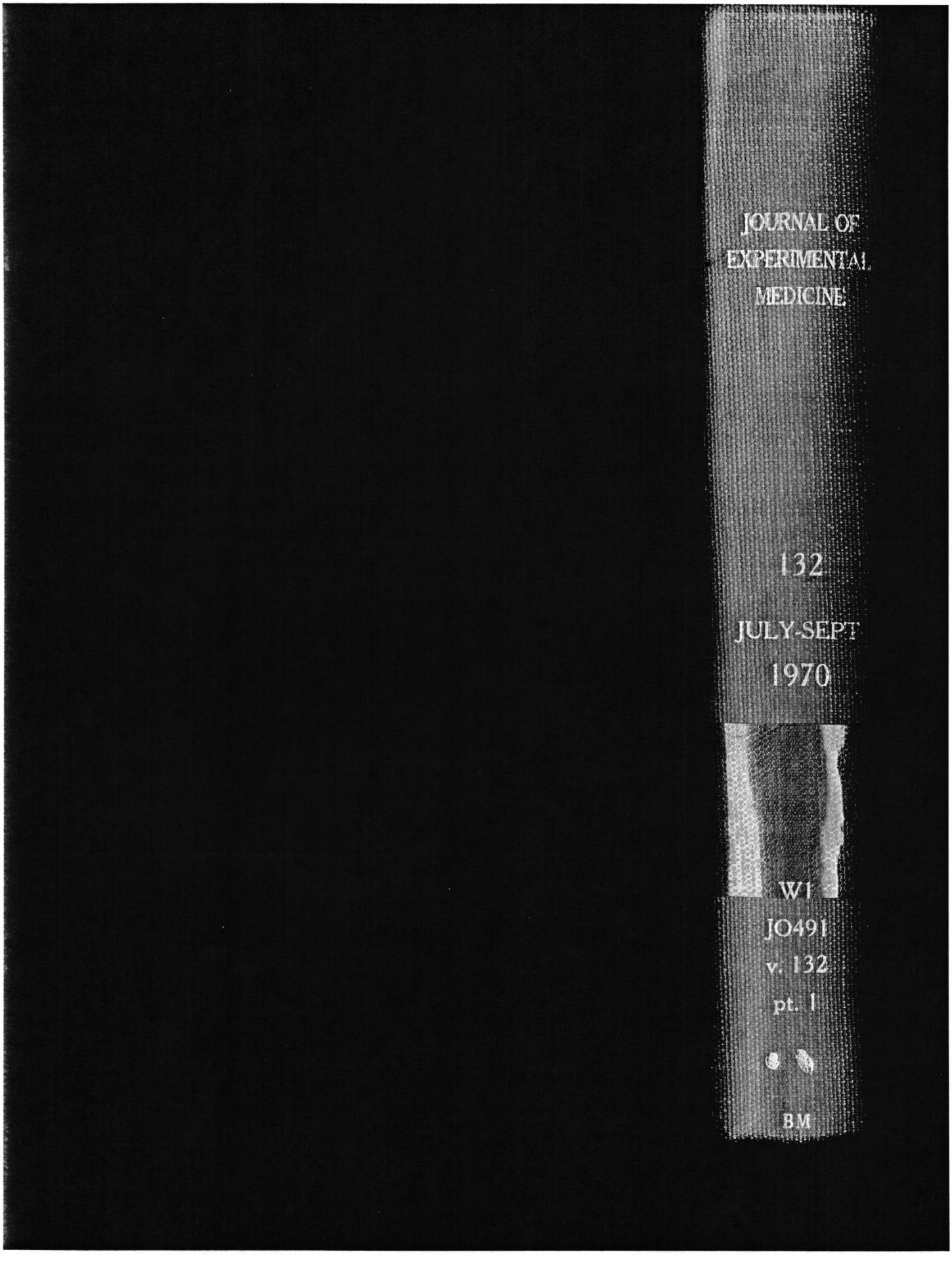
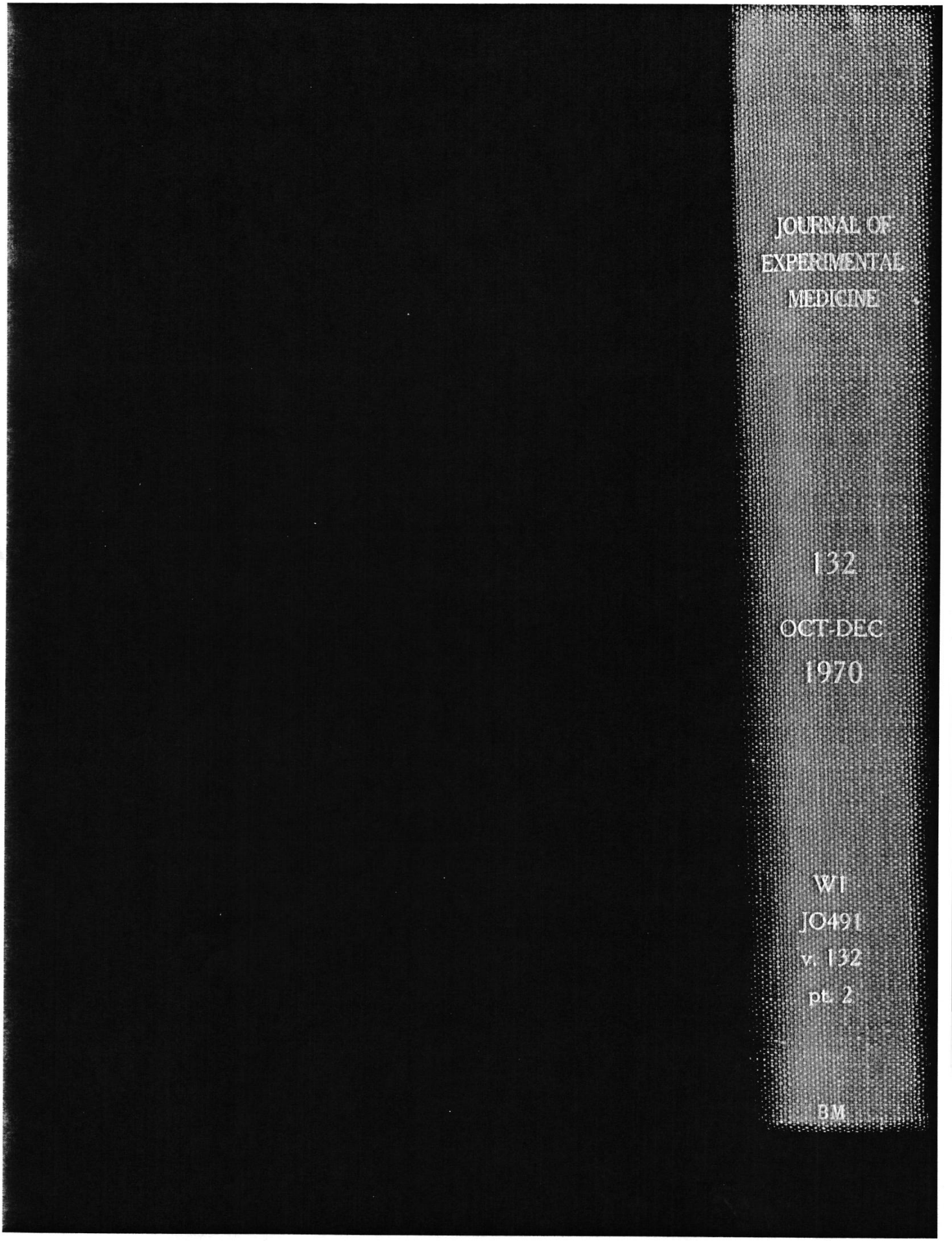


