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IDENTIFICATION OF TYROSINE AT THE ACTIVE SITE OF ARSONIC ANTIBODY[1]

DISCLAIMER

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Running Title:

Tyrosine in Arsonic Antibody

INTRODUCTION

An iodine-reactive residue is known to be at the active site of antibody directed against the azophenylarsonic acid group (1, 2). As the first step in the identification of this residue, the numbers and kinds of surface groups which can react with iodine were determined after extensive treatment of arsonic antibody[2] (3). Iodine was found to be substituted in all the available tyrosyl and histidyl residues and in addition significant numbers of methionyl and tryptophanyl residues were oxidized. Since in previous studies (4) loss of immunological activity was shown to be associated with the binding of iodine, these data suggested the presence of a tyrosyl or histidyl group at the active site.

The present work describes the positive identification of the active site group by (a) partial iodinations in which the modification of amino acid residues was correlated with loss in binding capacity and (b) the combined use of iodination and photooxidation.

METHODS AND MATERIALS

Preparation of Purified Arsonic Antibody--Antisera were prepared in New Zealand white rabbits by the injection of bovine gamma globulin coupled with diazotized arsanilic acid. After a single course of inoculations the animals were exsanguinated and sera containing more than 100 μg of antibody N per ml were pooled. Antibody directed only against the azophenylarsonic acid moiety was isolated from the pooled sera by precipitation with an azoantigen in which human fibrinogen was substituted as the

protein carrier. The washed immune precipitates were then dissolved in an 0.25 M solution of the haptene, p-nitrobenzenearsonic acid, and the resulting solution was applied to a DEAE column equilibrated with 0.02 M phosphate buffer, pH 7.2. Under these conditions the antibody appeared as a single peak at column volume and the eluate was shown to be at least 95% pure by equilibrium dialysis measurements. Detailed descriptions of the antigen syntheses and the purification procedures have appeared elsewhere (4).

Preparation of Purified Gamma Globulin--Pooled sera from normal rabbits was treated by the slow addition of saturated $(NH_4)_2SO_4$, pH 7.0, until the concentration of $(NH_4)_2SO_4$ reached 30%. The precipitate which formed after 24 hrs of standing was removed by centrifugation and discarded. The amount of $(NH_4)_2SO_4$ in the supernatant was then increased to 37% and the resulting precipitate was washed with 37% $(NH_4)_2SO_4$, dissolved in distilled water at pH 7.0, and reprecipitated at the same concentration of salt. This process was repeated two times with the exception that the final precipitation was carried out with a 35% solution of $(NH_4)_2SO_4$. Aliquots of the isolated gamma globulin were then separated on a DEAE column under exactly the same conditions used for the elution of the specific antibody.

Measurement of Antibody--The immunological activity was assayed by the dialysis of arsonic antibody against acetylated arsanilic acid in which the carboxyl carbon was radioactive. This equilibrium dialysis method has the advantage that it provides a direct measure of the binding capacity

of antibody and does not depend on a secondary manifestation of antigen-antibody binding such as the formation of insoluble complexes. The haptene was synthesized from arsanilic acid and C^{14}-labelled acetic anhydride by a modification of the method of Ehrlich (5). The dialyses were carried out in a lucite apparatus which was divided into two compartments by the insertion of a dialysis membrane. After 1 ml of the appropriate antibody or haptene solution was added to each compartment, the container was closed and rotated in the cold for 44 hrs. The antibody concentrations used varied from 1 to 2 x 10^{-5} M depending on the binding capacity; the haptene concentrations ranged from 1.5 x 10^{-5} M to 1.5 x 10^{-4} M. All solutions were made up in 0.2 M glycine buffer, pH 8.5 and 0.125 M NaCl.

To determine the amounts of free and bound haptene after dialysis duplicate 0.1 ml aliquots from each compartment were plated and counted in an end window gas chamber to an accuracy of better than 1%. Corrections were applied for self-absorption, background and non-specific absorption of the haptene on the membrane and walls of the container as previously determined in a series of control experiments (4). The protein content of the solutions in the antibody compartment were analyzed in triplicate by the micro-Kjeldahl N method.

