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Peptide Comparison of Two Histocompatibility-2 (H-2b and H-2d) Alloantigens†

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ABSTRACT: The peptide composition of two H-2 alloantigen glycoproteins (class I) of different antigenic profile was compared by several methods. In one set of studies, nonradioactively labeled class I alloantigen fragments from spleens of *H-2*^{b} and *H-2*^{d} strain animals were cleaved by CNBr and the resultant peptides compared by ion-exchange chromatography. Approximately 90% of the peptides were similar, and 10% were different. In another set of studies, peptides radiolabeled with either [^{3}H]- or [^{14}C]amino acids were prepared by CNBr cleavage and trypsin digestion of H-2b and H-2d alloantigens which had been purified by a combination of conventional methods as well as immunological techniques. Comparison of the double-label peptide patterns showed a sharing of about 80–90% of the peaks and differences in about 10–20%. Such findings confirm and extend previous data using different techniques and sources of antigen, and support the hypothesis that the H-2 antigenic sites are determined by primary amino acid structure. The great similarity of most of the peptides stresses the anticipated similarity to be expected from products of allelic forms of the same genetic region.

The murine H-2 histocompatibility transplantation alloantigens comprise a complex system of immunologically identifiable glycoproteins which are integrated into the cellular membrane. These antigens are controlled by a genetic region on the IXth mouse linkage group—a region which is exemplified by its extreme degree of polymorphism (Klein and Shreffler, 1971; Snell and Stimpfling, 1966).

Considerable information about the antigens has been obtained through the study of immunologically active glycoprotein fragments released from their membrane location by papain proteolysis. Two classes (class I and class II) of glycoprotein fragments (85% protein and 70% carbohydrate) carrying H-2 antigenic sites were isolated from each of two mouse strains (*H-2*^{b} and *H-2*^{d}), differing in their known H-2 specificity profile (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a,b). The glycoprotein fragments of each class appeared to be the gene product of one of the two postu-

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lated genes comprising the *H-2* complex (Snell *et al.*, 1971; Shreffler *et al.*, 1971).

Present evidence suggests that the H-2 glycoproteins express their antigenic sites in their primary amino acid structure. Some of the evidence for this concept comes from studies showing: (1) that removal of nearly 40% of the sugar residues result in no loss of antigenic reactivity (Nathenson and Muramatsu, 1972); and (2) that thin-layer peptide maps of class I H-2 glycoproteins of *H-2* different mice show similarity of 90% of the peptides and a difference in 10% (Shimada *et al.*, 1970).

The present paper presents further data on the peptide structure of the H-2b and H-2d class I glycoproteins by utilizing conventional and radiolabel techniques to compare peptides prepared by either CNBr cleavage alone or CNBr cleavage plus trypsin digestion.

Materials and Methods

Mice and Tumor Cells. C57BL/6 (*H-2*^{b}), DBA/2, and BALB/c (*H-2*^{d}) inbred mice were purchased from Jackson Laboratories, Bar Harbor, Maine, or obtained from the breeding colonies of Dr. Frank Lilly (Albert Einstein College of Medicine, Bronx, N. Y.). EL-4 tumor cells (*H-2*^{b}), a chemically induced lymphoma, were maintained in C57BL/6 mice and Meth-A tumor cells (*H-2*^{d}), a chemically induced fibrosarcoma, were maintained in BALB/c mice.

Preparation and Properties of Unlabeled H-2b and H-2d Alloantigens (Class I) from C57BL/6 and DBA/2 Mouse Spleens. Papain-solubilized H-2 alloantigens were purified from crude cell membranes of mouse spleens by $(NH_4)_2SO_4$ fractionation, Sephadex G-150, CM-Sephadex C-50, and DEAE-Sephadex A-25 column chromatography, and disc gel electrophoresis as previously shown (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a). The purified materials (class I) showed a single band on disc gel electrophoresis at pH 9.3 and 4.3. The H-2b glycoproteins were highly reactive for their characteristic specificities 5 and 33, and the H-2d glycoproteins were highly reactive for their characteristic specificities 4 and 3,42.

Preparation of Radiolabeled H-2 Alloantigens (Class I) from EL-4 (H-2^b*) and Meth-A (H-2*^{d}*) Tumor Cells.* PROTEIN LABELED H-2 ANTIGENS. Tumor cell suspensions were harvested from peritoneal cavities of mice, and, after removing the majority of contaminating red cells by differential centrifugation, were incubated for 16 hr at 37° (1×10^6 cells/ml) in culture medium (MEM minimum essential medium with 10% fetal calf serum but without arginine, leucine, and valine) containing [^{3}H]arginine (703 mCi/mmole), [^{3}H]leucine (58.2 Ci/mmole), and [^{3}H]valine (2.34 Ci/mmole) or [^{14}C]arginine (255 mCi/mmole), [^{14}C]leucine (240 mCi/mmole), and [^{14}C]-valine (312 mCi/mmole) (New England Nuclear Corp.). The radiolabeled H-2 alloantigens were solubilized by limited papain digestion from the crude cell membranes, and purified by Sephadex G-150 and DEAE-Sephadex A-25 column chromatography as described previously (Yamane and Nathenson, 1970a).