PFIZER EX. 1603
Page 1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PFIZER EX. 1603
Page 3

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PFIZER EX. 1603
Page 4

CONTENTS

No. 1, JULY 1, 1970

PFIZER EX. 1603
Page 5

PFIZER EX. 1603
Page 6

No. 4, OCTOBER 1, 1970

PFIZER EX. 1603
Page 7

PFIZER EX. 1603
Page 8

PFIZER EX. 1603
Page 9

PFIZER EX. 1603
Page 10

PFIZER EX. 1603
Page 11

THE JOURNAL OF EXPERIMENTAL MEDICINE
JULY 1, 1970
VOLUME 132, NUMBER 1

PFIZER EX. 1603
Page 12

QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

IV. Inhibition by Specific Haptens of the Reaction of Anti-hapten Antibody with its Anti-Idiotypic Antibody*

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(Received for publication 6 July 1970)

Myeloma proteins (1) and antibodies from individual animals (2, 3) have been shown to possess individually specific or "idiotypic" (4) antigenic determinants. For example, anti-salmonella antibodies from an individual rabbit have determinants that are not detectable in other immunoglobulins of that rabbit nor, ordinarily, in antibodies of the same specificity from other rabbits (3). Quantitative studies have shown that various fractions (up to 80%) of purified anti-benzoate antibodies, designated D (5), from a given donor rabbit are reactive with anti-idiotypic (anti-D) antisera (6, 7). Precipitin lines in the Ouchterlony test were obtained when as little as 2% of the donor (D) population was precipitable.

Our present investigations indicate that the reactions of anti-*p*-azobenzoate antibodies (D) with their anti-idiotypic antisera are strongly inhibited by benzoate derivatives, i.e. by specific haptens, whereas the reactions of D with anti-Fab antibodies are affected to a much smaller extent. The possible relationship of idiotypic determinants to the antigen-combining site of a donor antibody is discussed.

