[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 916]

The Serological Properties of Simple Substances. V. The Precipitation of Polyhaptenic Simple Substances and Antiserum Homologous to the *p*-(*p*-Azophenylazo)-phenylarsonic Acid Group and its Inhibition by Haptens

BY DAVID PRESSMAN, JOHN T. MAYNARD, ALLAN L. GROSSBERG, AND LINUS PAULING

To extend our studies of the reactions of antibodies with simple substances,[1,2,3,4] which so far have been concerned with antisera made by inoculating rabbits with sheep serum coupled with diazotized *p*-arsanilic acid (we hereafter refer to these antisera as anti-R sera), we have now investigated the reactions of antisera made by inoculation with sheep serum coupled with diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid (anti-R′ sera). These investigations, the results of which are reported in the present paper, included primarily quantitative studies of the precipitation of anti-R′ serum with simple substances containing two or more haptenic groups and the inhibition of these precipitation reactions by haptens; some work was also done on the precipitation reactions of anti-R′ and anti-R sera with ovalbumin coupled with diazotized arsanilic acid (R-ovalbumin) and with ovalbumin coupled with diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid (R′-ovalbumin).

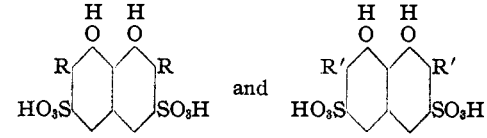
Experimental Methods

Protein Antigens.—The antigen used for inoculations was prepared by coupling diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid with crude sheep serum globulin at *p*H 9. The azoprotein was purified by the method of Landsteiner and van der Scheer.[5] It was found on analysis to contain 2.1% arsenic. The crude serum globulin was precipitated from sheep serum by the addition of an equal volume of saturated ammonium sulfate solution.

The protein test antigens were prepared similarly with use of purified ovalbumin. The number of haptenic groups per molecule (with assumed molecular weight 40,000 for ovalbumin) was calculated from the results of analyses for arsenic[6] to be 8.8 for R-ovalbumin and 8.4 for R′-ovalbumin.

Preparation of Antisera.—The antisera were prepared in rabbits as described previously[1] for the preparation of anti-R sera. The sera from several bleedings after several different courses of injections were pooled according to titer to three pools, A, B and C.

Simple Antigens and Haptens.—The simple substances used have been described previously[1,4,7] except for the substances



The R substance was prepared by Mr. Carol Ikeda by adding diazotized arsanilic acid in 25% excess to chromotropic acid in sodium carbonate solution. The product was purified by repeated solution in sodium hydroxide solution and precipitation with hydrochloric acid, followed by recrystallization from 50% ethanol solution. The R′ analog was made by Mr. David H. Brown by adding diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid in 50% excess to chromotropic acid in sodium carbonate solution containing pyridine. The product was purified by precipitation with hydrochloric acid and salt and solution in sodium hydroxide, followed by dialysis against water through cellophane sausage casing until the rate of dialysis of colored substance became negligible. This removed substances of smaller molecular dimension than the desired product, which does not pass through the membrane readily. The product was acidified and then precipitated with alcohol and washed with alcohol. These substances were analyzed chromatographically by Mr. Arthur Pardee as discussed previously.[1] The R substance was found to be essentially pure while the R′ substance contained 5 to 10% of a colored impurity. Carbon and hydrogen analyses indicated that the substances were sodium salts.

Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and permitted to stand one hour at room temperature and overnight in a refrigerator at 3°. The precipitates were then washed 3 times with 10-ml. portions of 0.9% saline solution, and the amounts of protein were determined by use of the Folin-Ciocalteu[8] reagent by a method to be described elsewhere.

The borate buffers were prepared by adding 0.16 *N* sodium hydroxide solution to 0.2 *M* boric acid in 0.9% sodium chloride solution. All dilutions of antigen and hapten were made with buffer of *p*H 8.0 unless otherwise noted.

Precipitation Reactions of Anti-R′ Serum and Simple Substances.—Precipitation tests first were made with the compounds VI, IX, XI, XIV, XX, XXIII, XXX, and XXXI.

(1) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, THIS JOURNAL, **64**, 2994 (1942).

