Acknowledgment

We wish to thank Dr. Norman Kondo and Miss Helen Holmes for obtaining several spectra in this work, and we also appreciate the consultation and assistance of Dr. Wai-Mun Huang.

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Role of the Net Electrical Charge of the Complete Antigen in Determining the Chemical Nature of Anti-*p*-azobenzenearsonate Antibodies*

Erwin Rüde,† Edna Mozes, and Michael Sela

ABSTRACT: Poly-ε-*N*-trifluoroacetyl-L-lysyl ribonuclease was prepared from ribonuclease and ε-*N*-trifluoroacetyl-α-*N*-carboxy-L-lysine anhydride. The free amino groups of the polypeptidyl protein were trifluoroacetylated, and the resulting molecules were conjugated through their tyrosine and histidine residues with the diazonium salt derived from arsanilic acid. After removal of the trifluoroacetyl groups, the basic *p*-azobenzenearsonate-poly-L-lysyl ribonuclease was used for immunization of rabbits. Most of the anti-*p*-azobenzenearsonate antibodies formed were found in the second fraction upon chromatography on DEAE-Sephadex under conditions which separate antibodies according to the net electrical charge of the antigens. In contrast, antibodies of the same specificity induced with the acidic *p*-azobenzenearsonate conjugates of rabbit serum albumin and hexa-L-tyrosine chromatographed mainly in the first fraction. Thus, the antibodies formed reflected the over-all net charge of the molecule, rather than the charge within the limited area around the *p*-azobenzenearsonate determinant in the immunogen, an area similar in its charge properties to similar areas in other *p*-azobenzenearsonate conjugates of proteins. It is concluded that the antigenic control of the antibody type formed (DEAE-Sephadex chromatography detection) occurs at the level of the complete antigenic molecule.

Antibodies from different rabbit antisera distribute unequally among two immunoglobulin G fractions obtained upon chromatography on DEAE-Sephadex (Sela *et al.*, 1963a). From a detailed study of rabbit antisera to lysozyme, obtained under a variety of conditions, it was concluded that the distribution of antibodies among the two chromatographic fractions is controlled by the chemical properties of the antigen, rather than by the course of immunization, the span of time elapsed between the immunization and the collection of the antisera, or the genetic makeup of the animals (Sela and Mozes, 1966; Sela, 1966). The antibodies to lysozyme were found predominantly, or exclusively, in the second chromatographic immunoglobulin G fraction on DEAE-Sephadex, and immunospecifically purified antilysozyme antibodies were found only under the second peak (Sela, 1967).

The extension of this investigation to antibodies produced by a variety of natural and synthetic antigens led to the conclusion that there is an inverse correlation

* From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel. *Received April 8, 1968.*

† Visiting scientist from the Max-Planck-Institute for Immunobiology, Freiburg, Germany.

between the net electrical charge on the antigen and that on the antibody it elicits (Sela and Mozes, 1966). This correlation persists also after simultaneous immunization with two antigens of opposite charge (Mozes and Sela, 1966). Antibodies to acidic antigens were found predominantly in the first chromatographic fraction on DEAE-Sephadex, whereas antibodies to basic antigens were mostly in the second fraction. Antibodies with specificity toward the same determinant (the dinitrophenyl group) belonged exclusively to either one or the other of the two immunoglobulin fractions, depending on the electrical charge of the macromolecular carrier to which the determinant was attached. These findings led to the conclusion that the biosynthesis of an antibody is controlled both by the specificity determinants and by other parts of the antigenic molecule (Sela and Mozes, 1966).

A similar correlation has been also observed between the net charge of antigens and the electrophoretic mobility of rabbit antibodies of the IgM[1] class (Robbins *et al.*, 1967a). The fractionation on DEAE-Sephadex is not limited to rabbit IgG, as similar results were obtained upon chromatography on DEAE-Sephadex of IgG preparations of goat, horse (Sela and Mozes, 1966), man, and mouse (E. Mozes and M. Sela, unpublished data).

