo-1-naphthol-3,6-disulfonate	0.07	550	61	66.5
Tetrahydrofolic acid	2.0	1,300	68	5.8
Pyridoxol	1.0	900	42	2.1
Riboflavin	0.37	300	41	3.7
Flavin mononucleotide	1.0	700	67	6.8
Flavin adenine dinucleotide	1.0	1,000	38	1.5
Nicotinamide	1.0	1,000	23	0.8
Nicotinamide adenine dinucleotide	1.4	1,700	17	0.3
Nicotinamide adenine dinucleotide phosphate	1.3	1,600	19	0.4
Barbital	300.0	33,000	13	0.02
Barbituric acid	10.0	12,400	18	0.05
Isobarbituric acid	5.0	5,800	27	0.2
Theophylline	10.0	6,000	82	1.6
Caffeine	10.0	5,000	90	3.8
Theobromine	50.0	3,200	65	1.3
O-Nitrophenyl galactose	10.0	1,200	14	0.4
Lactose	100.0	118,000	19	0.006
M-chlorocarbonyl cyanide phenylhydrazone	1.0	1,200	48	1.7
Isophthalate (meta)	3.8	5,100	N§	<0.02

* Ratio of inhibitor concentration to concentration of free [^{3}H]-ε-DNP-L-lysine (*c*).

‡ Inhibition of [^{3}H]-ε-DNP-L-lysine binding (see Table I footnote).

§ None detectable ($< 10\%$).

differ markedly in reactivity with 5-acetouracil caproate, another strange cross-reaction (3, 17). As is shown in Table V, a subset with high affinity for 5-acetouracil caproate had relatively high affinity for menadione, while another subset had much lower affinity for both of these ligands; the subsets were similar, however, in their affinities for ε-DNP-lysine and for 2,4-dinitronaphthol.

Because the binding of DNP and TNP ligands by conventional anti-DNP and anti-TNP antibodies is characterized by a large decrease in enthalpy (10), it was of interest to determine the effect of temperature on the binding of ε-DNP-L-lysine and of menadione by protein 315. As is shown in Fig. 3, the binding of both ligands by the myeloma protein was also markedly exothermal.

In view of the red shift and hypochromic change undergone by DNP and TNP ligands in the combining sites of protein 315 (2) and of conventional antibodies (16), similar changes were sought with menadione. When bound by protein 315, the naphthoquinone's absorption spectrum underwent a small red

TABLE III

Binding of Menadione and Some Related Substances by Protein 315

Inhibitor	Total (free) inhibitor concentration (I)	I/c*	Inhibition‡	K_I	Q'§
	M/L(× *10*$^{-4}$)		%	*L/M*(× *10*$^{-4}$)	
Menadione	1.0	480	95	52	30
^{14}C menadione‖	0.027	26	46	68	
1,4-Naphthoquinone	0.5	250	45	6	14
1,4-Naphthoquinone 2 sulfonic acid					<5
1,2-Naphthoquinone 4 sulfonic acid	0.1	113	15	4	<5
Phthiocol (1,4-naphthoquinone 2 methyl, 3 hydroxy)	1.0	1,430	<10	<0.2	
p-Benzoquinone	0.5	262	<10	<0.2	<5
Methyl-*p*-benzoquinone					
Dicumarol	1.0	900	62	4	<5
2,4-Dinitronaphthol	0.21	107	20	12	10

* Ratio of inhibitor concentration to concentration of free [^{3}H]-ε-DNP-L-lysine (*c*).

‡ Inhibition of [^{3}H]-ε-DNP-L-lysine binding.

§ Q' is percent quenching of protein fluorescence (290 nm activation, 345 nm emission) following addition of 2–4 nmol ligand (in the case of *p*-benzoquinone 13 nmol were used) to 0.42 nmol protein in a final vol of 1.2 ml.