(2) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *ibid.*, **64**, 3003 (1942).

(3) L. Pauling, D. Pressman, and C. Ikeda, *ibid.*, **64**, 3010 (1942).

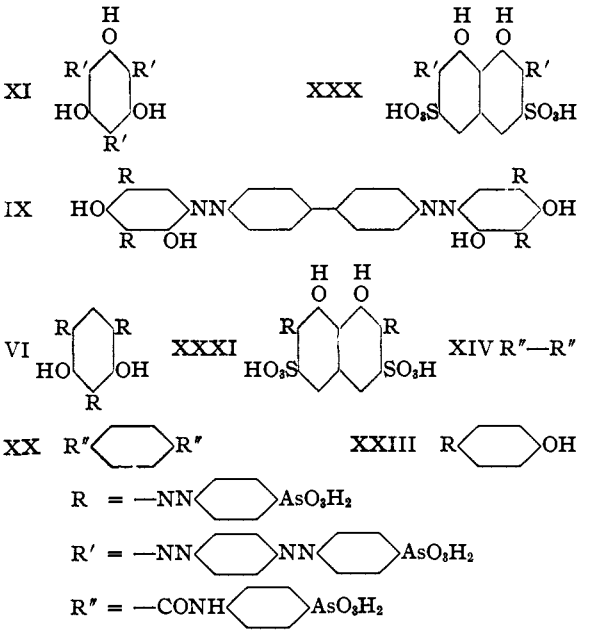
(4) D. Pressman, D. H. Brown, and L. Pauling, *ibid.*, **64**, 3015 (1942).

(5) K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, **55**, 781 (1932).

(6) By the method of E. B. Sandell, *Ind. Eng. Chem., Anal. Ed.*, **14**, 82 (1942).

(7) D. Pressman and D. H. Brown, THIS JOURNAL, **64**, 540 (1942).

(8) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).



Equal volumes of anti-R′ serum A and of antigen solution of concentrations varying from 200×10^{-6} to 12.5×10^{-6} M in two-fold dilutions were mixed. The experiments were run in triplicate, with use of 1 ml. each of antigen solution and antiserum for antigens XI and XXX and 3 ml. for all the others except antigen IX, for which only 0.5 ml. was used and only a single tube was set up at each concentration. Precipitates were obtained only with the antigens XI, XXX, and IX. A normal precipitation reaction, showing an optimum zone, was observed. The results are given in Table I.

TABLE I

PRECIPITATION OF ANTI-R′ SERUM A AND SIMPLE ANTIGENS

Antigen solution and antiserum, 1 ml. each. pH of supernates 8.1 for XI and XXX, 8.2 for IX. Control, antiserum and buffer, 5 μg.

	Moles of antigen added × 10^9				
	12.5	25	50	100	200
Antigen	Amount of antibody precipitated (μg.)[a]				
XI	233	612	(1172)	1055	(695)
XXX	(367)	(778)	1070	1053	748
IX[b]	[46]	[102]	[12]		

[a] The values given without parentheses are averages of triplicate analyses, with mean deviation of ±4%; those in parentheses are averages of duplicate analyses, and those in square brackets are single analyses only. [b] Run was made using 0.5-ml. portions. Reported values are for 1-ml. portions.

No precipitate would be expected with the monohaptenic substance XXIII. It is very interesting that no precipitates were obtained with the polyhaptenic substances VI, XIV, XX, and XXXI. These all gave precipitates with anti-R serum,[1] although not so large as given by antigen XI by a factor of three or four.

With anti-R′ serum B, which was obtained several months after anti-R′ serum A, a slight amount of precipitate was obtained with antigen VI. Antigen IX gave an equal amount of precipitate while antigen XII gave over thirty times as much. The results are in Table II.

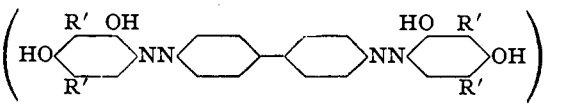


TABLE II

PRECIPITATION OF ANTI-R′ SERUM B AND SIMPLE ANTIGENS

Antigen solution and antiserum, 1 ml. each. pH of supernates 8.1. Control, antiserum and buffer, 0 μg.