The fractions from the DEAE-Sephadex A-25 chromatographic runs containing the H-2 alloantigens were then pooled and further purified by immunological means to assure complete homogeneity with respect to their H-2 antigenic properties with an *H-2*^{d} anti-*H-2*^{b} antiserum for the H-2b alloantigens from EL-4 cells (BALB/c anti-EL-4 serum detecting H-2 specificities 2,5,22,33) and an *H-2*^{b} anti-*H-2*^{d} antiserum for the H-2d alloantigens from Meth-A cells (C57BL/6 anti-Meth-A serum detecting H-2 specificities 3,4,8,10,13,31). The resultant antigen–antibody complexes were separated from nonreacted materials by Sephadex G-150 column chromatography (Cullen and Nathenson, 1971). The antigen–antibody complex region from the column was pooled, dialyzed against distilled water, and lyophilized. This antigen–antibody complex was then treated with CNBr and trypsin without separation of the radiolabeled antigen from the unlabeled antibody.

Radioactivity was proven to be in arginine, leucine, and valine residues by amino acid analysis. ^{3}H- and ^{14}C-labeled amino acids were equally well incorporated into the H-2 alloantigens. The ratio of the radioactivity of arginine and valine with respect to leucine was 0.7:0.7:1.0 in the H-2d alloantigen preparation from Meth-A cells, and 0.25:0.8:1.0 in the H-2b alloantigen from EL-4 cells.

CARBOHYDRATE LABELED H-2 ALLOANTIGENS. An EL-4 or Meth-A cell suspension was incubated for 16 hr at 37° (1×10^6 cells/ml) in a culture medium containing 25 μCi/ml of [^{14}C]glucosamine (52 mCi/mmole). The carbohydrate-labeled H-2 alloantigenic fragments were solubilized by limited papain digestion, purified by Sephadex G-150 column chromatography, and finally obtained as antigen–antibody complexed materials (Muramatsu and Nathenson, 1970a). Approximately 20% of the radioactivity was in sialic acid, and 80% in glucosamine (Muramatsu and Nathenson, 1970b).

Cyanogen Bromide Cleavage, Trypsin Digestion, and Peptide Column Chromatography. The cyanogen bromide cleavage was carried out as described previously (Yamane and Nathenson, 1970b). The trypsin digestion was performed as follows. Lyophilized samples of CNBr cleaved antigen were dissolved in about 100 μl of 0.1 M NH_4HCO_3 buffer (pH 8.5) and TPCK-trypsin (Worthington Biochemical Co.) was added. The ratio of sample to trypsin (as protein) was 20:1. After digestion at 37° for 18 hr, the reaction mixture was lyophilized.

Peptide column chromatography was conducted under the conditions previously published (Yamane and Nathenson, 1970b), using the general methods described by Hirs (1967) and Hill and Delany (1967), except that when radiolabeled materials were analyzed, the effluent in each tube was dried, and the residue dissolved in 1.0 ml of 0.1 N NaOH solution which was transferred to 15 ml of Triton X-100 Omnifluor (New England Nuclear Corp.) solution and counted in a Beckman LS-233 liquid scintillation counter adjusted for double label counting.

Neuraminidase Digestion. [^{14}C]Glucosamine labeled H-2 alloantigen and neuraminidase (50 units/10^3 cpm of antigen) were reacted in a small volume (100 μl) of 0.05 M Tris-HCl (pH 7.0) containing 0.01 M $CaCl_2$. After the incubation at 37° overnight, the samples were lyophilized.

Chemicals. Papain (twice crystallized) from Sigma Chemical Co., TPCK-treated trypsin (trypsin-TPCK) from Worthington Biochemical Co., CNBr from J. T. Baker Chemical, Inc., Sephadex and DEAE-Sephadex from Pharmacia Chemical, Inc., were used. All other chemicals were of reagent grade. Neuraminidase from *Vibrio cholerae* was purchased from General Biochemicals, Chagrin Falls, Ohio.

