Extrinsic Cotton Effect and Immunological Properties of the *p*-Azobenzenearsonate Hapten Attached to a Helical Amino Acid Copolymer*

Abigail Conway-Jacobs,† Bilha Schechter, and Michael Sela

ABSTRACT: The *p*-azobenzenearsonate (Ars) hapten was coupled to two synthetic carriers. One was an ordered copolymer possessing the repeating sequence L-tyrosyl-L-alanyl-L-glutamic acid [$(TAG)_n$], and an α-helical conformation under physiological conditions. The other one was a random copolymer of L-tyrosine, L-alanine, and L-glutamic acid [$(TAG)_r$]. The *p*-azobenzenearsonate group was attached, on the average, to one of every 26 tyrosine residues in the ordered copolymer, and to one in every 7 tyrosine residues in the random copolymer. Under conditions at which the ordered copolymer is in a helical conformation, its *p*-azobenzenearsonate derivative exhibits a large positive ellipticity band in the visible region, with a maximum at 420 nm. In contradistinction, the azo conjugate of the random copolymer exhibits only a small negative ellipticity band in this region. All rabbits immunized with Ars-$(TAG)_r$ responded with a high titer of anti-Ars antibodies ($\sim$1 mg/ml), whereas those injected with Ars-$(TAG)_n$ gave a very weak anti-Ars response ($\sim$1 μg/ml). Both modified copolymers reacted, nevertheless, with anti-Ars antibodies, as shown by their capacity to inhibit the inactivation of arsanilated bacteriophage. Addition of monovalent anti-Ars antibody fragments (Fab) to Ars-$(TAG)_r$ increased the ellipticity of its small band. The addition of these same fragments to Ars-$(TAG)_n$ resulted in the inversion of the positive band at 420 nm, with a new negative peak at 465 nm.

These data indicate that the Fab may "suck out" the *p*-azobenzenearsonate moiety from its conformation within the ordered copolymer and convert it into another conformation, recognized by Fab. It is suggested that the inability to induce significant amounts of anti-Ars antibodies by means of the azo conjugate of the helical copolymer may be related to the unique conformation or environment of the *p*-azobenzenearsonate hapten.

Antibodies to arsanilazo haptens have been studied ever since the beginning of modern immunochemical research (Landsteiner, 1945). The *p*-azobenzenearsonate group has been coupled to various proteins (*e.g.*, to ovomucoid or egg white, Hooker and Boyd, 1932; to globulin, Haurowitz, 1936). as well as to synthetic macromolecules (*e.g.*, to poly-L-tyrosine, Leskowitz, 1963; Borek and Stupp, 1966). The resulting conjugates elicited an immune response specific for the "arsanilazo" hapten. Pressman and Grossberg (1968) reviewed in detail the properties of anti-arsanilazo antibodies.

Attachment of haptens to immunogenic carriers leads to conjugates which usually are capable of provoking anti-hapten antibodies in experimental animals. In the present study we describe a case in which it seems that the hapten attached interacts with its own carrier in such a way as to be inaccessible to the site of antibody biosynthesis.

The *p*-azobenzenearsonate (denoted as "arsanilazo" and abbreviated as Ars[1]) group was coupled to two related polypeptide carriers. One is an α-helical ordered copolymer with the repeating sequence (L-tyrosyl-L-alanyl-L-glutamyl)$_n$ (J. Ramachandran, A. Berger, and E. Katchalski, in preparation; Ramachandran, 1967; Sela *et al.*, 1967) and the other is a random copolymer of L-tyrosine, L-alanine, and L-glutamic acid. In the course of our investigation it became apparent that the arsanilazo haptens on the two carriers had radically different properties. In contrast to the strong antibody response to the arsanilazo moiety of the Ars-$(TAG)_r$, the antibody response to the arsanilazo group of the helical Ars-$(TAG)_n$ was too weak to be detected by a precipitin reaction. Both the random $(TAG)_r$ and the ordered $(TAG)_n$ are good immunogens, and arsanilazo conjugates led in both cases to the formation of anti-carrier antibodies.

Kagan and Vallee (1969) have described a characteristic circular dichroism spectrum due to the presence of a *p*-azobenzenearsonate group coupled to tyrosine in carboxypeptidase. Similary, we have found that the absorption spectra of Ars-$(TAG)_n$ and Ars-$(TAG)_r$ are optically active and display extrinsic Cotton effects between 300 and 550 nm. This property enabled us to study the structural properties of the two conjugates, and the events taking place upon their interaction with antibodies. The two arsanilazo derivatives differ qualitatively as well as quantitatively in their circular dichroic properties. Moreover, the interaction between the Fab fragments derived from anti-arsanilazo antibodies and the helical

* From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel. *Received August 10, 1970.* This study was supported by Agreement 06-010 with the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

† Recipient of an American Cancer Society postdoctoral fellowship (PF-473).