‖ Direct binding with this ligand yielded a K (assoc.) value of 5.1×10^5 M^{-1} (Fig. 1).

shift (340–350 nm) and a hypochromic change (22%) (Fig. 4). Hypochromia, but no red shift, was observed when menadione was bound by rabbit anti-2,4-DNP antibodies, and by tryptophan at high concentration (as the free amino acid). These changes are concordant with the similar but more marked changes observed when DNP (and especially TNP) ligands form complexes with tryptophan (free amino acid). All of these changes in absorption spectra were more readily apparent in difference spectra (lower panels of Fig. 4 A, B, C). With normal rabbit γ-globulin as control there was no change in menadione's absorption spectrum.

DISCUSSION

Protein 315, a mouse IgA myeloma protein with considerable affinity for DNP and TNP ligands, was tested for ability to bind approximately 57 benzenoid, heterocyclic, and other substances. A number of purine and pyrimidine derivatives were bound weakly ($<1 \times 10^5$ L/M), but only menadione (vitamin K_3) was bound with an affinity (5×10^5 L/M) that approximates the values commonly observed with benzenoid ligands and their antibodies. Other ligands could have been overlooked because the assay, chosen for convenience, detected only substances whose binding interfered with the binding of the reference ligand ([^{3}H]-ε-DNP-L-lysine).

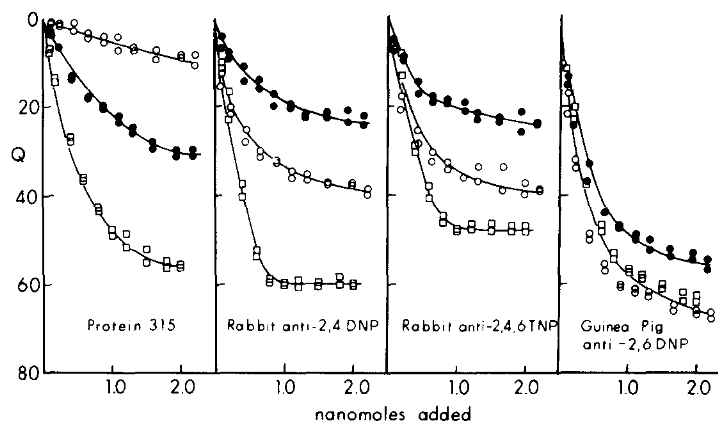

FIG. 2. Fluorescence quenching titrations of protein 315 and some antinitrophenyl antibodies with ε-2,4-DNP-L-lysine (□), menadione (●), and 2,4-dinitronaphthol (○). In each titration a total of 0.2 ml of ligand (1×10^{-5} M/L) was added to 1.0 ml containing 50 μg purified antibody or myeloma protein. The final value at end of the titration is called Q′ (see Tables III and V). Temperature, 4°C.

TABLE IV

*Affinity of MOPC-315 and Conventional Antinitrophenyls for Menadione and 2,4-Dinitronaphthol**

Ligands	Protein 315		Rabbit anti-2,4-DNP		Rabbit anti-2,4,6-TNP‡		Guinea pig anti-2,6-DNP‡	
	$K (\times 10^{-5}$ L/M)	a	$Ko (\times 10^{-5}$ L/M)	a	$Ko (\times 10^{-5}$ L/M)	a	$Ko (\times 10^{-5}$ L/M)	a
Menadione	5.6§	0.8	1.6	0.5	4.0	0.5	19	0.5
2,4-Dinitronaphthol	1.1‖	1.0	8.0	0.3	75	0.5	130	0.4
Ko-Menadione/ Ko-Dinitronaphthol	5.0		0.2		0.05		0.2	

* Affinity values based on fluorescence quenching titrations in Fig. 2. K, intrinsic association constant; Ko, average intrinsic association constant; a, heterogeneity index for K values estimated from Sips plots (1.0 represents homogeneous sites; heterogeneity increases as values approach zero). Essentially the same values were given in a preliminary report (6).

‡ These purified antibodies were generously provided by Dr. J. R. Little.