Iodination of Antibody--Arsonic antibody was iodinated by a modification of the method of McFarlane (6) in which iodine monochloride is the iodinating agent and the reaction is carried out in 0.2 M glycine, pH 8.5. A complete description of the apparatus and the procedure used may be found in (3). In the experiments to determine the effect of

iodination on binding capacity radioactive iodine monochloride was not employed because of the difficulty of counting C^{14} in the presence of I^{131}. Iodine incorporation was measured in these cases by carrying out duplicate experiments in which highly purified gamma globulin was substituted for the arsonic antibody and the ICI was equilibrated with radioactive iodine. The iodine uptake was then calculated from the specific activity of the samples and the assumptions of a N content of 16% and a molecular weight of 160,000. To determine specific activities, the iodinated samples were precipitated in the presence of 0.13% $NaHSO_3$ and 7% CCl_3DOOH (TCA); the precipitates were washed three times with 7% TCA, dissolved in dilute NaOH and aliquots removed for counting and N analysis by the micro-Kjeldahl method. Radioactivity was assayed in a deep well scintillation counter and corrected for background, decay and sample volume. It was shown that under these conditions the iodine uptake of the globulin preparations was identical within experimental error to that of purified arsonic antibody and that the yields were reproducible (4).

Photooxidation of Antibody--The procedure used for catalytic photooxidation was developed by Ray and Koshland (7) on the basis of earlier work by Weil and his co-workers. The apparatus consisted of a Warburg bath with a lucite bottom through which passed the light from a bank of 150 watt spotlights mounted below. The light intensity was controlled by use of a rheostat and volt meter which maintained a 126 volt input to the lights. Reaction flasks constructed with a flat polished glass bottom were attached to the Warburg shaker and immersed 1/2 inch into the water

bath maintained at 11-12° C. The shaker was set to give a full stroke at the rate of 80 per min.

Since the rate of photooxidation was found to vary with the volume, concentration of reactants and ionic strength of the reaction solution (7), the photooxidations were carried out under a constant set of conditions. A 1.9 ml volume of a solution 3.3×10^{-5} M in antibody and 0.02 M in phosphate buffer, pH 7.2, was pipetted into the reaction flask. One-tenth milliliter of 0.2% methylene blue, freshly prepared in phosphate buffer, was added, the solution was thoroughly mixed and the flask was fitted to the stopper mounted on the manometer stand. After suitable exposure to light, the contents of the flask were transferred so that the methylene blue could be removed by dialysis or precipitation with TCA. The photooxidations and all manipulations from the time of addition to the final removal of the methylene blue were performed in a darkened room and the reaction mixture kept ice-cold at all times. In those instances when more than 10 mg of an antibody were required for experiments subsequent to photooxidation, an appropriate number of reaction solutions were photooxidized simultaneously under the conditions described above. Controls were included to assay the effect of methylene blue in the dark and light exposure in the absence of the dye.

Analyses of Amino Acid Residues--The amino acid analyses were carried out on 4 mg samples which were either dialyzed exhaustively against dilute phosphate buffer or precipitated with TCA according to the procedures described above. In the case of the photooxidized samples, the TCA

precipitates were subjected to an additional wash with ice-cold 95% alcohol which removed most of the adsorbed methylene blue. Acid hydrolyses were performed according to the standard procedure; basic hydrolyses by the $Ba(OH)_2$ method of Ray and Koshland (7). The dried hydrolysates were dissolved in 5 ml of the appropriate buffer and 2 ml aliquots were applied to the long and short column of the automatic analyzer. The recoveries obtained for the amino acids in each chromatogram were expressed relative to a leucine value of 89 residues per mole of antibody. The use of this correction was supported when the sum of the residues so obtained (3) gave a molecular weight for arsonic antibody of 156,700, a value in good agreement with the average of 160,000 determined for rabbit antibody by various physical measurements (8 - 11).

The modification of tyrosyl, histidyl, methionyl and tryptophanyl residues by iodination was determined from the differences obtained between the amino acid recoveries of treated and untreated antibody. The acid hydrolysates were used to assay the reaction of histidyl groups with iodine; the basic hydrolysates were required to measure tyrosyl, methionyl and tryptophanyl reactivity because of the deiodination of di-iodotyrosine, the reduction of methionine sulfoxide and the destruction of tryptophan during HCl hydrolysis. The validity of this difference method was established by the demonstration (3) that the presence of oxidized or iodinated derivatives did not affect the recoveries of the unmodified residues with the exception of di-iodohistidine which was partially deiodinated in acid to regenerate histidine. Since an average of 18% was found to be deiodinated

over a wide range of di-iodohistidine concentration, the observed loss in histidine could be corrected to obtain the number of modified residues.