Materials and Methods

The following methods and materials have been described previously (6, 7): preparation and specific purification of anti-*p*-azobenzoate antibodies of the IgG class; polymerization of these antibodies with glutaraldehyde for the purpose of immunization; preparation and labeling with ^{125}ICl of F(ab')$_2$ fragments of purified anti-benzoate antibodies (each such preparation containing a 20-fold excess of nonspecific F[ab']$_2$ fragments); preparation of goat antiserum specific for rabbit fragment Fc; and determination of allotypes of rabbit sera. Anti-allotype antisera were the generous gift of Dr. Sheldon Dray.

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PFIZER EX. 1603
Page 13

Percentages of ^{125}I-F(ab')$_2$ fragments of D antibodies precipitable were determined by an indirect method utilizing excess goat antibody directed to the Fc fragment of rabbit IgG to precipitate complexes of ^{125}I-F(ab')$_2$ fragments with anti-D antibodies. The method used corresponds to that reported previously (7) with two exceptions. First, ovalbumin (50 μg per test) was used in place of bovine serum albumin to minimize adherence of the labeled protein to glass. Serum albumin was not used because it is known to bind various small molecules which were tested as inhibitors. Second, controls were run by utilizing anti-dinitrophenyl (anti-DNP) antiserum in place of anti-D. (Formerly anti-ovalbumin was used as a control). Percentages of radioactivity precipitated in control experiments did not exceed 2.8%. The control value was subtracted in each series from percentage values obtained with anti-D antiserum.

When small molecules such as haptens were tested as competitors of the reaction of F(ab')$_2$ fragments of D with anti-D, the competitor was first incubated with the labeled F(ab')$_2$ fragments and 50 μg of ovalbumin for 2 hr at 37°C, pH 8. Anti-D antiserum was added, incubation was continued for 1 hr, and goat anti-rabbit–Fc was then added to the mixture. After standing for 3 days in the refrigerator, the percentage of radioactivity precipitated was determined with a gamma scintillation counter (7). A minimum of 3000 counts was recorded for each precipitate-supernatant pair. Experiments were carried out in triplicate. All haptens and other small molecules tested as competitors were crystallized from water or from an ethanol-water mixture, with the exception of *p*-nitrobenzene sulfonic acid, and used as the sodium salts. Solutions were adjusted to pH 8.

Preparation of Anti-Idiotypic (Anti-D) Antibodies.—Specifically purified anti-benzoate antibodies (D) from individual rabbits were injected into recipients of allotype matched to the donor with respect to the following specificities: a1, a2, a3, b4, b5, b6, b9, c7, c21.

Rabbit 9Q was immunized with purified D antibody from rabbit AZ5, according to the second protocol given in reference 6; 3–5 mg of monomeric D were used for the initial inoculations with Freund's adjuvant, and polymerized D was used for subsequent intravenous inoculations. A pool of antiserum from rabbit 9Q was made from several bleedings which showed strong antibody activity by the method of indirect precipitation.

Purified D antibodies from rabbits A4 and I-14 were injected exclusively in the polymerized form into recipients 14E and 10X, respectively, and D antibody from rabbit A5 was injected into 3 recipients, 7A, 7C, and 7D. Two subcutaneous inoculations of 3 mg each in complete Freund's adjuvant were given 3 wk apart. These were followed by intravenous inoculations at intervals of 2–4 wk. Bleedings were taken 5–8 days after an injection. Antisera from individual recipients which gave strong reactions by the method of indirect precipitation were pooled. Evidence that antibodies produced were directed to idiotypic determinants, and not to allotypic or hidden determinants, has been described in detail for D antibodies of rabbits A5 and AZ5 (7, 8). This evidence includes the failure of sera taken from donor rabbits prior to immunization to inhibit reactions of anti-D with ^{125}I-F(ab')$_2$ fragments of D, the loss of inhibitory capacity of whole D serum after precipitation of antibenzoate antibodies, and the specificity of the reactions when various anti-D sera and D antibodies were utilized.

Similar results were obtained in a large number of preliminary control experiments with the D antibodies of rabbits I-14 and A4.

The percentages of F(ab')$_2$ fragments of specifically purified anti-*p*-azobenzoate antibodies that were precipitable by anti-D antisera, using the indirect method, were: rabbit AZ5, 57 ±3%; rabbit A5, 37 ±2%; rabbit I-14, 31 ±1%; rabbit A4, 23 ±1%.