	Moles of antigen added × 10^9				
	8	16	32	63	126
Antigen	Amount of antibody precipitated (μg.)[a]				
XII	24	160	676	919	476
IX	0	19	29	12	
VI	0	26	27	2.2	

[a] Averages of triplicate analyses.

Precipitation of Anti-R′ Serum and Anti-R Serum by R′-ovalbumin and R-ovalbumin.—In order to see whether the failure of some R-antigens to form appreciable precipitates with anti-R′ serum extended also to azoproteins, precipitation tests were carried out with anti-R′ serum and anti-R serum and the two azo-ovalbumins, with the results given in Table III. It is seen that each of the antigens R′-ovalbumin and R-ovalbumin formed precipitates with each of the antisera. Although the number of haptenic groups per molecule was nearly the same for the two azo-ovalbumins (8.4 for R′, 8.8 for R), the antigen with the long groups R′ was found to precipitate much more antibody from each antiserum than that with the short groups R. The ratio of R′-precipitable antibody to R-precipitable antibody in each of the antisera was about 4, whereas the ratio in the case of the dyes is about 30 for anti-R′ serum and, as previously determined, 3 to 4 for anti-R serum.[1]

The Effect of Hydrogen Ion Concentration on the Precipitation of Anti-R′ Serum and Antigen XXX.—Precipitation experiments were carried out at five different hydrogen ion concentrations from pH 7.6 to 9.1 as controlled by borate buffer and also in normal saline solution at pH 7.9, with the results given in Table IV. Optimum precipitation was found to occur at about pH 7.9, and the replacement of buffer by saline solution was found to have small effect. The antigen concentration at which the maximum amount of antibody is precipitated becomes lower with increasing pH. These observations are similar to those made[2] with anti-R serum and antigen VI.

The Relative Inhibiting Powers of Different Haptens.—To extend our studies of the inhibiting powers of haptens, hapten inhibition experiments similar to those made with anti-R serum[4] were carried out with anti-R′ serum. In Table V there are given the results obtained with antigens XXX and XII and anti-R′ serum C and the haptens XXI, XXXII, XXXIII, XXXIV, and XXXV.

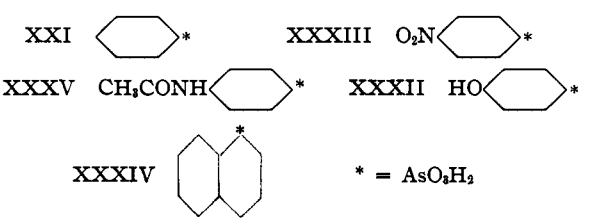


TABLE III

PRECIPITATION OF ANTI-R′ SERUM B AND ANTI-R SERUM U BY R′-OVALBUMIN (8.4 HAPTENIC GROUPS PER MOLECULE) AND R-OVALBUMIN (8.8 HAPTENIC GROUPS PER MOLECULE)

2 ml. each of R-ovalbumin solution and antiserum or 1 ml. each of R′-ovalbumin solution and antiserum. Data are given on basis of 1 ml. of antiserum. *p*H of supernates 8.1 to 8.2. Blank of serum and buffer, 0.0 μg. B is amount of antibody in precipitate (μg.). Average of triplicate analyses with mean deviation ±2.7%. A is amount of antigen in precipitate (μg.). Average of duplicate analyses with mean deviation ±3.0% for amount greater than 50 μg. and ±11% otherwise. The small values are unreliable.

Amount of antigen (μg.)	46		93		185		370		730		1460	
	B	A	B	A	B	A	B	A	B	A	B	A
R′-Ovalbumin, anti-R′ serum	330	22	813	54	1343	118	1248	145	840	140		
R′-Ovalbumin, anti-R serum	298	51	603	53	848	75	898	119	633	120		
R-Ovalbumin, anti-R′ serum			100	20	211	34	330	46	332	40	198	28
R-Ovalbumin, anti-R serum			77	14	171	16	230	30	181	31	67	23

TABLE IV

THE EFFECT OF HYDROGEN ION CONCENTRATION ON PRECIPITATION OF ANTI-R′ SERUM A AND ANTIGEN XXX

Antigen solution, 1 ml.; antiserum and buffer, ½ ml. each.