Studies of photooxidized phosphoglucomutase and chymotrypsin showed that methionyl, histidyl, tryptophanyl and tyrosyl groups were destroyed and the products of the reaction were not stable to hydrolysis (7). However, since no regeneration of histidine (12) occurred in acid and no regeneration of methionine (7), tryptophan and tyrosine (12) in base, the residues in arsonic antibody modified by photooxidation could be determined by the same scheme of analysis of acid and basic hydrolysates used to identify iodinated groups.

EXPERIMENTAL RESULTS

The effect of increasing iodination on the reactive groups in arsonic antibody is shown in Table I. It may be seen that the tyrosyl residues were most susceptible to destruction under the experimental conditions employed and the only groups to react significantly during the addition of small amounts of reagent. The imidazole rings were iodinated more slowly as would be expected from the comparative iodination of the free amino acids. Finally methionyl and tryptophanyl residues were oxidized only after more than 71 atoms of iodine were added per molecule of antibody.

The observed amino acid modifications are compared in Table II with the measurements of iodine uptake and binding capacity. The correlation with iodine uptake revealed that the number of atoms incorporated per antibody molecule was always twice the sum of the residues iodinated within the limits of experimental error. Thus, essentially every substituted

residue contained two iodine atoms even when additional unreacted groups were available. These data supported the earlier observations of Li (13) that monoiodinated tyrosine reacts more rapidly with iodine than tyrosine itself.

The correlation with the measurements of binding capacity showed that 87.7% of the active sites were destroyed by the modification of 26 tyrosyl and 3 histidyl residues. These results eliminated the possibility of a methionyl or tryptophanyl group being the iodine-reactive residue at the active site of arsonic antibody and strongly indicated the presence of tyrosine. However, final proof depended on the demonstration that the reaction of the few histidyl residues was not responsible for the observed loss in immunological activity.

From previous studies, particularly those of Weil and his co-workers (14, 15), the histidyl residues in proteins were known to be susceptible to destruction by catalytic photooxidation while the tyrosyl residues were modified very slowly if at all. This information suggested that the iodine-reactive histidines in arsonic antibody could be selectively modified by photooxidation prior to iodination so that the effect of tyrosyl loss alone could be assayed in the subsequent iodination. The optimal period of photooxidation was chosen from measurements of the loss of hisitdyl groups as a function of exposure time shown in Fig. 1. It was evident from the nonlinearity of the semi-log plot that all the histidines in arsonic antibody were not destroyed at the same first order rate. However, the shape of the curve obtained suggested two general degrees of

reactivity; approximately 11 of the 16 histidyl groups were rapidly oxidized while the remainder appeared relatively inaccessible to the reagent. The 30 minute exposure used represented, therefore, the time required for the reaction of those histidyl residues readily accessible to modification by photooxidation and presumably also by iodination.

The effect of photooxidation and subsequent iodination on the amino acid content and the binding capacity of arsonic antibody is shown in Table III and Fig. 2 respectively. The photooxidative step produced the expected destruction of 11 histidyl groups and in addition altered 2 tyrosyl and 10 methionyl residues. These modifications were accompanied by a 33% loss in binding capacity as illustrated in Fig. 2 by the decrease in r, the average number of binding sites per molecule, from 1.91 for the control to 1.26 for the photooxidized sample. The cause of this loss was not immediately apparent. The amount was greater than would be expected from the number of tyrosyl residues modified unless a tyrosine at the active site was selectively photooxidized. On the other hand, the loss was only one-third that expected if a histidine were at the active site, since the subsequent data demonstrated that the three iodine-reactive histidines were oxidized. It is possible that the loss resulted from the oxidation of a histidyl or methionyl residue present at the active site but not accessible to iodine even though the overall rate of histidyl or methionyl photooxidation did not parallel the rate at which activity was destroyed. It is also possible that the loss was due to changes in the tertiary structure since the presence of haptene did not protect against the effects of photooxidation.