[1] Abbreviations used are: Ars, the *p*-aminobenzenearsonate group, also denoted "arsanilazo"; $(TAG)_n$, an ordered repeating sequence copolymer of L-tyrosyl-L-alanyl-L-glutamic acid; $(TAG)_r$, a random copolymer of L-tyrosine, L-alanine and L-glutamic acid; Ars-$(TAG)_n$, the arsanilazo conjugate of $(TAG)_n$; Ars-$(TAG)_r$, the arsanilazo conjugate of $(TAG)_r$; Fab, papain-produced fragment of immunoglobulin, defined by the nomenclature summarized in *Bull. World Health Organ.* (1964), *30*, 447; RSA, rabbit serum albumin.

polymer Ars-$(TAG)_n$ results in a dramatic change of its circular dichroic properties. This may reflect changes in the conformation or environment of the arsanilazo hapten within the helical immunogen.

Materials and Methods

N-Benzyloxycarbonyl(*p*-azobenzenearsonate)-L-tyrosine was a gift from Mr. M. Becker. *p*-Arsanilic acid was a product of Eastman Organic Chemicals, Rochester, N. Y. Rabbit serum albumin was a product of Mann Research Laboratories, New York, N. Y.

A random copolymer of L-tyrosine, L-alanine, and L-glutamic acid, $(TAG)_r$,[1] was synthesized (Conway-Jacobs, *et al.*, 1970), as described previously (Sela *et al.*, 1962). It had an average molecular weight of 15,000 and a residue molar ratio of Tyr:Ala:Glu of 1.0:1.5:1.5. An ordered copolymer of L-tyrosine, L-alanine, and L-glutamic acid, $(TAG)_n$, with a molecular weight of 75,000, was prepared by J. Ramachandran (Ramachandran, 1967; J. Ramachandran, A. Berger, and E. Katchalski, in preparation; Sela *et al.*, 1967). The reaction of $(TAG)_n$, $(TAG)_r$, and RSA[1] with the diazonium salt derived from *p*-arsanilic acid was carried out according to the procedure described by Tabachnick and Sobotka (1959). Molar ratios of *p*-diazonium-benzenearsonate salt added, per mole of carrier and per mole of tyrosine, are given in Table I. The resulting compounds were exhaustively dialyzed against water and were then passed through a column of Sephadex G-25 (Pharmacia, Uppsala), in order to remove any traces of low molecular weight by-products. The amount of arsenic in the conjugates was determined according to Merz (1959). The amount of *p*-azobenzenearsonate groups coupled was determined from the absorption spectra of the conjugates in 0.1 N NaOH according to Tabachnick and Sobotka (1959). The results obtained by both methods were in good agreement. The number of *p*-azobenzenearsonate groups coupled to each carrier is given in Table I.

Immunization Procedure. Two groups of rabbits were immunized with $(TAG)_n$ and with Ars-$(TAG)_r$ by injecting 10 mg intramuscularly in complete Freund's adjuvant (Difco Laboratories) as described by Schechter *et al.* (1966). Four injections were given at 2-week intervals. Two other groups were immunized with Ars-$(TAG)_r$ and Ars-$(TAG)_n$ by injecting 3 mg in complete Freund's adjuvant into each of the four footpads of each animal. Some of the rabbits injected with Ars-$(TAG)_n$ were boosted intramuscularly with 2 mg of Ars-$(TAG)_n$ in complete Freund's adjuvant. A fourth group of rabbits was immunized in the same way, but injecting only 0.1 mg instead of 3 mg of Ars-$(TAG)_n$ into the footpads. All rabbits were bled weekly for 6 months. Studies were performed on sera of individual rabbits.

Immunospecific Isolation of Antibodies. The immunospecific isolation of antibodies from serum was achieved by means of adsorption on the water-insoluble immunoadsorbents, Ars-RSA-cellulose or $(TAG)_n$-cellulose, according to the procedure described by Robbins *et al.* (1967).

Preparation of Fab. The isolated antibodies were cleaved with papain as described by Porter (1959). After exhaustive dialysis against distilled water, the precipitate which formed (mainly Fc) was removed by centrifugation. The Fab solution was then concentrated by ultrafiltration (Hofsten and Falkbring, 1961).

TABLE I: Characterization of Arsanilazo Conjugates.