In the earlier studies it has not yet been established whether the chromatographic behavior of antibodies depends on the net electrical charge of the complete antigenic macromolecule, or whether they are a function of the net charge in the area surrounding the antigenic determinant. In order to answer this query we have prepared from bovine pancreatic ribonuclease an immunogen containing a big excess of positive charges, in the form of poly-L-lysyl chains, on the *outside* of the molecule, and several acidic *p*-azobenzenearsonate determinants bound to tyrosine and histidine residues *within* the molecule. The results of an investigation of the rabbit antisera to Ars-pLysRNase, leading to the conclusion that the type of the antibodies formed is controlled by the net electrical charge on the complete immunogen, are presented below.

Materials and Methods

Bovine pancreatic RNase (type I-A, five-times crystallized) was purchased from Sigma, St. Louis, Mo., and bovine serum albumin from Armour, Chicago.

Poly-L-lysyl bovine serum albumin was synthesized analogously to polylysyl rabbit serum albumin (Sela *et al.*, 1963b). BSA (2 g) in 0.05 M sodium phosphate buffer (pH 7.0) (200 ml) was treated at 0° with ε-*N*-trifluoroacetyl-α-*N*-carboxy-L-lysine anhydride (2 g) in anhydrous dioxane (100 ml). The ε-*N*-trifluoroacetyl groups were removed from the resulting poly-ε-*N*-trifluoroacetyl-L-lysyl BSA with 1 M piperidine at 4°, and the pLysBSA obtained was isolated as described earlier (Sela *et al.*, 1963b).

p-Azobenzenearsonate Bovine Serum Albumin. Arsanilic acid (5 mmoles) was dissolved in 0.6 N HCl (25 ml) and was treated at 4° with a solution of sodium nitrite (5 mmoles) in water (5 ml). After stirring for 20 min the solution of the diazonium salt (0.167 M) was used for coupling.

ArsBSA was prepared (Tabachnick and Sobotka, 1960) by treating 125 mg of BSA dissolved in 0.01 M sodium tetraborate (12.5 ml) at pH 9.0 and 4° with the above diazonium salt (1.25 ml of 0.167 M solution). The reaction mixture was maintained at pH 9.0 by the addition of 2 N NaOH in a pH-Stat.

Poly-ε-N-trifluoroacetyl-L-lysyl RNase was prepared as described by Frensdorff and Sela (1967). RNase (1.5 g) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) (150 ml) was treated at 0° with ε-*N*-trifluoroacetyl-α-*N*-carboxy-L-lysine anhydride (1.5 g) in anhydrous dioxane (75 ml). After dialysis against water, the water-insoluble fraction of the reaction product was separated off by centrifugation and discarded. The water-soluble fraction was lyophilized (0.68 g). The trifluoroacetyl groups were removed from a small sample by treatment with 1 M piperidine. The number of lysine residues attached to RNase in the resulting pLysRNase was determined by amino acid analysis (Spackman *et al.*, 1958).

Trifluoroacetylation of the above pTFALysRNase was carried out, using ethyl thioltrifluoroacetate as reagent (Schallenberg and Calvin, 1955) to block the free amino groups (ε-amino groups of unreacted lysine residues and α-amino termini of the peptide chains attached to RNase). pTFALysRNase (660 mg) was suspended in a mixture of water (75 ml) and dioxane (25 ml). After adjusting the pH to 9.9 with 5 N NaOH most of the material dissolved. Ethyl thioltrifluoroacetate (3.6 ml) was then slowly added at room temperature to the vigorously stirred solution over a period of 40 min. The pH was kept at 9.9, using a pH-Stat for the addition of 5 N NaOH. The uptake of alkali stopped completely 10 min after the last addition of the thioester. The pH was then lowered to 7.0, and the solution was dialyzed exhaustively against water. The precipitate formed during dialysis was lyophilized, yielding 610 mg of the trifluoroacetylated product. The content of free amino groups, as determined by the Van Slyke (1923) method, was 1.29% N before and 0.16% N after the trifluoroacetylation.

p-Azobenzenearsonate-poly-L-lysyl RNase. The trifluoroacetylated pTFALysRNase (600 mg) was suspended in a mixture of water (90 ml) and formamide (70 ml). After vigorous stirring most of the material dissolved. An ice-cold 0.167 M solution of the diazonium salt derived from arsanilic acid (10 ml) was then added dropwise at 4° for about 30 min. During this period, and for an additional hour, the pH was maintained at