The subsequent addition of 40 iodine atoms per molecule to the photooxidized antibody did not cause any detectable destruction of the remaining histidyl residues as shown in the last two columns of Table III, while the identical treatment of a non-photooxidized control resulted in the loss of 1.6 histidines. Furthermore, iodination of histidyl groups was not obtained when the amount of reagent added, 72 iodine atoms per molecule of photooxidized antibody, was sufficient to destroy 3 histidyl residues. It would appear, therefore, that those histidyl groups whose reaction with iodine coincided with a 90% destruction of immunological activity were altered by the preliminary photooxidative treatment. However, the addition of 40 iodine atoms per molecule modified 19 tyrosyl residues and reduced the average number of sites from 1.26 to 0.32 per molecule, a 74.6% loss in the activity remaining after photooxidation. These data in conjunction with the previous demonstration (1, 2, 4) that haptene prevented the loss of activity by iodination established that a tyrosyl residue is at the active site of arsonic antibody.

DISCUSSION

Previous studies (3) indicated that iodine is not a highly specific amino acid reagent under conditions of extensive iodination since significant numbers of tyrosyl, histidyl, methionyl and tryptophanyl residues were destroyed. However, the present analyses of partially iodinated material showed that iodine reacts preferentially with the tyrosyl side chains in arsonic antibody. Tyrosyl residues alone were modified by the incorporation of the first 8 iodine atoms per molecule and only 3

histidyl groups were destroyed in addition to 26 tyrosines by an increase in iodine uptake to 56 atoms per molecule. Furthermore, the delayed oxidation of methionyl and tryptophanyl residues in the presence of excess reagent suggested that these groups do not become available for reaction until the tertiary structure is disrupted by the iodination of tyrosyl residues. A similar pattern of amino acid modification was obtained by the successive iodination of a pool of immunologically inert gamma globulin, as shown in Table IV. These results indicate that the relative accessibility of tyrosyl, histidyl, methionyl and tryptophanyl residues to iodine is not detectably altered by the differences in sequence and/or conformation which determine immunological specificity.

The active sites of antibodies other than the anti-p-azobenzenearsonate have been shown to contain an iodine-reactive residue. Grossberg et al. (16) found that iodination reduced the binding capacity of anti-p-azobenzoate, anti-3-azopyridine and anti-p-phenyltrimethylammonium antibodies and the loss could be partially prevented by the prior addition of the respective homologous haptene. Katsura (17) has obtained similar results with diphtheria antitoxin. The presence of a tyrosine at the active center of each of these antibodies is certainly likely on the basis of the present studies of the iodination of arsonic antibody and gamma globulin. However, final identification can only be made by the use of methods, such as photooxidation, which eliminate the possible involvement of the other iodine-reactive groups.

The number of iodine-reactive residues at each active site of

arsonic antibody was determined previously (4) by calculating the probable losses in binding capacity if there were one or two groups present and if the iodination of any one of these destroys the binding at that site. For this calculation the assumption was made that any substituted residue contains two iodine atoms independently of the extent of iodination. When the theoretical values were compared with the observed losses in binding capacity, a close correspondence was found with the losses that would be expected if only one reactive group were at each site. The data obtained in the present studies provide experimental evidence for the di-substitution of iodinated residues and thus support the previous conclusion that only one iodine-reactive group, i.e. tyrosine, is involved in determining the specificity of arsonic antibody.

CONCLUSIONS

The modification of amino acid residues in arsonic antibody was followed as a function of iodine uptake. The tyrosyl side chains were most susceptible to destruction by iodination, histidyl groups reacted more slowly and the oxidation of methionyl and tryptophanyl residues was not observed until most of the available tyrosines were reacted. Two iodine atoms were found to be substituted in each iodinated residue independently of the extent of iodination.

The correlation of the observed amino acid modifications with losses in binding capacity showed that essentially all immunological activity was destroyed when only tyrosyl and histidyl residues were iodinated. By the combined use of photooxidation, which selectively altered the histidyl

residues, and iodination which effected a significant decrease in binding capacity by the reaction of only tyrosyl groups, it was possible to identify a tyrosyl residue as the iodine-reactive group known to be at the active site of arsonic antibody.