Arsanilazo Derivative	Moles of *p*-Diazonium Salt of Benzenearsonate Added per Mole of Tyr	Added per Mole of Carrier	Coupled per Mole of Tyr	Coupled per Mole of Carrier
Ars-$(TAG)_n$	1:10	20.7	1:26	8
Ars-$(TAG)_r$	1:7	3.8	1:7	3.8
Ars-RSA		30		29

Phage Inactivation Technique. Preparation of arsanilazo-polytyrosyl-bacteriophage T_4, inactivation of the phage, and inhibition of the inactivation were carried out according to the procedure described by Becker *et al.* (1970).

Spectral and Circular Dichroic Measurements. Ultraviolet and visible spectra were measured at 22° in a Cary 15 recording spectrophotometer. Circular dichroic measurements in the 300–550-nm range were performed at 27° in a Cary Model 60 recording spectropolarimeter with circular dichroic attachment in a cell with a path length of 1 cm. All solutions were prepared in 0.15 M sodium chloride–0.02 M sodium phosphate (pH 7.4). The absorbance of solutions in the visible region where circular dichroism was measured did not exceed 1.8 optical density units. Ellipticity calculated for the arsanil moiety is defined as $[\theta]_\lambda = M\theta/10cl$, with units of deg $(cm)^2$ per dmole, where θ is the observed ellipticity in degrees, M is the molecular weight per arsanilazo group (10,000 for Ars-$(TAG)_n$ and 4390 for Ars-$(TAG)_r$), l is the path length in centimeters, and c is the concentration in grams per milliliter. Molecular ellipticities are not corrected for the refractive index of the solvent (Djerassi, 1960).

Concentrations of Ars-$(TAG)_n$, Ars-$(TAG)_r$, and papain fragments were determined spectrophotometrically at 280 nm, using the following extinction coefficients for 1-mg/ml solutions and a path length of 1 cm: Ars-$(TAG)_n$, 4.0; Ars-$(TAG)_r$, 3.5; and Fab, 1.4.

Results

Immunological Properties. The presence of antibodies specific to the arsanilazo hapten was tested by reacting anti-Ars-$(TAG)_r$ and anti-Ars-$(TAG)_n$ antisera with Ars-RSA. The reaction with Ars-$(TAG)_n$ and Ars-$(TAG)_r$ was also checked. The ten rabbits which were immunized with Ars-$(TAG)_r$ all responded with a good production of anti-arsanilazo antibodies (Figure 1), whereas sera from 15 rabbits which were immunized by various techniques with Ars-$(TAG)_n$ did not precipitate with Ars-RSA. Using the modified bacteriophage technique, anti-Ars-$(TAG)_r$ antiserum could inactivate Ars-polytyrosylphage at a dilution of 1 to 10^6, whereas inactivation by anti-Ars-$(TAG)_n$ antiserum could be detected only at a 20-fold dilution (Table II).

Ars-$(TAG)_n$ and Ars-$(TAG)_r$ were also tested for their capacity to inhibit the inactivation of Ars-polytyrosyl-phage by anti-Ars-$(TAG)_r$ antibodies. Both arsanilazo derivatives were good inhibitors and equally effective (50% inhibition at

TABLE II: Inactivation of Arsanilazo-Polytyrosyl-Bacteriophage T_4 by Various Anti-arsanilazo Antisera.

Rabbit No.	Antiserum	Anti-arsanilazo Antibodies[a] (mg/ml of Serum)	Anti-carrier Antibodies[a] (mg/ml of Serum)	Inactivation of Arsanilazo-Polytyrosyl-Bacteriophage: Serum Dilution	Inactivation of Arsanilazo-Polytyrosyl-Bacteriophage: % Inactivation
A-10	Ars-$(TAG)_r$	1.7	0.4	$1:10^3$	95
				$1:10^6$	50
A-15	Ars-$(TAG)_r$	0.9	0.2	$1:10^3$	50
B-24	Ars-$(TAG)_n$	<0.05	0.2	1:20	35
B-25	Ars-$(TAG)_n$	<0.05	0.15	1:20	50
B-27	Ars-$(TAG)_n$	<0.05		1:20	40
B-30	Ars-$(TAG)_n$	<0.05		1:20	20
	Normal rabbit serum			1:20	0

[a] As determined by the quantitative precipitin analysis.

6×10^{-8} M arsanilazo group, Figure 2). Arsanilic acid was a poor inhibitor (22% at 10^{-4} M arsanilic acid), whereas *N*-benzyloxycarbonyl(*p*-azobenzenearsonate)-L-tyrosine was an effective inhibitor (Figure 2). Antibodies of antiarsanilazo specificity, obtained by immunization with Ars$(TAG)_r$ and isolated on an Ars-RSA immunoadsorbent, gave upon immunodiffusion (Ouchterlony, 1953) one line of complete identity when reacted with Ars-$(TAG)_r$, Ars-$(TAG)_n$, and Ars-RSA.