RESULTS

The effects of specific haptens and other small molecules on the reactions of ^{125}I-F(ab')$_2$ fragments of D antibodies with anti-D antisera are shown in Tables

PFIZER EX. 1603
Page 14

TABLE I

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit AZ5 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab')$_2$ *precipitated, % of control*§			
p-(*p'*-hydroxy)-phenylazobenzoate	57 (1)	61 (5)	74 (4)	22
benzoate	73 (4)	90 (4)	95 (1)	1.0
p-nitrobenzoate	69 (2)	70 (3)	82 (2)	11.5
m-nitrobenzoate	74 (3)	86 (3)	85 (4)	0.4
o-nitrobenzoate	90 (1)	98 (1)	89 (2)	<0.1
p-aminobenzoate	88 (1)	96 (1)	97 (2)	0.9
m-aminobenzoate	93 (3)	91 (1)	96 (3)	0.3
o-aminobenzoate	76 (1)	85 (1)	93 (2)	1.5
p-bromobenzoate	75 (1)	86 (1)	78 (2)	5.0
m-bromobenzoate	81 (2)	98 (1)	93 (4)	1.3
o-bromobenzoate	95 (1)	103 (1)	96 (1)	0.1
p-chlorobenzoate	64 (2)	77 (2)	91 (2)	3.7
m-chlorobenzoate	78 (7)	83 (3)	89 (2)	0.8
o-chlorobenzoate	81 (3)	97 (1)	87 (5)	0.2
p-iodobenzoate	64 (2)	75 (2)	90 (1)	
o-iodobenzoate	87 (1)	97 (1)		
p-methylbenzoate	71 (2)	81 (1)	91 (1)	2.6
m-methylbenzoate	87 (2)	97 (3)	102 (2)	0.7
o-methylbenzoate	92 (2)	100 (1)	102 (1)	0.1
Sodium acetate	94 (1)	99 (1)	99 (1)	
Potassium iodide	98 (1)	102 (2)	101 (3)	
Potassium bromide	97 (1)	102 (1)	95 (1)	
p-(*p'*-dimethylamino)-phenylazobenzene sulfonate	112 (1)	104 (1)	94 (2)	

* Anti-D serum was from rabbit 9Q. The indirect method of precipitation was used. Each test contained 0.5 μg ^{125}I-F(ab')$_2$ of the D antibody, 9.5 μg nonspecific F(ab')$_2$, and 50 μg ovalbumin in a final volume of 0.31 ml. 10 μl of anti-D serum and 0.4 ml goat anti-rabbit Fc were then added. In the absence of competitors 57 ± 3% of the radioactivity was precipitated with anti-D serum and 1.2% in the control utilizing 10 μl anti-DNP antiserum. Experiments were in triplicate with average deviations given in parentheses.

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

I–IV. Each table reports data obtained with a different donor–recipient pair of rabbits. The last column in each table presents relative binding affinities of haptens, obtained by Pressman et al. (9), who measured inhibition of specific precipitation by haptens.

It is evident that specific haptens inhibit the reactions of anti-D sera. By far the best inhibitor in each system was *p*-(*p'*-hydroxy)-phenylazobenzoate. This

PFIZER EX. 1603
Page 15

compound also combines with greater affinity with anti-*p*-azobenzoate antibodies than any of the other compounds tested. The percentage inhibition observed in the four systems ranged from 43 to 69% when *p*-(*p'*-hydroxy)-phenylazobenzoate was present at a final concentration of 1.6×10^{-3} M.

In three of the four systems (Tables I–III), all para-substituted benzoate derivatives tested were quite effective as inhibitors, and were more effective than unsubstituted benzoate. This is consistent with the greater binding affinities of *p*-substituted benzoate derivatives. At a final concentration of $1.6 \times$

TABLE II

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit A4 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab') precipitated, % of control§			
p-(*p'*-hydroxy)-phenylazobenzoate	43 (1)	42 (1)	86 (1)	22
Benzoate	92 (<1)	95 (<1)	106 (1)	1
p-nitrobenzoate	71 (<1)	92 (<1)	90 (2)	11.5
m-nitrobenzoate	87 (1)	102 (1)	101 (2)	0.4
o-nitrobenzoate	101 (<1)	101 (1)	103 (1)	<0.1
p-aminobenzene arsonic acid	105 (1)	103 (<1)	100 (1)	
p-nitrobenzene sulfonic acid	100 (<1)	102 (1)	102 (2)	
Sodium acetate	91 (1)	95 (<1)	98 (1)	
Potassium iodide	96 (1)	100 (1)	94 (2)	
Potassium bromide	84 (<1)	97 (1)	95 (<1)	
p((*p'*-dimethylamino)-phenylazobenzene sulfonate	98 (1)	99 (<1)	77 (<1)	

* Anti-D serum was from rabbit 14E. The indirect method of precipitation was used (see first footnote of Table I).

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

10^{-3} M, *p*-nitrobenzoate decreased the percentage of ^{125}I-F(ab')$_2$ fragments bound to 68–90% of the control value.