§ By equilibrium dialysis with [^{14}C]menadione K = 5.1×10^5 L/M, Fig. 1.

‖ By competitive equilibrium dialysis $K_I = 1.2 \times 10^5$ L/M. Table III.

Three observations emphasize that menadione is bound specifically, rather than nonspecifically through hydrophobic bonds at an apolar patch on the surface of the protein. (*a*) A variety of other relatively hydrophobic ligands (dansyl, pipsyl, 1,8-anilinonapthalene sulfonate) are not bound to a significant extent by protein 315 (2). (*b*) Analogs of menadione with small structural

TABLE V

*Affinities of Subsets of Guinea Pig Anti-2,4-DNP Antibodies for Menadione and other Ligands**

Antibody	Ligand					
	ε-DNP-L-lysine		2,4-Dinitronaphthol		Menadione	
	Q′	Ko	Q′	Ko	Q′	Ko
Subset 1‡	69.7	ca. 2×10^8	66.0	5×10^7	50.6	1.0×10^6
Subset 2§	66.6	ca. 2×10^8	62.0	1×10^7	34.2	1.3×10^5

* Based on fluorescence quenching titrations, calculated on the basis of Qmax = 90. Q′ is percent quenching of protein fluorescence after addition of 1–4 nmol ligand to 0.35 nmol protein in a final vol of 1.2 ml (see legend, Table III).

‡ This subset of purified anti-DNP was precipitated by 5-acetouracil-protein and has a high affinity for 5-acetouracil caproate (16).

§ This subset of purified anti-DNP was not precipitable by 5-acetouracil-protein and has barely measurable affinity for 5-acetouracil caproate (16).

modifications that hardly change the overall apolar character (phthiocol, with addition of an OH group at carbon 3, and 1,4-naphthoquinone, in which the CH_3 at carbon 2 is deleted; see Fig. 5, below) are bound much less well. (*c*) The binding of menadione is markedly temperature-dependent (enthalpy driven), whereas binding due simply to hydrophobic bonds is characteristically independent of temperature (19).

The intrinsic association constant for the reversible binding of menadione was the same when estimated in the presence or absence of DNP-lysine, showing that menadione binding is not due to "unmasking" a site by a conformational change induced by the binding of ε-DNP-lysine. These findings also suggest that the reduced binding of ε-DNP-lysine in the presence of menadione is due to competitive blocking via steric hindrance rather than to a conformational change in the site for DNP-lysine brought about by binding of menadione at a distant site.

A cross-reaction between structures as different as DNP and menadione would hardly have been predicted from past experience with hapten binding by antibodies (20, 21): hence it has been designated a strange cross-reaction (5). However, examination of models shows that with superimposition of their benzene rings, there can be considerable overlapping of DNP and menadione (Fig. 5). In addition, the molecules have similar charge distribution: electron-withdrawing oxygen atoms establish positively charged rings capable of serving as acceptors in charge-transfer complexes. In view of these similarities their cross-reaction seems not to be so strange after all.

It is thus not surprising that the DNP/menadione cross-reaction is also evident with many antibodies raised by conventional procedures in rabbits and guinea pigs to various nitrophenyls (anti-2,4-DNP; anti-2,6-DNP, anti-2,4,6-TNP) (Fig. 2). It also occurs (at lower affinities) with protein 460 another mouse IgA myeloma protein with anti-DNP activity (22, 23). The