FOOTNOTES

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[2] The antiazophenylarsonic acid antibody will be referred to henceforth in this paper as arsonic antibody.

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TABLE I

Destruction of amino acid residues in arsonic antibody by iodination

Iodine added	Amino acid residues recovered per 160,000 g of antibody			
	Tyrosyl[1]	Histidyl[2]	Methionyl[1]	Tryptophanyl[1]
atoms/mol.				
0	58.8	16.4	12.8	18.3
4.5	57.1	16.5	13.0	-
8.9	55.1	16.4	13.4	-
17.8	51.2	16.3	13.0	-
26.7	48.3	15.9	12.7	-
35.6	43.1	15.4	13.0	18.7
71.2	32.6	14.1	13.0	18.7
260.	14.7	10.3	3.2	12.5

[1] Determined after 16 hr $Ba(OH)_2$ hydrolysis

[2] Determined after 20 hr HCl hydrolysis

TABLE II

Correlation of destruction of amino acid residues in arsonic antibody with iodine uptake and loss in binding capacity

Residues lost per 160,000 g of antibody			Iodine uptake	Loss in binding capacity
Tyrosyl	Histidyl[1]	Sum of tyrosyl + histidyl		
			atoms/mol. antibody	%
1.7	0	1.7	3.57	7.7
3.7	0	3.7	7.63	12.3
7.6	0.1	7.7	15.7	26.0
10.5	0.6	11.1	22.1	44.9
15.7	1.2	16.9	30.5	56.6
26.2	2.8	29.0	55.5	87.7

[1] Corrected for 18% deiodination of di-iodohistidine in HCl hydrolysis

TABLE III

Amino acid residues in arsonic antibody modified by (a) photooxidation and (b) iodination subsequent to photooxidation

Amino acid	Untreated[a]	Photooxidized[b]		Photooxidized and iodinated[c]	
	Residues per mole[d]	Residues per mole[d]	Residues lost	Residues per mole[d]	Residues lost
	Acid hydrolysates[e]				
lys	69.6	69.7		68.9	
hist	16.4	4.93	11.5	4.96	0
arg	45	45		45	
	Basic hydrolysates[f]				
pro	117	118		122	
met	12.2	2.49	9.7	2.29	0
Ileu[g]	50.3	49.6		49.0	
leu	89	89		89	
tyr	58.3	56.2	2.1	37.3	19.0
phe	43.0	42.8		43.0	

[a] Combined with methylene blue, but not photooxidized

[b] Photooxidized for 30 min in presence of 0.01% methylene blue

[c] Iodinated by addition of 40 I atoms per mol. of photooxidized antibody

[d] The molecular weight of arsonic antibody was assumed to be 160,000

[e] Hydrolyzed in HCl for 20 hrs. Values corrected to arg = 45 residues per mole. Each value represents an average of 3 determinations

[f] Hydrolyzed in $Ba(OH)_2$ for 16 hrs. Values corrected to leu = 89 residues per mole. Each value represents an average of at least 2 determinations

[g] Sum of Ileu and allo-Ileu

TABLE IV

Destruction of amino acid residues in rabbit gamma globulin by iodination

Iodine uptake	Residues per 160,000 g of globulin							
	Tyrosyl[1]		Histidyl[2]		Methionyl[1]		Tryptophanyl[1]	
	Recovered	Lost	Recovered	Lost[3]	Recovered	Lost	Recovered	Lost
atoms/mol. antibody								
0	57.0	0	18.0	0	13.0	0	18.6	0
3.6	55.0	2.0	-	-	12.4	0	-	-
7.6	52.3	4.7	18.0	0	13.2	0	-	-
15.7	49.3	7.7	18.0	0	13.0	0	-	-
22.1	45.7	11.3	17.7	0.4	13.2	0	-	-
30.5	42.0	15.0	16.4	1.9	13.2	0	-	-
55.5	33.1	23.9	15.9	3.0	12.8	0	19.5	0
102.1	13.4	43.6	10.7	8.9	3.0	10.0	12.4	6.2

[1] Determined after 16 hr $Ba(OH)_2$ hydrolysis

[2] Determined after 20 hr HCl hydrolysis

[3] Corrected for 18% deiodination of di-iodohistidine in HCl hydrolysis

FIGURE LEGENDS

Fig. 1. Modification of histidyl residues in arsonic antibody by photooxidation.