*p*H of supernates	Amount of antigen added (μg.) 8.3	12.5	18.7	29.6	44.4	66.7
	Amount of antibody precipitated (μg)[a]					
7.6	89	139	218	266	(305)	259
7.9	161	200	288	356	331	306
7.9[b]	114	197	297	362	372	320
8.3	130	176	226	235	(217)	176
8.7	132	157	139	114	(89)	(70)
9.1	121	141	105	83	58	56

[a] Averages of triplicate analyses with mean deviation ±6%; duplicate analyses in parentheses. [b] Run made with saline solution replacing borate buffer.

Experiments were also made with antigen XXX and anti-R′ serum A and the twenty-six haptens listed in Table VI, in which the results are recorded.

TABLE V

INHIBITION BY HAPTENS OF PRECIPITATION OF ANTI-R′ SERUM C AND ANTIGEN XII AND XXX AT OPTIMUM ANTIGEN CONCENTRATION

Antigen solution, hapten solution, and antiserum, 1 ml each.

Antigen	Hapten	Moles of hapten × 10^9 0	25	50	100	200
		Amount of antibody precipitated (μg.)[a]				
XXX, 33 μg.	XXI	295	252	251	(209)	169
	XXXII		222	195	165	(120)
	XXXIII		224	188	145	109
	XXXIV		211	162	140	79
	XXXV		204	159	134	101
XII, 50 μg.	XXI	404	(310)	(295)	213	167
	XXXII		266	224	170	133
	XXXIII		235	(184)	119	70
	XXXIV		248	186	133	87
	XXXV		226	175	121	76

[a] Averages of analyses in triplicate, with mean deviation ±4%; duplicate analyses in parentheses.

Discussion

The data given in Tables IV, V, and VI are similar to those found for anti-R serum and reported in earlier papers of this series. In particular the data given in Table V provide some further support for the prediction of our simple quantitative theory of hapten inhibition[2,4] that the order of inhibiting power of a series of haptens is for a given antiserum the same for different antigens; for antigens XXX and XII and anti-R′ serum the same order was found for the five haptens listed in Table V except for interchange of haptens XXXIII and XXXIV.

With use of the procedure described in the preceding paper,[4] values of the constant K' representing the strength of the hapten-antibody bonds were derived from the initial slopes of the curves of amount of precipitate plotted against the number of moles of hapten. The average for the two azohaptens was placed equal to 1. These values are given in the second column of Table VI. In the third column are the values of K' (listed as K'^*) for anti-R serum as found previously.[4]

It is seen, as was found also for anti-R serum in the preceding investigation,[4] that there is no correlation between these values of K' and the values of the second ionization constant of the arsonic acids, which are in the fourth column.

The most striking feature of the K' values is their independence of the position of the substituent in the substituted phenylarsonic acids. It was found in the earlier study with anti-R serum that for a given substituent the *o*-substituted phenylarsonic acid had a small value of K', the *m*-substituted acid a larger one, and the *p*-substituted acid a still larger one, with the value of K' increasing by about 100% at each step. This phenomenon was attributed to steric hindrance of the group (methyl, amino, nitro) in the ortho or meta position; it was assumed that the pocket formed as the antibody folded about a *p*-azophenylarsonic acid group of the immunizing azoprotein would fit a *p*-substituted group closely, and a *m*-substituted or *o*-substituted group less closely. But the data of Table VI for anti-R′

TABLE VI

INHIBITION OF PRECIPITATION OF ANTI-R′ SERUM A AND ANTIGEN XXX BY HAPTENS

Antigen solution, 1.25 ml. (43.5 μg.); antiserum, 0.75 ml.; hapten solution, 1 ml.