[1] The nomenclature used is that recommended by the *World Health Organ.* (1964). Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Ars, *p*-azobenzenearsonate; pLys, poly-L-lysyl; Ars-pLysRNase, poly-L-lysyl ribonuclease to which *p*-azobenzenearsonate groups were attached; pTFALysRNase, poly-ε-*N*-trifluoroacetyllysyl RNase; BSA, bovine serum albumin; pLysBSA, poly-L-lysyl BSA; ArsBSA, BSA to which *p*-azobenzenearsonate groups were attached; RSA, rabbit serum albumin; ArsRSA, RSA to which *p*-azobenzenearsonate groups were attached; Ars-(Tyr)$_6$, *p*-azobenzenearsonate conjugate of hexa-L-tyrosine.

pH 8.5 with 2 N NaOH, in a pH-Stat. Stirring was continued overnight at 4°. After exhaustive dialysis against 0.1 M NaCl, followed by water, most of the reaction product precipitated.

To remove the trifluoroacetyl groups, the suspension was concentrated to about 20 ml by ultrafiltration (Hofsten and Folkbring, 1955) and cooled with ice, 2 M aqueous piperidine (20 ml) was added, and the mixture was stirred for 24 hr at 4°. The resulting clear solution was neutralized with 2 N acetic acid and dialyzed against 0.1 M NaCl, followed by water. A small amount of material precipitated out of solution during the above procedure and was removed by centrifugation. The supernatant fluid was lyophilized, yielding 530 mg of Ars-pLysRNase. This preparation was soluble both in 0.9% NaCl and in distilled water.

Arsenic Determination. The content of arsenic was determined by elementary analysis according to Merz (1959).

Amino Acid Analysis. This was conducted in a Spinco Model 120 B amino acid analyzer (Spackman *et al.*, 1958). All hydrolyses were carried out in 6 N HCl in sealed, evacuated ampoules for 22 hr at 116°. The enrichment of the protein derivatives with lysine residues was calculated assuming that RNase contains the theoretical number of 4 arginine residues/molecule (Hirs *et al.*, 1956; Smyth *et al.*, 1962), and that BSA contains 22 arginine and 17 histidine residues per molecule (Tanford *et al.*, 1955).

Spectrophotometric measurements were made on a Zeiss Model PMQII spectrophotometer, at approximately 25°, with quartz cells of 1-cm light path.

Immunization Procedure. A group of 6 rabbits was immunized by two injections, at a 3-week interval, with 10 mg of Ars-pLysRNase, emulsified in complete Freund's adjuvant (Difco, Mich.) at multiple intradermal sites. Animals were bled 10 days after the second injection and subsequently at weekly intervals, and the antisera were pooled.

Column Chromatography. The antisera investigated and the isolated antibodies were chromatographed on DEAE-Sephadex A-50 medium (Pharmacia, Uppsala) columns (33 × 2.3 cm.) after an 18-hr dialysis against 0.02 M potassium phosphate buffer (pH 8.0) (Sela and Mozes, 1966). After the elution of the protein under the first peak with the above buffer (180 ml), a gradient of increasing molarity to 0.3 M potassium phosphate buffer was started. The chromatographies were performed at 4°. The pooled fractions were concentrated by ultrafiltration (Hofsten and Folkbring, 1955).

Quantitative Precipitin Tests. The antibody content in the two chromatographic fractions was determined by the quantitative precipitin test using the homologous antigen Ars-pLysRNase, as well as the cross-reacting antigens pLysBSA and ArsBSA, according to Sela *et al.* (1962). The amount of antibody precipitated at the equivalent point was calculated after making the necessary corrections for the contribution of the antigen absorbancy at 280 mμ, using $E^{1\%}_{1\,cm}$ 14.0 for rabbit IgG (Porter, 1957).

Immunospecific Isolation of Antibodies. Antibodies to *p*-azobenzenearsonate were isolated by adsorption from the anti-Ars-pLysRNase sera, with an immunoadsorbent prepared by the chemical reaction of ArsBSA and bromoacetylcellulose, followed by elution with 0.1 M acetic acid (Robbins *et al.*, 1967b). The physical adsorption of ArsBSA to bromoacetylcellulose was carried out in 0.1 M phosphate–citrate buffer (pH 4.6).