No major changes in the conformation of $(TAG)_n$ occurred upon attachment of the arsanilazo group, as shown by the fact that antiserum reacted in the precipitin test with Ars-$(TAG)_n$ to the same extent as unmodified $(TAG)_n$ (Figure 3). Moreover, antisera obtained upon immunization with Ars-$(TAG)_n$, while essentially devoid of anti-Ars antibodies, contained anti-$(TAG)_n$ antibodies (Table II).

Circular Dichroic Properties. The absorption and circular dichroic spectra between 300 and 550 nm of the various arsanilazo compounds are shown in Figure 4. At pH 7.4, 0.02 M sodium phosphate, 0.15 M NaCl, *N*-benzyloxycarbonyl-(*p*-azobenzenearsonate)-L-tyrosine and Ars-$(TAG)_r$ each exhibit a small negative ellipticity band with maximum amplitude at 415 and 455 nm, respectively. Ars-$(TAG)_n$, on the other hand, exhibits a strong positive ellipticity band with a peak at 430 nm.

Reaction with Fab. Fab fragments derived from anti-$(TAG)_n$ antibodies (4.8×10^{-4} M) were reacted with Ars-$(TAG)_n$ (10^{-4} M). No change was observed in the circular dichroic spectrum of Ars-$(TAG)_n$. Fab fragments derived from antibodies with specificity directed toward arsanilazo groups (anti-Ars-$(TAG)_r$ antiserum adsorbed on Ars-RSA-cellulose) were reacted with Ars-$(TAG)_n$ in a ratio of 6×10^{-4} M Fab to 4.2×10^{-4} M arsanilazo group. In this case a change in the polarity of the circular dichroic spectrum was observed, giving rise to a new trough with a maximum at 465 nm (Figure 5). Thus, while the position of the peak of the positive ellipticity band at 430 nm occurs approximately at the maximum of the corresponding absorption spectrum, the peak of the negative

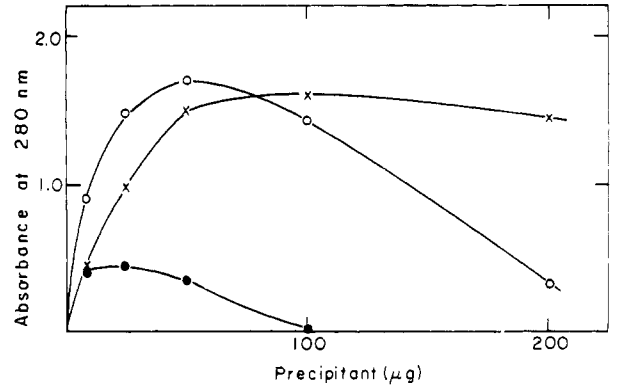

FIGURE 1: The precipitin reaction between anti-Ars-$(TAG)_r$ antiserum (1 ml) and $(TAG)_r$ (●); Ars-$(TAG)_r$, (○); and Ars-RSA, (×).

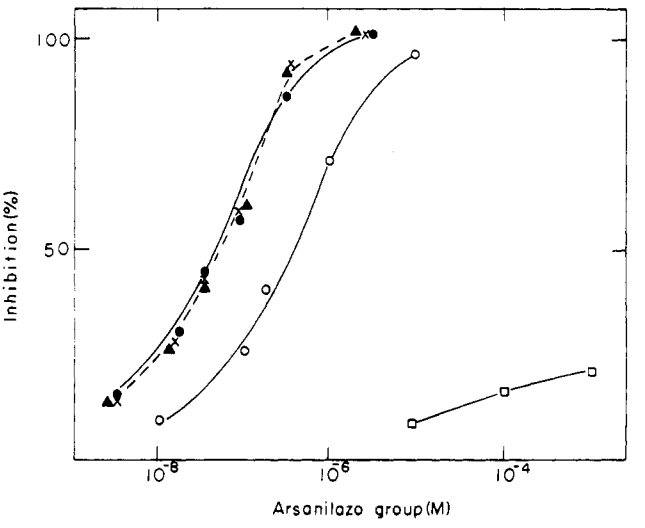

FIGURE 2: Inhibition by various arsanilazo derivatives of the inactivation of Ars-polytyrosyl-bacteriophage T_4 with anti-Ars-$(TAG)_r$ antiserum. The inhibitors are: (●) Ars-RSA; (×) Ars-$(TAG)_r$; (▲) Ars-$(TAG)_n$; (○) *N*-benzyloxycarbonyl(*p*-azobenzenearsonate)-L-tyrosine; (□) arsanilic acid.