	K′	K′*[a]	$K_{A_2} \times 10^9$[b]	Moles of hapten added × 10^9: 6.25	12.5	25	50	100	200
				Amount of antibody precipitated (μg.)[c]					
Methylarsonic acid[d]	0.00								
o-Aminophenylarsonic acid	.10	0.13	2.2			485	468	420	382
m-Aminophenylarsonic acid	.11	.29	2.4			471	447	385	(360)
p-Carboxyphenylarsonic acid	.14	.29	3.6			435	(411)	392	315
p-Aminophenylarsonic acid	.15	.44	1.2			438	(412)	347	302
Phenylarsonic acid	.15	.26	3.3			438	397	350	295
p-Hydroxyphenylarsonic acid	.16	.60	3.9			432	395	337	232
p-Methylphenylarsonic acid	.18	.80	2.1			444	393	293	211
m-Methylphenylarsonic acid	.19	.53	1.5			417	372	300	209
o-Methylphenylarsonic acid	.20	.13	1.4			425	310	263	189
β-Naphthylarsonic acid	.21	.66	3.4			423	387	261	171
p-Chlorophenylarsonic acid	.25	.80	5.6			421	373	255	184
p-Bromophenylarsonic acid	.27	.80	6.5			419	347	232	157
p-Iodophenylarsonic acid	.31	.80	5.7			382	310	199	110
p-Benzoylaminophenylarsonic acid	.35	.80		476	443	369	258		
p-Nitrophenylarsonic acid	.39	1.40	13.5	465	416	353	252		
o-Nitrophenylarsonic acid	.39	0.28	2.9			(345)	253	161	114
m-Nitrophenylarsonic acid	.40	.75	18.2			311	247	(144)	103
p-(*p*-Aminobenzoylamino)phenylarsonic acid	.46	.89		(428)	(410)	311	212		
p-Acetaminophenylarsonic acid	.48	1.02		407	365	319	239		
p-(*p*-Nitrobenzoylamino)phenylarsonic acid	.50	0.89		384	338	299	(202)		
α-Naphthylarsonic acid	.59	.23	2.2			253	191	128	79
1,4-Aminonaphthylarsonic acid	.65	.17	0.7			258	162	117	63
p-(*p*-Hydroxyphenylazo)phenylarsonic acid	.94	.98		(419)	311	213	124		
p-(*p*-Aminophenylazo)phenylarsonic acid	1.06	1.02		(290)	215	125	62		
VI	1.60			(155)	(65)	(32)	(13.2)		

Average value with buffer in place of hapten, 537 μg. Blank with 2 ml. buffer and 1 ml. antiserum, 3 μg. *p*H of supernates 8.05 to 8.10.

[a] Values for anti-R serum found previously.[4] [b] Second dissociation constant of the arsonic acid.[6] [c] Averages of triplicate analyses with mean deviation of ±4%; duplicate analyses in parentheses. [d] Values 518 and 532 μg. protein obtained at 400 and 800 × 10^{-9} moles hapten, respectively.

serum do not show this effect; the values of the *o*, *m*, and *p*-substituted acids lie very close together for the methyl, amino, and nitro compounds. This observation was verified by similar tests with another pool of anti-R′ serum.

An interpretation of these results which we suggest is the following. It was pointed out in a theoretical discussion[9] that in general all effective antigen–antibody bonds should have about the same strength, and that in consequence antibodies to antigens containing strong groups should show low specificity, and those to antigens containing weak groups should show high specificity. We may consider that in order to form a sufficiently strong bond with the short haptenic group —NN⟨⬡⟩AsO_3H_2 the antibody would have to fit it closely, whereas an equally effective antibody to the longer haptenic groups —NN⟨⬡⟩NN⟨⬡⟩AsO_3H_2 might fit the group only very loosely—so loosely as to permit the easy attachment of ortho- and meta-substituted phenylarsonic acids. This looseness of fit is also indicated by the large values of K' shown by α-naphthylarsonic acid and 1,4-aminonaphthylarsonic acid.

Aside from this, the values of K' for the haptens with anti-R′ serum show essentially the same reasonable dependence on the structure of the hapten as found for anti-R serum. The values of K' for the *p*-substituted phenylarsonic acids depend on the substituent in the order HOC_6H_4NN > CH_3CONH > NO_2 > C_6H_5CONH > I > Br > Cl > CH_3 > OH > H, NH_2 > COOH. This order is nearly the same as that found for anti-R serum, which was NO_2 > CH_3CONH > HOC_6H_4NN > C_6H_5CONH > Cl, Br, I, CH_3 > OH > NH_2 > COOH > H, the only differences being the interchange of the azo and nitro groups, the change in position of phenylarsonic acid itself, and the small differences found for methyl, chloro, bromo, and iodo groups with anti-R′ serum. These

(9) L. Pauling, THIS JOURNAL, 62, 2643 (1940).

changes in order may not be characteristic of anti-R and anti-R′ serum, since different pools of the same antiserum are observed to give somewhat different results.