Fig. 2. Effect of photooxidation and subsequent iodination on the binding capacity of arsonic antibody. Curve C represents both untreated antibody and antibody combined with methylene blue and then diolyzed in the dark. Curve 1 represents antibody photooxidized for 30 min in the presence of methylene blue. Curve 2 represents the same antibody after the addition of 40 I atoms per molecule.

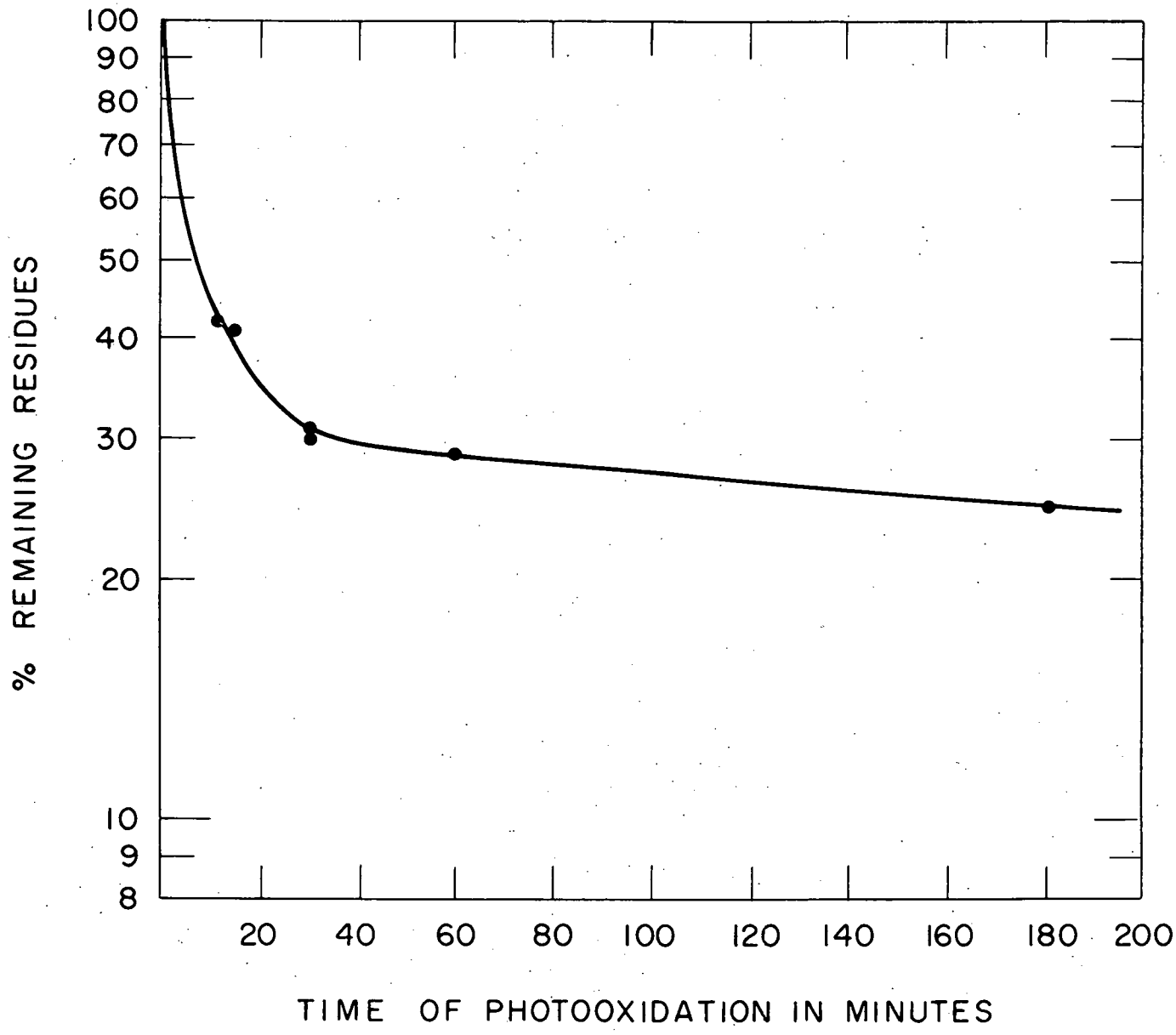
100
90
80
70
60
50
40
30
20
10
9
8
% REMAINING RESIDUES
20
40
60
80
100
120
140
160
180
200
TIME OF PHOTOOXIDATION IN MINUTES