Results

It was desired to prepare an immunogen in which the negatively charged *p*-azobenzenearsonate groups would be bound to tyrosine and/or histidine residues within the protein carrier, whereas the positively charged lysine peptides would be attached on the outside of the carrier, so as to bring about a strong net positive charge. For this purpose, bovine pancreatic RNase was chosen as the carrier molecule. As diazonium salts may react also with amino groups of proteins to give unstable diazoamino compounds (Gelewitz *et al.*, 1954; Howard and Wild, 1957; Tabachnick and Sobotka, 1959, 1960), care was taken to treat the protein with the diazonium salt derived from arsanilic acid only after all its amino groups were reversibly protected.

For this purpose RNase was treated first with ε-*N*-trifluoroacetyl-α-*N*-carboxy-L-lysine anhydride (Sela *et al.*, 1963b) and the amino groups still present in the resulting poly-ε-*N*-trifluoroacetyllysyl RNase (these included the terminal α-amino groups of the poly-ε-*N*-trifluoroacetyllysyl side chains as well as any unreacted ε-amino groups of RNase) were then blocked by trifluoroacetylation (Goldberger and Anfinsen, 1962) using ethyl thioltrifluoroacetate as reagent (Schallenberg and Calvin, 1955). The completely trifluoroacetylated poly-L-lysyl RNase, which is insoluble in water but soluble in a mixture of formamide and water, was then coupled with the diazonium salt derived from arsanilic acid. After coupling, the trifluoroacetyl groups were removed by treatment with 1 M aqueous piperidine. The resulting *p*-azobenzenearsonate-poly-L-lysyl ribonuclease (Ars-pLysRNase) tends to aggregate and to become insoluble if the degree of substitution with lysine residues or *p*-azobenzenearsonate groups is higher than that indicated in Table I.

Six rabbits were immunized with Ars-pLysRNase, and the antisera collected were pooled and chromatographed on DEAE-Sephadex. The antibody content of the two chromatographic fractions was ex-

TABLE I: Characterization of the Protein Conjugates.

	Lysine Residues Attached/Protein Molecule	Arsenic Atoms/ Protein Molecule
pLysRNase	21	
Ars-pLysRNase	22	6.8
pLysBSA	97	
ArsBSA		21

TABLE II: Distribution of Anti-*p*-azobenzenearsonate Antibodies upon Chromatography on DEAE-Sephadex.

Immunogen	Charge Properties	First Chromatographic Fraction (%)	Second Chromatographic Fraction (%)	References
ArsRSA	Acidic	85[a]	15	Mozes (1967)
Ars-$(Tyr)_6$	Strongly acidic	100	0	Borek *et al.* (1967)
Ars-pLysRNase	Basic	24	76	This study

[a] Calculated as described previously (Sela and Mozes, 1966).

amined by quantitative precipitin reactions with the homologous antigen, Ars-pLysRNase, as well as with the cross-reacting antigens pLysBSA and ArsBSA. Of the antibodies precipitable with the homologous antigen, 70% were found under the second peak. Similarly, 79% of the antibodies precipitable with pLysBSA were found under the second peak. This chromatographic fraction has been reported previously to contain antibodies to basic proteins (Sela and Mozes, 1966). Antibodies precipitable with ArsBSA were also found to the extent of 60% under this second peak. The amount of antibodies precipitated in the second chromatographic fraction by Ars-pLysRNase, pLysBSA, and ArsBSA was, respectively, 0.75, 0.28, and 0.62 mg per 10 mg of IgG.

An amount of 140 mg of anti-*p*-azobenzenearsonate antibodies was isolated immunospecifically by making use of an immunoadsorbent prepared from bromoacetylcellulose and ArsBSA. Immunoadsorbents prepared from a *p*-azobenzenearsonate derivative of human serum albumin and bromoacetylcellulose have been used with success previously for the isolation of anti-*p*-azobenzenearsonate antibodies (Robbins *et al.*, 1967b; Borek *et al.*, 1967).