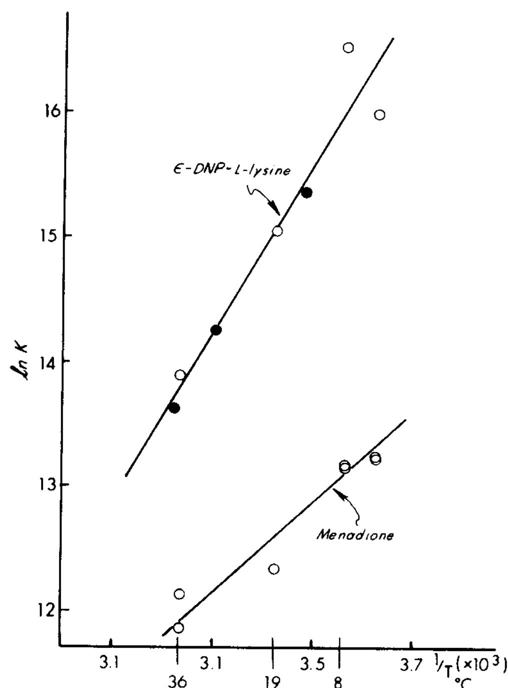

FIG. 3. Temperature dependency of binding ε-DNP-lysine and menadione by protein 315. K values, the intrinsic association constants at various temperatures, were determined by fluorescence quenching (○) or equilibrium dialysis (●). ΔH, the enthalpy change on binding, was −13,500 and −7,230 cals/mol ligand bound for ε-DNP-lysine and menadione, respectively.

gross similarity in shift of absorption spectrum of menadione and of DNP when bound specifically by protein 315 and by antinitrophenyl antibodies probably derives from the environment at the sites which these ligands occupy; they might reflect contact with tryptophan residues with, perhaps, formation of charge-transfer complexes. Several tryptophans in the V regions of the protein's light and heavy chains are candidates for such complexes (24, 25). Interactions with tryptophan have also been suggested as a possible basis for the otherwise unexplained marked changes in enthalpy associated with the specific binding of DNP and of menadione to protein 315 and to conventional antibodies (26).

It is possible that not all conventional anti-DNP molecules bind menadione. Anti-DNP subsets that were separated (17) on the basis of their ability to engage in another strange cross-reaction, with 5-acetouracil, differed in their reaction with menadione. The subset that reacted best with 5-acetouracil also reacted best with menadione and with dinitronapthol. These results emphasize the importance of antibody diversity for comparisons of the combining sites