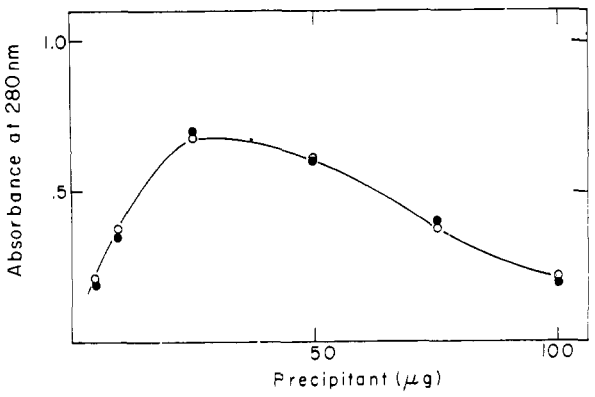

FIGURE 3: The precipitin reaction between anti-$(TAG)_n$ antiserum (0.8 ml) with $(TAG)_n$ (●) and Ars-$(TAG)_n$ (○).

band was shifted toward a longer wavelength. The shape of the resulting curve is similar to that of Ars-$(TAG)_r$ alone or when combined with Fab.

When Ars-$(TAG)_r$ was treated with anti-arsanilazo Fab fragments in a ratio of 11.5×10^{-4} M arsanilazo group to 6×10^{-4} M Fab, there was a threefold increase in the value of the trough of Ars-$(TAG)_r$ at 455 nm (Figure 5). An amplitude larger than the one observed could probably be obtained with a higher ratio of Fab to Ars-$(TAG)_r$. A similar effect was observed when *N*-benzyloxycarbonyl(*p*-azobenzenearsonate)-L-tyrosine was treated with anti-arsanilazo Fab fragments.

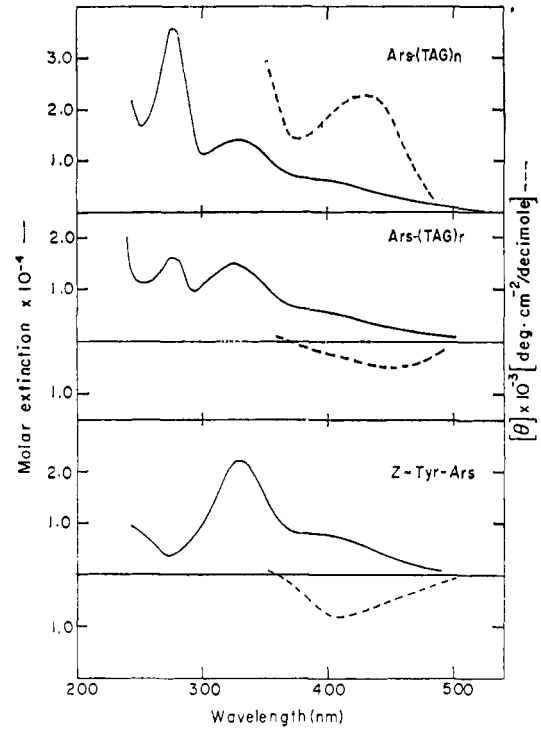

FIGURE 4: Absorption and circular dichroic spectra in the visible region of Ars-$(TAG)_n$, Ars-$(TAG)_r$, and *N*-benzyloxycarbonyl-(*p*-azobenzenearsonate)-L-tyrosine (Z-Tyr-Ars) in 0.15 M sodium chloride–0.02 M phosphate buffer (pH 7.4). Ellipticity is calculated for the molecular residue weight of the arsanilazo group.

Discussion

Diazonium compounds were shown recently to be capable of generating, in some enzymes, extrinsic Cotton effects which may serve as suitable probes of their conformation (Vallee, 1970; Kagan and Vallee, 1969; Fairclough and Vallee, 1969, 1970; W. D. Behnke and B. L. Vallee, in preparation). In the present study, the optical properties of the *p*-azobenzenearsonate chromophore facilitate a better understanding of its structural properties when coupled to two polypeptides, the helical $(TAG)_n$ and the random $(TAG)_r$. In addition, the optical properties of these two arsanilazo derivatives enable us to follow circular dichroic changes of the arsanilazo moiety upon interaction with a specific antibody combining site.

The ellipticity bands of both the arsanilazo compounds arise from arsanilazotyrosyl residues. Nevertheless, the circular dichroic spectrum of Ars-$(TAG)_n$ differs from that of Ars-$(TAG)_r$ (Figure 4). This difference is most likely due to the distinct conformational properties of the two carriers.