FIGURE 1

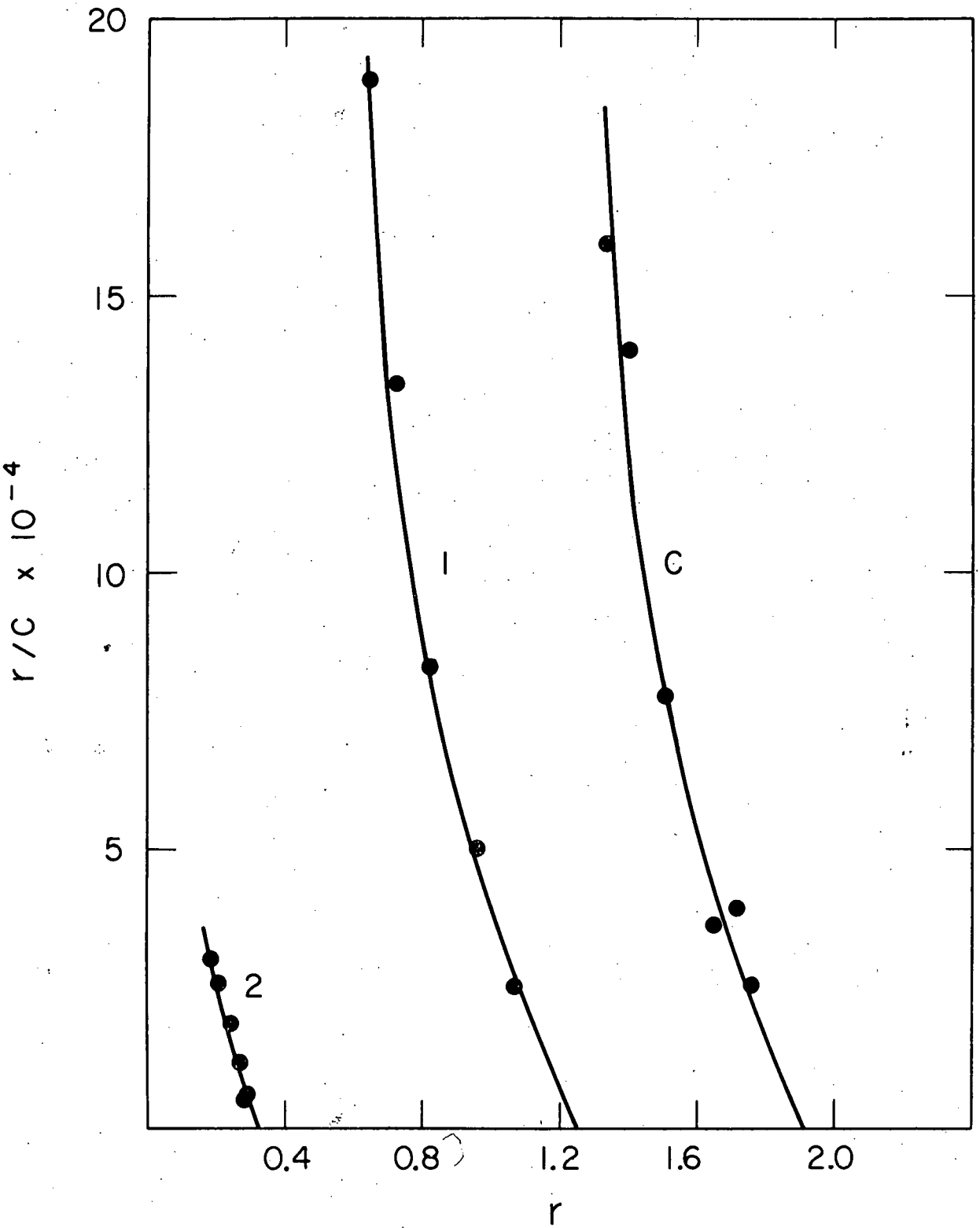

FIGURE 2