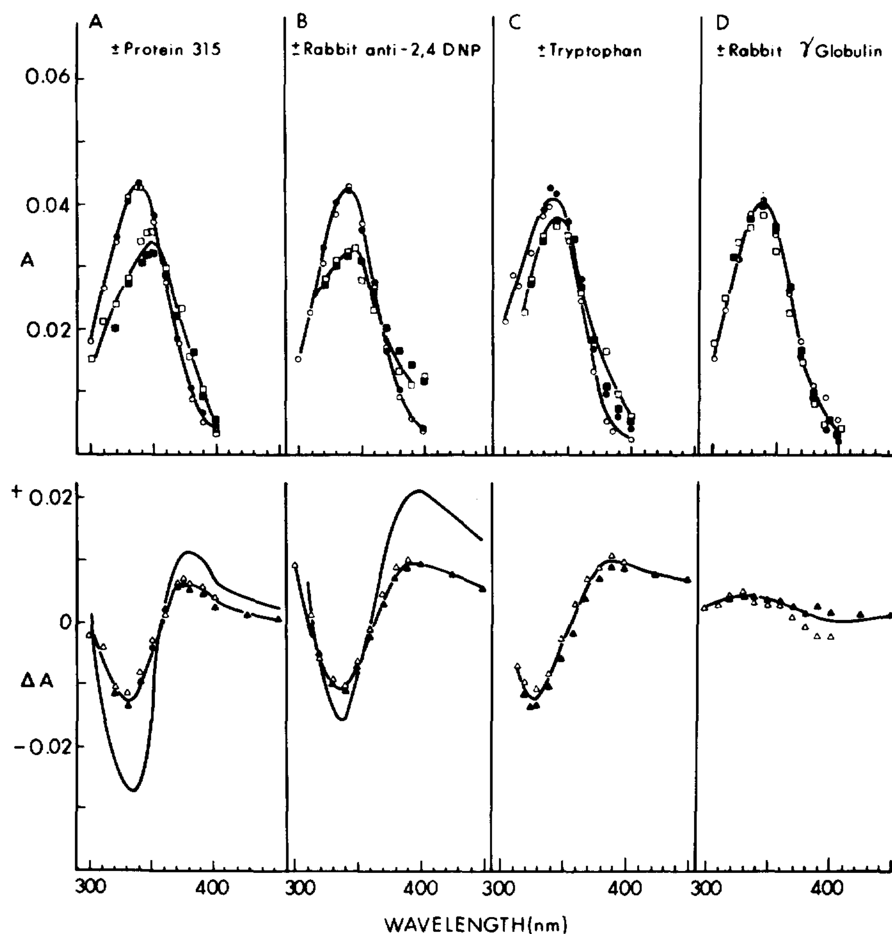

FIG. 4. Change in absorption spectrum of menadione when bound by protein 315, rabbit anti-2,4-DNP antibodies, and tryptophan (as free amino acid). Spectra in the upper panels were determined vs. buffer. Difference spectra in lower panels were obtained in tandem cuvets with menadione plus appropriate protein (or tryptophan) in the one beam and free menadione and free protein in the reference beam (15). Hydrogen (○, □, △) and tungsten (●, ■, ▲) lamps gave the same results for free menadione (○, ●), bound menadione (□, ■), and for the difference spectra (△, ▲). Protein concentrations in mg/ml were: protein 315, 2.7; rabbit anti-DNP antibody, 4.4; nonspecific rabbit γ-globulin (pseudoglobulin), 3.6; tryptophan concentration was ca. 0.02 M/L. Total menadione concentration was 1.37×10^{-5} M/L, except for the curves without symbols in lower panels A and B where it was increased to 6×10^{-5} M/L (symbols for H_2 and tungsten lamps omitted for simplification). Difference spectra were obtained with a slide wire that gave full-scale pen deflection for an absorbancy of 0.100. PBS buffer; 14°C.

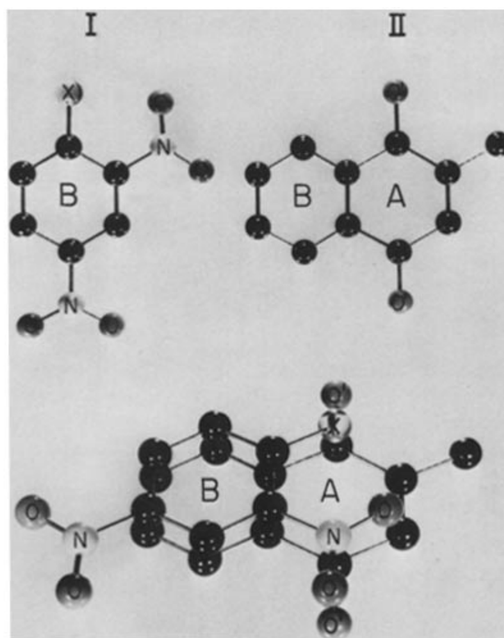

FIG. 5. Models of 2,4-dinitrophenyl (I), with X representing an unspecified atom (C, N, or other), and of menadione (II). B refers to benzene rings, A to the quininoid moiety of menadione. In the lower photograph, DNP is superimposed on the benzene ring of menadione and rotated 60° to maximize overlap. Phthiocol (see text) is menadione with an OH on carbon 3; 1,4-naphthaquinone (see text) is menadione without the CH_3 on carbon 2 (the carbons are numbered clockwise with carbon 1, substituted with 0, at the top of ring A). The models were kindly constructed to scale by Mitchell Schwartz, using accepted bond angles and distances; hydrogen atoms are omitted.

on a given myeloma protein with those in a heterogeneous population of conventionally raised antibody molecules. For example, the finding by Glaser and Singer (27) of a circular dichroism difference between the specific complexes formed by ϵ-DNP-lysine with protein 315 and with conventionally induced mouse anti-DNP antibodies leaves open the possibility that a subset of the induced, heterogeneous antibody population could resemble protein 315 in this property. Karush has observed that different isoelectric-focusing fractions of antilactosyl antibodies from a single rabbit cause substantially different spectral shifts of bound azophenyl lactoside, even when the fractions have the same affinity for this ligand (28).