For benzoate derivatives with the same substituent group at various positions on the benzene ring, the order of inhibitory capacity in nearly all instances is $p > m > o$, which is the same as the decreasing order of combining affinities (9). The only exceptions are the amino derivatives (Table I); however a similar anomaly obtains with respect to their combining affinities. This was attributed (9) to the capacity of an *o*-amino substituent to form a hydrogen bond with the carboxylate group of benzoate ion.

A few tests were carried out in two additional systems in which the donor antibody again was from rabbit A5 and the recipients were rabbits 7A and 7C.

PFIZER EX. 1603
Page 16

In these experiments *p*-(*p'*-hydroxy)-phenylazobenzoate gave significant inhibition (47 and 50%, respectively, at a concentration of 1.6×10^{-3} M) but *p*-nitrobenzoate had no effect on the reaction.

In general, small molecules other than benzoate derivatives had no significant effect on the percentage of ^{125}I-F(ab')$_2$ fragments precipitated. The only exception was methyl orange, *p*-(*p'*-dimethylamino)-phenylazobenzene sulfonate,

TABLE III

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit A5 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab') precipitated, % of control§			
p-(*p'*-hydroxy)-phenylazobenzoate	51 (<1)	67 (3)	90 (6)	22
Benzoate	95 (3)	98 (1)	109 (1)	1.0
p-nitrobenzoate	78 (<1)	83 (1)	95 (1)	11.5
m-nitrobenzoate	88 (2)	95 (1)	99 (1)	0.4
o-nitrobenzoate	95 (1)	96 (1)	98 (2)	<0.1
p-aminobenzoate	90 (1)	94 (4)	99 (2)	0.9
m-aminobenzoate	91 (2)	99 (<1)	99 (1)	0.3
o-aminobenzoate	94 (2)	99 (2)	101 (2)	1.5
p-iodobenzoate	74 (1)	89 (5)	97 (<1)	
m-iodobenzoate	87 (2)	95 (<1)	94 (1)	0.9
o-iodobenzoate	92 (3)	96 (2)	96 (1)	
Sodium acetate	102 (2)	107 (3)	105 (2)	
Potassium bromide	106 (2)	106 (1)	109 (3)	
p-aminobenzene arsonate	95 (4)	96 (2)	85 (1)	
p-(*p'*-dimethylamino)-phenylazobenzene sulfonate	95 (4)	96 (2)	85 (1)	

* Anti-D serum was from rabbit 7D. The indirect method of precipitation was used (see first footnote of Table I).

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

which gave weak but consistent inhibition in two of the four systems but, surprisingly, inhibited only at the lowest concentration tested (5×10^{-5} M). We have at present no explanation for this result.

Other substances related structurally to benzoate, *p*-aminobenzene arsonate and *p*-nitrobenzene sulfonate, were inactive. Iodide, bromide, and acetate salts were also ineffective as inhibitors.

Additional experiments were carried out to determine whether *p*-(*p'*-hydroxy)-phenylazobenzoate inhibited the reaction of F(ab')$_2$ derived from D antibodies with an equivalent amount of goat anti-rabbit Fab antiserum. These experiments were carried out in triplicate with two systems (D from rabbits A5

PFIZER EX. 1603
Page 17

and A4). The amount of goat antibody used was just sufficient to precipitate a maximal quantity of the labeled antigen. Each test utilized 5 μg of ^{125}I-labeled F(ab')$_2$ derived from D antibody and 75 μg of unlabeled nonspecific F(ab')$_2$. In the absence of hapten, 86% of the labeled F(ab')$_2$ fragments from rabbit A4 was precipitated by the goat antibody. There was no significant change in the presence of 6×10^{-4} M *p*-(*p'*-hydroxy)-phenylazobenzoate, but the value decreased to 75% when the hapten concentration was 1.6×10^{-3} M. In the case of D antibodies from rabbit A5, 88% of the ^{125}I-F(ab')$_2$ fragments were precipi-

TABLE IV

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit 114 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab') *precipitated, % of control*§			
p-(*p'*-hydroxy)-phenylazobenzoate	32 (2)	39 (1)	61 (1)	22
Benzoate	95 (2)	101 (1)	108 (2)	1
p-nitrobenzoate	72 (1)	72 (2)	86 (1)	11.5
m-nitrobenzoate	85 (2)	90 (2)	112 (1)	0.4
o-nitrobenzoate	98 (<1)	106 (1)	98 (2)	<0.1
Sodium acetate	99 (2)	101 (1)	106 (1)	
Potassium bromide	93 (<1)	98 (3)	95 (2)	
Potassium iodide	100 (1)	101 (1)	101 (2)	
p-nitrobenzene sulfonate	95 (4)	98 (1)	101 (3)	
p-(*p'*-dimethylamino)-phenyl-azobenzene sulfonate	84 (1)	91 (2)	68 (3)	

* Anti-D serum was from rabbit 10X. The indirect method of precipitation was used (see first footnote of Table I).