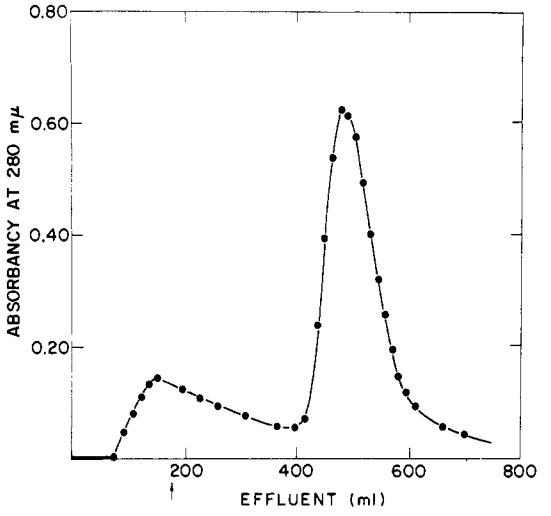

FIGURE 1: Chromatography on DEAE-Sephadex A-50 of immunospecifically isolated antibodies (100 mg) to *p*-azobenzenearsonate from an antiserum to Ars-pLysRNase. Elution from the column was started with 0.02 M potassium phosphate buffer (pH 8.0). The arrow indicates the start of a molarity gradient to 0.3 M.

The chromatography of the isolated anti-*p*-azobenzenearsonate antibodies (100 mg) on DEAE-Sephadex is shown in Figure 1. The second chromatographic fraction contains 76% of the antibodies. This should be contrasted (Table II) with anti-*p*-azobenzenearsonate antibodies obtained upon immunization of rabbits with a *p*-azobenzenearsonate conjugate of hexa-L-tyrosine [Ars-$(Tyr)_6$] (Borek *et al.*, 1967). Similarly, a *p*-azobenzenearsonate conjugate of rabbit serum albumin (ArsRSA) led to the formation in rabbits of anti-*p*-azobenzenearsonate antibodies, most of which were found in the first chromatographic fraction (Mozes, 1967).

Discussion

It has been established previously that the formation of antibodies possessing different chromatographic properties on DEAE-Sephadex is a function of the net electrical charge on the antigen (Sela and Mozes, 1966). The two immunoglobulin G fractions obtained upon chromatography on DEAE-Sephadex yield, after papain cleavage (Porter, 1959), two fragments II and one fragment III, and two fragments I and one fragment III, respectively (Sela *et al.*, 1963a). The two fractions are different only in those areas of the molecules which correspond, respectively, to fragments I and II (denoted now Fab), as the peptide maps of fragments III (denoted now Fc) derived from either of the two fractions were identical (Seijen and Gruber, 1963). Significant differences were found in the electrophoretic mobility of bands from light chains derived from antibodies with different net charge and from normal rabbit immunoglobulin G fractions which were separated on DEAE-Sephadex (Mozes *et al.*, 1967). No such clear differences were seen upon a similar comparison on polyacrylamide gels of the Fd fragments obtained by cyanogen bromide cleavage of isolated antibodies and of DEAE-Sephadex fractions of normal rabbit IgG (Segal *et al.*, 1968).

The present study was undertaken to elucidate whether the correlation found is between the type of antibody formed and the net electrical charge of the intact antigenic molecule or whether the antibody type

depends only upon the net charge of a certain limited area around the antigenic specificity determinant. We have shown previously (Sela and Mozes, 1966) that, whereas antibodies to serum albumins distribute between the two chromatographic fractions, antibodies against poly-L-lysyl rabbit serum albumin (Arnon *et al.*, 1965) were found essentially exclusively in the second fraction. This finding suggested that, using a careful chemical approach, it should be possible to change the net electrical charge of the complete antigenic molecule by attaching peptides of lysine on its outside, whereas the more limited area around a negatively charged determinant (the *p*-azobenzenearsonate group) could be relatively unchanged. For this purpose special care was taken to prevent the diazonium salt (derived from arsanilic acid) from reacting with any amino groups in the antigenic carrier used.

It is seen clearly in Table II that the anti-*p*-azobenzenearsonate antibodies obtained upon immunization with the positively charged Ars-pLysRNase were found predominantly in the second chromatographic fraction on DEAE-Sephadex. This is in contradistinction to anti-*p*-azobenzenearsonate antibodies obtained after immunization with ArsRSA, which has in the neutral pH range a negative charge, or with the strongly acidic *p*-azobenzenearsonate-hexa-L-tyrosine. In the last two cases the antibodies were found mainly, or exclusively, in the first chromatographic fraction.

It may be thus concluded that the antigen control of the antibody type formed, as detected by DEAE-Sephadex chromatography, occurs at the level of the complete antigenic molecule. It seems that it is the net electrical charge of the intact antigen which plays a role in the process of selection by the antigen, at cellular or subcellular level, of the loci at which the antibody is synthesized.

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