The importance of the phenyl group is shown by the fact that methylarsonic acid has practically no inhibitory power.[10] On the other hand, the haptens $HOC_6H_4NNC_6H_4AsO_3H_2$ and $H_2NC_6H_4NNC_6H_4AsO_3H_2$, which are very like the immunizing haptenic group $—NNC_6H_4NNC_6H_4AsO_3H_2$, show very strong inhibition.

The very strong inhibition shown by the trihaptenic substance VI is probably due to the entropy effect of the three haptenic groups.

The fact that this substance and the other polyhaptenic substances containing R groups give at most only slight precipitates with anti-R′ serum we attribute to steric repulsion of antibodies; their strong inhibitory action shows that a strong bond can be formed with one antibody molecule. Presumably a group R with part of the nucleus to which it is attached occupies the cavity of an anti-R′ antibody; the remaining groups R then protrude from the antibody by such a small distance that another antibody cannot approach closely enough to form a bond. This steric effect is similar to that invoked earlier[3] in explanation of the observed effective bivalence of trihaptenic and tetrahaptenic antigens. The formation of an appreciable amount of precipitate by the exceptional substance IX we attribute to the large size of its molecules, in which arsonic acid groups are separated by six benzene rings. The same explanation—large distances between haptenic groups—applies to the precipitation of anti-R′ serum by R-ovalbumin (Table II).

(10) See also F. Haurowitz and F. Breinl, *Z. physiol. Chem.*, **214**, 111 (1933).

This investigation was carried out with the aid of a grant from The Rockefeller Foundation.

Summary

Quantitative data are reported for the precipitation reactions of polyhaptenic simple substances and antisera prepared by inoculating rabbits with azoproteins made from *p*-(*p*-azophenylazo)phenylarsonic acid. Measurements were made of the inhibitory effect of each of twenty-six haptens on one antigen-antibody reaction, and interpreted to give values of the bond-strength constant of the haptens with the antibody. These values are discussed in relation to the structure of the hapten molecules and in comparison with the values previously found for an antiserum homologous to the *p*-azophenylarsonic acid haptenic group. An explanation in terms of steric hindrance is given of the failure of some polyhaptenic substances to give precipitates with the antiserum.

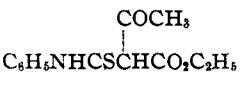
PASADENA, CALIFORNIA RECEIVED DECEMBER 23, 1942

[COMMUNICATION NO. 906 FROM THE KODAK RESEARCH LABORATORIES]

Investigation of Pyrazole Compounds. III.[1] The Condensation of α-Carbethoxyacetothioacetanilide with Hydrazines

BY A. WEISSBERGER AND H. D. PORTER

By the condensation of phenyl isothiocyanate with sodium acetoacetic ester suspended in ether, Worrall obtained the sodium salt of α-carbethoxyacetothioacetanilide I.[2] The free ester on heating with two moles of hydrazine formed a compound which may be formulated as 3-anilino-5-pyrazolone II.[3] The over-all reaction is represented in III (R = H).



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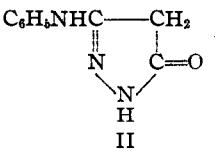

II

In the present work we have modified Worrall's procedure in that instead of isolating the free ester I, we added one molecular proportion of hydrazine to the alcoholic reaction mixture containing the sodium salt of I. A compound was obtained of the composition of 3-anilino-5-pyrazolone, with a melting point 268–270° (dec.). The same material resulted when Worrall's procedure was followed strictly, but this author reports a melting point of 255–256° (dec.). The difference does not appear to be caused by the technique of measurement and may indicate polymorphism.[1]

(1) Investigation of Pyrazole Compounds, II, THIS JOURNAL, **65**, 52 (1943).

(2) Worrall, *ibid.*, **40**, 418 (1918).

(3) Worrall, *ibid.*, **44**, 1551 (1922).