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

tated in the absence of the hapten. There was no significant change in the presence of 6×10^{-4} M hapten; at a hapten concentration of 1.6×10^{-3} M the average value decreased to 84%. Thus, there is little effect of the hapten on the reactions of D antibodies with goat anti-rabbit Fab.

Effect of Order of Addition of Hapten and Anti-D Antiserum.—The order of addition of reagents was varied in order to determine whether hapten could exert its inhibitory activity when added subsequent to the anti-D serum. The results, shown in Table V, indicate that the hapten was equally effective as an inhibitor when added subsequent to the anti-D, provided that 12 hr were allowed to elapse before to the addition of anti-Fc. When the anti-Fc was added 1 or 2 hr after the hapten, the inhibitory capacity was reduced slightly. Thus the hapten is capable of displacing anti-D and the reaction is essentially reversible.

PFIZER EX. 1603
Page 18

DISCUSSION

The data to be discussed were obtained with rabbit anti-idiotypic antibodies (anti-D) prepared against specifically purified rabbit anti-*p*-azobenzoate antibodies (D) from four individual rabbits. The results indicate that the combination of D antibodies with hapten specifically inhibits their reaction with anti-D antisera.

There is a direct relationship between the affinity of a hapten for anti-*p*-azobenzoate antibody and the inhibitory capacity of that hapten. With the hapten of greatest affinity used, *p*-(*p'*-hydroxy)-phenylazobenzoate, the degree

TABLE V

Effect of Order of Addition of Hapten and Anti-D on the Inhibitory Capacity of the Hapten

Experiment	Order of addition	^{125}I-F(ab')$_2$ precipitated
		% of control‡
1	D; anti-D (2 hr, 37°C); anti-Fc	100
2	D; hapten (2 hr, 37°C); anti-D (2 hr, 37°); anti-Fc	51 (<1)
3	D; anti-D (2 hr, 37°C); hapten (1 hr, 37°); anti-Fc	62 (<1)
4	D; anti-D (2 hr, 37°C); hapten (2 hr, 37°); anti-Fc	64 (<1)
5	D; anti-D (2 hr, 37°C); hapten (12 hr, 5°); anti-Fc	50 (1.5)

* These experiments were carried out by the indirect method of precipitation with anti-benzoate antibody (D) from rabbit A4 and anti-D from rabbit 14E. The method is given in the first footnote of Table I. The hapten used was *p*-(*p'*-hydroxy)-phenylazobenzoate at a final concentration of 1.6×10^{-3} M. Mixtures were allowed to stand for 2 days at 5°C after the addition of goat anti-rabbit Fc.

‡ Values refer to the amount of radioactivity precipitated relative to that obtained in the absence of hapten. Numbers in parentheses are average deviations from the mean of triplicate experiments.

of inhibition at the highest hapten concentration tested varied from 43 to 69%. In contrast, very little if any inhibition was observed with ortho-substituted benzoate derivatives, which combine with relatively low association constants (9). For benzoate derivatives substituted with a single chloro-, bromo-, iodo-, or nitro-group, the order of inhibitory capacity, in the reaction of D with anti-D, was $p > m > o$. This is the same as the order of decreasing combining affinities reported by Pressman et al. (9), who measured relative association constants by determining the capacities of haptens to inhibit the specific precipitation of anti-hapten antibodies by protein–hapten conjugates. The relatively high affinity of *p*-substituted benzoate derivatives is attributable to the fact that the hapten is conjugated through a *p*-azo linkage to the carrier protein used for immunization. Ortho and meta derivatives combine with lower affinity than unsubstituted benzoate; this is attributable to steric interference (9). The particularly high affinity of *p*-(*p'*-hydroxy)-phenylazobenzoate can be ascribed to the conjugation of azobenzoate groups to tyrosine residues in the carrier pro-

PFIZER EX. 1603
Page 19

tein. Thus *p*-(*p'*-hydroxy)-phenylazobenzoate closely resembles a determinant in the actual immunogen.

The amino group is the only substituent for which the order $p > m > o$, did not consistently obtain. However, a similar anomaly was also noted in studies of relative combining affinities (9) and was ascribed to the unique capacity of the amino group in the ortho position to form a hydrogen bond with an oxygen atom of the adjacent carboxylate group.

With one exception compounds other than benzoate derivatives were ineffective as inhibitors. The exception was methyl orange, *p*-(*p'*-dimethylamino)-phenylazobenzene sulfonate, which exhibited significant inhibitory capacity in two of the four systems studied, but only at the lowest concentration of methyl orange tested. Other anions investigated failed to inhibit the reactions of anti-D antisera. These included derivatives of benzene arsonate and benzene sulfonate, which are related structurally to benzoate.