The ordered helical copolymer of (L-tyrosyl-L-alanyl-L-glutamyl)$_n$ has been investigated and compared with the random copolymer of L-tyrosine, L-alanine, and L-glutamic acid by Ramachandran *et al.* (Ramachandran, 1967; J. Ramachandran, A. Berger, and E. Katchalski, in preparation). These investigators concluded that under physiological conditions of pH and ionic strength the $(TAG)_n$ exhibits an α-helical conformation which is stabilized by hydrogen bonds of the polypeptide backbone, as well as by side-chain interactions involving hydrogen bonds between carboxylate oxygens and phenolic hydroxyl groups. The $(TAG)_r$ was shown to exist mostly in a disordered form.

The reaction of the diazonium salt derivative of arsanilic acid with the two copolymers under similar conditions led to a more extensive substitution in the case of the random copolymer than in the helical one (Table I). This may reflect the above-mentioned side-chain interactions which decreased the availability of the tyrosine phenolic groups. Introducing an arsanilazo group to a tyrosine residue in $(TAG)_n$ may re-

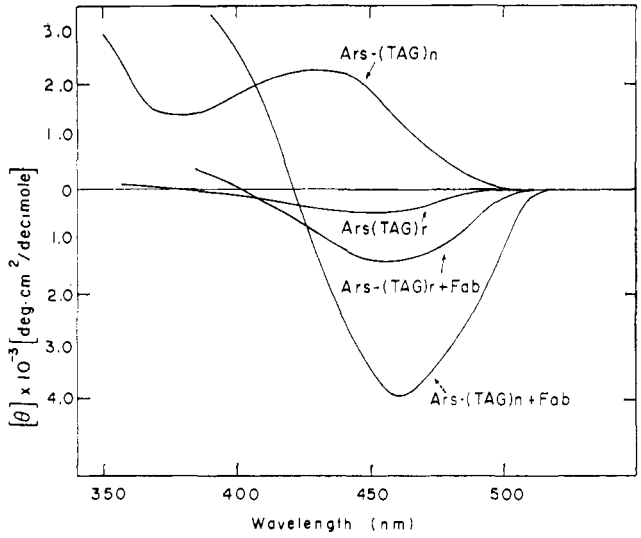

FIGURE 5: Circular dichroic spectra of arsanilazo derivatives in the presence or absence of anti-arsanilazo Fab fragments.

sult in a loss of a side-chain interaction, but the helical structure of $(TAG)_n$ is not grossly disturbed as only one out of 78 amino acids is substituted (Table I). Supporting evidence for the persistence of the helical structure in Ars-$(TAG)_n$ comes from immunological studies, namely that precipitin curves of anti-$(TAG)_n$ antiserum with $(TAG)_n$ and Ars-$(TAG)_n$ are identical (Figure 3).

In the interpretation of circular dichroic spectra due to a chromophore such as the *p*-azobenzenearsonate group used in this study, it is usually assumed that the degree of ellipticity and its sign are determined by the interaction of the chromophore with its environment. The circular dichroic spectra of Ars-$(TAG)_n$ and Ars-$(TAG)_r$ indicate that the arsanilazo groups on the two copolymers exist in different environments. In Ars-$(TAG)_n$, the hapten moieties are bound to an ordered carrier possessing a repeating structure. Each of the arsanilazo groups might interact with the carrier in a manner similar to every other arsanilazo group. Immobilization of the azo chromophore within the periodic structures could give rise to the strong positive ellipticity band at 420 nm. On the other hand, any helical regions still present in the random copolymer could be partially or completely destroyed upon introducing one arsanilazo group into every seventh tyrosine residue. Thus, the arsanilazo groups of Ars-$(TAG)_r$ may exist in a disordered form, similar to that of *N*-benzyloxycarbonyl-(*p*-azobenzenearsonate)-L-tyrosine.

The ellipticity band at 420 nm of the helical Ars-$(TAG)_n$ disappears when the polymer is dissolved in distilled water. This is in agreement with the observation by Ramachandran (1967) that $(TAG)_n$ has, in the neutral pH range, a helical conformation only in the presence of salt, whereas it possesses a random coil conformation in the absence of salt.

The change in the conformation of the arsanilazo group on $(TAG)_n$ due to its interaction with an antibody combining site could be followed by circular dichroic measurements. Monovalent Fab fragments of anti-arsanilazo antibodies were used in these experiments in order to prevent formation of immune precipitates. Ars-$(TAG)_n$ in the presence of anti-arsanilazo Fab exhibited an inversion in the polarity of the positive ellipticity band at 430 nm (Figure 5) A new trough was formed, with a maximum at 465 nm. The shape of the new curve is similar to that of Ars-$(TAG)_r$ alone or in the presence of anti-arsanilazo Fab, indicating that there was a change in the conformation of the arsanilazo hapten due to its interaction with an antibody combining site of related specificity. The direct interaction between the arsanilazo group and the new environment of the combining site may also contribute to the amplitude of this curve.