Because of the pronounced structural difference between DNP and menadione, the binding of both ligands by the same immunoglobulin has been taken as support for the idea that individual immunoglobulin molecules are multispecific (1, 29). From the structural overlap revealed by Fig. 5, however, it would seem that a stronger argument for multispecificity would be provided

by ligands with so little structural similarity that they would be more likely to bind to different, entirely nonoverlapping sites within the combining region (V_L and V_H) of the same immunoglobulin. One way to detect such nonoverlapping ligands for a protein that binds DNP and menadione would be to repeat the screening exercise described in this paper with [^{14}C]menadione (in place of radiolabeled DNP-lysine) as the reference ligand. It may thus be possible to identify a molecule (call it X) that inhibits the binding of menadione but not that of DNP-lysine: X and DNP-lysine might then occupy entirely separate but neighboring sites within the immunoglobulin's combining region.

In order to be significant biologically, multispecificity must involve stimulation of a given lymphocyte by structurally dissimilar immunogens, not just binding of different ligands by a particular Ig. That antibody-forming cells can be stimulated in secondary immune responses by similar cross-reacting ligands (say, 2,4-DNP and 2,4,6-TNP) is strongly implied by the phenomenon of "original antigenic sin" (30–32); and recent findings of Varga et al (33) suggest that a given clone of antibody-forming cells can also be stimulated by structures as dissimilar as DNP and menadione. However, it is not clear why in primary immune responses it is so difficult to detect cross-stimulation by such similar cross-reacting ligands as DNP and TNP (34). The contributions of cross-stimulation and multispecificity to the triggering of antibody-forming cells remain to be established.

SUMMARY

To explore the possibility that the affinity of some myeloma proteins for 2,4-dinitrophenyl (DNP) ligands is the consequence of a "strange" (i.e., unexpected) cross-reaction for more natural ligands, a variety of substances (primarily derivatives of purines, pyrimidines, naphthaquinone) were tested for ability to block the binding of [^{3}H]-ϵ-DNP-L-lysine by protein 315, an IgA mouse myeloma protein with high affinity for DNP ligands. The most impressive inhibiting activity was observed with 2-methyl-1,4-napthaquinone (menadione, vitamin K_3). The affinity (intrinsic association constant) of protein 315 for menadione was 5×10^5 L/M (at 4°C). Because the same affinity was measured in direct-binding assays (e.g., equilibrium dialysis) and in an indirect one based on the assumption of competitive binding with DNP-lysine, it is likely that menadione and DNP bind at overlapping sites in the protein's combining region. This conclusion is supported by molecular models which reveal some common structural features in these ligands. Hence it is not surprising that antinitrophenyl antibody preparations, raised by conventional immunization procedures (anti-2,4-DNP; anti-2,6-DNP; anti-2,4,6-TNP) also bind menadione with considerable affinity. As with DNP ligands, when menadione binds to protein 315 or to conventional antinitrophenyl antibodies, some of the protein's tryptophan fluorescence is quenched, there is a change in the ligand's

absorption spectrum (hypochromia and/or red shift), and the binding is temperature-dependent (exothermal).

Note Added in Proof.—Additional evidence in support of the idea that DNP and vitamin K_3 are bound at overlapping sites on protein 315 is provided by a recent "paired labeling" experiment in which the protein was iodinated with ^{125}I and ^{131}I in presence and absence of various ligands: ε-DNP-lysine and menadione blocked to the same extent the iodination of what appears to be the same tyrosine residue of protein 315's heavy chain (O. A. Roholt, B-K. Seon, A. L. Grossberg, and D. Pressman. *Transplant. Proc.* In press).

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