There appear to be two possible explanations for these results, each of which seems equally consistent with the data. (*a*) The region of the combining site of the anti-benzoate antibody is part of a major idiotypic determinant, reactive with a large fraction of the anti-D antibodies. In this case the presence of hapten could sterically inhibit the reaction with anti-D. (*b*) Combination of hapten with the active site of the antibody results in a conformational change which alters idiotypic determinants not necessarily confined to the region of the active site.

In connection with hypothesis *b*, it may be relevant that the presence of hapten had little effect on the reaction of Fab fragments of purified anti-benzoate antibodies with goat anti-rabbit Fab. In addition, in experiments reported elsewhere (10), it was found that hapten similarly does not affect the reaction of Fab fragments with anti-allotype antisera directed to determinants on either the light or heavy chains. Thus, if conformational changes do occur in the presence of hapten, they do not drastically alter the antigenic structure of the Fab fragment, and are probably confined to regions near the active site. We have so far been unable to design an experiment to distinguish between the alternatives of conformational change and steric hindrance by hapten.

The experiments on order of addition did not contribute data relevant to this question. The effect of hapten was the same whether it was added before, or 2 hr after the addition of anti-D antiserum to ^{125}I-F(ab')$_2$ fragments of D molecules (Table V). In this respect, therefore, the reaction of D with anti-D is reversible. This effect is being utilized in attempting to purify specifically the subpopulation of anti-benzoate antibodies that reacts with anti-D.

That a conformational change may occur in the antigen-antibody reaction was suggested by Ishizaka and Campbell (11) on the basis of measurements of optical rotation during the interaction of bovine serum albumin with its rabbit antibody. However, the increase in the negative optical rotation value caused by the interaction might be ascribable to changes in either the antigen or the anti-

PFIZER EX. 1603
Page 20

body; also, the structure of the complexes, as well as internal structural changes in either protein, might contribute to the optical rotation.

Steiner and Lowey (12) failed to observe any change in optical rotation upon interaction of rabbit anti-dinitrophenyl antibody with specific haptens. Such data cannot, however, rule out the possibility of small conformational changes.

Warner et al. (13) observed an increase of 1–3% in sedimentation coefficient upon interaction of rabbit anti-lactoside antibody with specific hapten, which was interpreted as indicating that the antibody had assumed a slightly more compact configuration. The effect was not observed with anti-dinitrophenyl antibodies (14). Changes in sedimentation rate were noted with bivalent haptens which could alter configuration through a cross-linking mechanism.

Grossberg et al. (15) observed that the interaction of hapten with specific antibody protected the antibody to some extent against the proteolytic action of chymotrypsin and concluded that a conformational change had occurred. An alternative possibility, which they considered unlikely, is that one point of attack of the chymotrypsin on antibody is the region of the active site, and that hapten sterically protects this region against the action of the enzyme.

It thus appears uncertain whether combination with monovalent hapten can induce a significant structural alteration in an antibody molecule. It should be noted, however, that it is well documented that the presence of hapten can retard the denaturation of antibody by 4 M guanidine (16) or partially protect antibody from dissociation into H and L chains after reduction of interchain disulfide bonds (17).

The failure of hapten to prevent completely the combination of D with anti-D suggests that the D population may be heterogeneous with respect to the contribution of the active site to idiotypic determinants. An alternative hypothesis is that the active site is equally important in all D antibodies, but that anti-D antibody competes with hapten for combination with the active site; partial inhibition could result from such competition. The latter hypothesis is given some support by the observation that haptens of highest affinity were the most effective inhibitors of the reaction of D with anti-D. It is not inconceivable that complete inhibition could result if the hapten combined with sufficiently high affinity.

It should be noted that competition between hapten and anti-D could occur whether the hapten exerts its effect through a conformational change or by direct steric interference. In either instance the following equilibria would obtain (where D is anti-benzoate antibody and H is hapten):

$$\begin{array}{l} \text{D} + \text{Anti-D} \rightleftharpoons \text{D-Anti-D complex} \\ + \\ \text{H} \\ \downarrow\uparrow \\ \text{D-H} \end{array}$$

Competition would be observed if the D-H complex is unreactive with anti-D.

PFIZER EX. 1603
Page 21

It is relevant that with the highest concentrations of the more effective haptens, essentially all of the combining sites of the D antibody were occupied. The following calculation can be made by assuming an association constant of 10^5 liters/mole (a typical value for a *p*-substituted benzoate derivative [18] and a hapten concentration of 10^{-3} M).

$$Ab + H \rightleftharpoons Ab\,H$$

$$K = \frac{b}{A \cdot f},$$

where b and f are the bound and free hapten concentrations and A is the concentration of combining sites remaining unoccupied at equilibrium.

$$10^5 \cdot 10^{-3} = \frac{b}{A}$$

$$100 = \frac{b}{A}$$

Thus, under these hypothetical conditions, 99% of the combining sites of the antibody would be occupied.