The arsanilazo moiety in Ars-$(TAG)_r$ is a potent determinant (Figure 1), whereas in Ars-$(TAG)_n$ it is very poor (Table II). The lack of an anti-arsanil response in the latter case is not due to lack of immunogenicity of Ars-$(TAG)_n$ as this substituted helical copolymer provoked antibodies of $(TAG)_n$ specificity (Table II).

Competition between antigenic determinants involving the *p*-azobenzenearsonate hapten has been reported for arsanilated ferritin, where the presence of the arsanilazo group impaired the formation of anti-ferritin antibodies (Adler, 1964). Amkraut *et al.* (1966) found that when a doubly substituted carrier was used for immunization, the dinitrophenyl groups suppressed the antibody response to *p*-azobenzenearsonate. It seems unlikely that antigenic competition could explain the extremely low level of anti-arsanilazo antibodies obtained after immunization with the helical Ars-$(TAG)_n$ as both $(TAG)_n$ and $(TAG)_r$, in spite of their distinct properties, cross-react with each other immunologically (15% cross-reaction; Conway-Jacobs, *et al.*, 1970), and they are closely related chemically. It would have to be assumed that the $(TAG)_n$ carrier competes successfully with the arsanilazo group in Ars-$(TAG)_n$ even though we have shown that $(TAG)_r$ does not thus compete with the same hapten in Ars-$(TAG)_r$.

The most probable reason for the extremely poor antibody response against the arsanilazo hapten in Ars-$(TAG)_n$ seems to be its unavailability to the biosynthetic site, due to the environment in which this hapten finds itself within the helical polymer. This special environment was reflected in the circular dichroic properties of the substituted copolymers, as described above. It is quite possible that this explanation may hold also for another recently reported case of lack of response to a hapten which was attached to an antigen composed of repeating units, namely the picolinimidyl derivative of tobacco mosaic virus (Slobin, 1970).

It is of special interest in this connection that a derivative of carboxypeptidase A in which a *p*-azobenzenearsonate group is attached to the tyrosine residue within the active site of the enzyme led to almost no anti-arsanilazo antibody response, when used for immunization of rabbits (D. Givol, M. Sela, H. M. Kagan, J. F. Riordan, and B. L. Vallee, unpublished data). Kagan and Vallee (1969) have previously shown that the same arsanilazo-carboxypeptidase exhibited a unique circular dichroic spectrum in the visible region, which disappeared upon the denaturation of the enzyme. Thus, both in the case of Ars-$(TAG)_n$ and arsanilazo-carboxypeptidase A there is a correlation between a special environment of the arsanilazo group reflected in circular dichroic properties and a lack of antibody response. The *p*-arsanilazo conjugate of the helical copolymer was not capable of provoking the formation of antibodies of arsanilazo specificity, but was nevertheless able to react with anti-arsanilazo antibodies. The capacity of a molecule to react with the combining site of an antibody is not, therefore, parallel with its ability to elicit the formation of antibodies of similar specificity (Sela *et al.*, 1962).

The fact that heterologous anti-arsanilazo antibodies interact with Ars$(TAG)_n$ (Figure 2) suggests that a change in the conformation of the arsanilazo moiety is induced by such an interaction. This might be considered similar to the conformational change induced by antibodies described by Crumpton and Small (1967) and by Crumpton (1966). In the present case, anti-arsanilazo antibodies may either shift a preformed equilibrium in which a small fraction of the hapten is exposed, or they may "suck out" the buried hapten. This could also be shown for Ars-carboxypeptidase, which although it did not elicit an anti-arsanilazo immune response could, nevertheless, react with anti-Ars-$(TAG)_r$ antibodies when tested by the capacity of the modified enzyme to inhibit the inactivation of arsanilated phage by the antibodies.

In conclusion, the inability to induce more than microgram quantities of antibodies against the arsanilazo group of Ars-$(TAG)_n$ may be related to the unique conformation or environment of this hapten within Ars-$(TAG)_n$. A change in the conformation of such an antigenic determinant may be induced by its interaction with an antibody combining site of the corresponding specificity.