Our findings contrast with those of Kelus and Gell (5) who showed that anti-proteus antibodies (D) react with anti-D even when the D antibodies are combined with proteus. They concluded that the active site itself was not an important idiotypic determinant. Two possibilities may be suggested to reconcile our findings. First, since their reactions were carried out with bivalent D antibody, one of the combining sites of some of the D antibodies complexed with proteus may have been unoccupied and available for reaction with anti-D. Second, their anti-D antibody was elicited by immunization with proteus–anti-proteus complexes. It is conceivable, therefore, that some of the anti-D antibody population was specific for the complex, i.e., for D antibodies in which the combining sites were occupied. This could not apply to the entire anti-D population since it also reacted with D in the absence of proteus.

SUMMARY

Rabbit anti-idiotypic antibodies were prepared by injection of specifically purified anti-*p*-azobenzoate antibodies (D) from individual donor rabbits. Benzoate derivatives were found to be strong inhibitors of the reactions of D with anti-D antisera. There was a close correlation between the combining affinities of the benzoate derivatives used and their effectiveness as inhibitors. Compounds tested that are chemically unrelated to benzoate were ineffective. The results indicate either that the combining site of anti-benzoate antibody is part of an important idiotypic determinant, which is sterically blocked by hap-

PFIZER EX. 1603
Page 22

ten, or that the hapten induces a conformational change which alters idiotypic determinants not involving the active site. Such conformational changes, if they occur, must be restricted since hapten has little effect on the reactions of $F(ab')_2$ fragments of anti-benzoate antibodies with antisera directed to rabbit fragment Fab and no detectable effect on reactions with antibodies directed to allotypic determinants.

BIBLIOGRAPHY

1. Slater, R. J., S. M. Ward, and H. G. Kunkel. 1955. Immunologic relationships among the myeloma proteins. *J. Exp. Med.* **101:**85.
2. Kunkel, H. G., M. Mannik, and R. C. Williams. 1963. Individual antigenic specificities of isolated antibodies. *Science (Washington).* **140:**1218.
3. Oudin, J., and M. Michel. 1963. Une nouvelle forme d'allotypie des globulines γ du sérum de lapin apparemment liée à la jonction et à la specificité anticorps. *C. R. Seances Acad. Agr. Fr.* **257:**805.
4. Oudin, J. 1966. The genetic control of immunoglobulin synthesis. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **166:**207.
5. Kelus, A. S., and P. G. H. Gell. 1968. Immunological analysis of rabbit anti-antibody systems. *J. Exp. Med.* **127:**215.
6. Daugharty, H., J. E. Hopper, A. B. MacDonald, and A. Nisonoff. 1969. Quantitative investigations of idiotypic antibodies. I. Analysis of precipitating antibody populations. *J. Exp. Med.* **130:**1047.
7. Hopper, J. E., A. B. MacDonald, and A. Nisonoff. 1970. Quantitative investigations of idiotypic antibodies. II. Nonprecipitating antibodies. *J. Exp. Med.* **131:**41.
8. MacDonald, A. B., and A. Nisonoff. 1970. Quantitative investigations of idiotypic antibodies. III. Persistence and variations of idiotypic specificities during the course of immunization. *J. Exp. Med.* **131:**583.
9. Pressman, D., S. M. Swingle, A. L. Grossberg, and L. Pauling. 1944. The serological properties of simple substances. VIII. The reactions of antiserum homologous to the *p*-azobenzoic acid group. *J. Amer. Chem. Soc.* **66:**1731.
10. Spring, S. B., A. Nisonoff, and S. Dray. 1970. *J. Immunol.* In press.
11. Ishizaka, K., and D. H. Campbell. 1959. Biologic activity of soluble antigen-antibody complexes. *J. Immunol.* **83:**318.
12. Steiner, L. A., and S. Lowey. 1966. Optical rotatory dispersion studies of rabbit γG-immunoglobulin and its papain fragments. *J. Biol. Chem.* **241:**231.
13. Warner, C., V. Schumaker, and F. Karush. 1970. The detection of a conformational change in the antibody molecule upon interaction with hapten. *Biochem. Biophys. Res. Commun.* **38:**125.
14. Warner, C., and V. Schumaker. 1970. Detection of a conformational change in an anti-hapten antibody system upon interaction with divalent hapten. *Biochemistry.* **9:**451.
15. Grossberg, A. L., G. Markus, and D. Pressman. 1965. Change in antibody conformation induced by hapten. *Proc. Nat. Acad. Sci. U.S.A.* **54:**942.

PFIZER EX. 1603
Page 23