References

Adler, F. L. (1964), *Progr. Allergy 8*, 41.
Amkraut, A. A., Garvey, S. J., and Campbell, D. H. (1966), *J. Exp. Med. 124*, 293.
Becker, M., Conway-Jacobs, A., Wilchek, M., Haimovich, J., and Sela, M. (1970), *Immunochemistry 7*, 741.
Borek, F., and Stupp, Y. (1966), *Immunochemistry 3*, 339.
Conway-Jacobs, A., Schechter, B., and Sela, M. (1970), *Israel J. Med. Sci. 6*, 440.
Crumpton, M. J. (1966), *Biochem. J. 100*, 223.
Crumpton, M. J., and Small, P. A., Jr. (1967), *J. Mol. Biol. 26*, 143.
Djerassi, C. (1960), Optical Rotatory Dispersion, New York, N. Y., McGraw-Hill.
Fairclough, G. F., Jr., and Vallee, B. L. (1969), 158th National Meeting of the American Chemical Society, New York, N. Y., Abstract No. BIOL 248.
Haurowitz, F. (1936), *Hoppe-Seyler's Z. Physiol. Chem. 245*, 23.
Hofsten, B. V., and Falkbring, S. O. (1961), *Anal. Biochem. 1*, 437.
Hooker, S. D., and Boyd, W. C. (1932), *J. Immunol. 23*, 465.
Kagan, H. M., and Vallee, B. L. (1969), *Biochemistry 8*, 4223.
Landsteiner, K. (1945), The Specificity of Serological Reactions, rev ed, Cambridge, Mass., Harvard University.
Leskowitz, S. (1963), *J. Exp. Med. 117*, 909.
Merz, W. (1959), *Mikrochem. Acta 4*, 640.
Ouchterlony, O. (1953), *Acta Pathol. Microbiol. Scand. 32*, 231.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Pressman, D., and Grossberg, A. (1968), The Structural Basis of Antibody Specificity, New York, N. Y., Benjamin.
Ramachandran, J. (1967), *7th Intern. Congr. Biochem. (Tokyo)*, J-144, 982.
Robbins, J. B., Haimovich, J., and Sela, M. (1967), *Immunochemistry 4*, 11.
Schechter, I., Schechter, B., and Sela, M. (1966), *Biochim. Biophys. Acta 127*, 438.
Sela, M., Fuchs, S., and Arnon, R. (1962), *Biochem. J. 85*, 223.
Sela, M., Schechter, B., Schechter, I., and Borek, F. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 537.
Slobin, L. (1970), *Nature (London) 225*, 698.
Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234*, 1726.
Vallee, B. L. (1970), *8th Intern. Congr. Biochem., Interlaken, Switzerland*, 56.

Studies on the Carbohydrate Portion of Membrane-Located Mouse *H-2* Alloantigens*

Takashi Muramatsu and Stanley G. Nathenson

ABSTRACT: Membrane-located *Histocompatibility-2* (*H-2*) alloantigens labeled with radioactive sugars were solubilized by papain digestion and purified from three types of murine tumors: MTC (mastocytoma, $H\text{-}2^d$), Meth-A (fibrosarcoma, $H\text{-}2^d$), and EL-4 (lymphoma, $H\text{-}2^b$). An antibody–antigen binding reaction between the *H-2* alloantigen and alloantibody was utilized as the final purification step. It was established that galactose, mannose, fucose, glucosamine, and sialic acid are integral components of immunologically active *H-2* alloantigen glycoproteins of tumor cells of all *H-2* strains examined. About 4–6% of [^{3}H]fucose and somewhat smaller proportions of the other sugars that were incorporated into crude membranes were present in the papain-solubilized *H-2* alloantigens. Radioactive glycopeptides prepared by pronase digestion of carbohydrate-labeled *H-2* alloantigens from the three tumor cells formed single sharp peaks upon Sephadex G-50 column chromatography and each showed an elution volume corresponding to a molecular weight of 3300. In contrast to the high degree of homogeneity of *H-2* glycopeptides with respect to size, glycopeptides prepared from crude membranes or from cell surfaces formed broad peaks upon Sephadex G-50 column chromatography. Upon DEAE-Sephadex column chromatography, the *H-2* glycopeptides from Meth-A cells ($H\text{-}2^d$) or EL-4 cells ($H\text{-}2^b$) were separated into two major sharp peaks. The chromatographic double-label patterns were almost identical when preparations from each source were mixed before chromatography. Glycopeptides from crude membrane preparations of the two tumor cells formed broad peaks upon DEAE-Sephadex column chromatography.

These results demonstrate the close similarities of the *H-2* glycopeptides from the two tumor cells differing both in cell type and *H-2* specificity profile and the unique properties of *H-2* glycopeptides with respect to size and charge when compared to the heterogeneous array of glycopeptides from the whole membrane of these tumor cells.

The *histocompatibility-2* (*H-2*) alloantigens are the major transplantation antigens of the mouse, and are located on the cell surface membrane. The *H-2* alloantigens have been solubilized from their membrane site by papain digestion and purified by a series of fractionation procedures. The purified materials were found to be glycoproteins containing

* From the Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461. *Received June 22, 1970.* This research was supported in part by Grant AI-07289 from the U. S. Public Health Service and Grant GB-7924 from the National Science Foundation. S. G. N. is a recipient of a research development award from the U. S. Public Health Service (AI-11569).