Results

Analysis of CNBr-Cleaved Peptides from Antigens Isolated from Mouse Spleens. The peptides produced by reduction and alkylation of H-2 glycoproteins followed by CNBr cleavage were separated by column chromatography on PA-35 resin and detected by ninhydrin. Reproducible small differences were found between the elution profiles of the peptides pre-

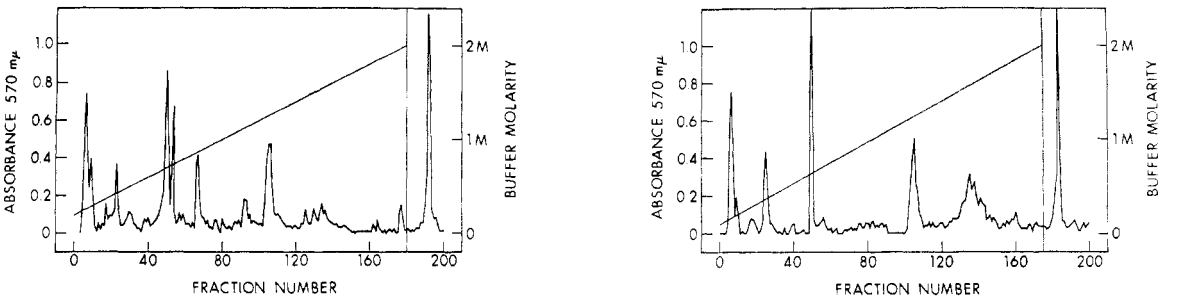

FIGURE 1: PA-35 resin peptide column chromatography of CNBr-cleaved peptides of H-2b alloantigen (class I) from C57BL/6 mouse spleens (a, left) and of H-2d alloantigen (class I) from DBA/2 mouse spleen (b, right). The H-2b (400 μg) and H-2d (420 μg) alloantigens were reduced, alkylated, and treated with CNBr and then the samples were applied to the PA-35 column. The peptide mixture was eluted by the procedures outlined in Materials and Methods and each peptide peak was detected by ninhydrin method. Recovery of ninhydrin-positive material eluted with respect to material applied to column averaged 70–80%.

pared from H-2b alloantigen (class I) from C57BL/6 mouse spleens and the H-2d alloantigen (class I) from DBA/2 mouse spleens (Figure 1a and -b). About seven to nine major ninhydrin-positive peaks were detected in both sources; two of the peptide peaks, those eluting at tubes 52 and 67 were specific for the H-2b alloantigen, while a cluster of peptide peaks eluting at tubes 135–140 was specific for the H-2d alloantigen. The other peaks were identical in position for both antigen preparations. It is probable that not all the ninhydrin positive peaks represent homogeneous peptides, especially those eluting at tubes 60 and 180 which could represent aggregated peptides, possibilities pointed out elsewhere (Adelstein and Kuehl, 1970). The chromatographic profiles of the peptide peaks, nonetheless, were consistently reproducible in triplicate runs. The finding of about 7–9 peptides is consistent with the 8–10 peptides theoretically predictable from the methionine

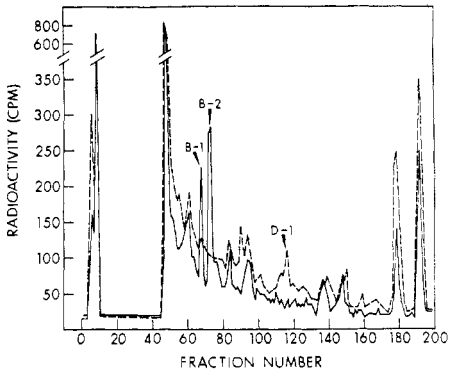

FIGURE 2: Double-label PA-35 peptide column chromatographic comparison of peptides from CNBr-cleaved and trypsin-digested ^{14}C-labeled H-2b-alloantigen (class I) from EL-4 (*H-2^b*) and ^{3}H-labeled H-2d alloantigen (class I) from Meth-A (*H-2^d*) tumor cells. Radioactive antigen–antibody purified H-2b antigen (20,000 cpm) labeled with the three [^{14}C]amino acids: [^{14}C]arginine, [^{14}C]-luecine, and [^{14}C]valine, and H-2d antigen (20,000 cpm) labeled with the three [^{3}H]amino acids: [^{3}H]arginine, [^{3}H]leucine, and [^{3}H]-valine were combined, and the mixture reduced, alkylated, treated by CNBr, and digested by TPCK-trypsin. Then the sample was applied to the PA-35 resin column which was eluted by the procedures outlined in Materials and Methods. The effluent samples were counted with a scintillation counter. (—) ^{14}C radioactivity in H-2b alloantigen from EL-4 cells; (– –) ^{3}H radioactivity in H-2d alloantigen from Meth-A cells. B-1 and B-2 peaks were specific for the H-2b alloantigen and the D-1 peak was specific for the H-2d alloantigen. About 75–80% of the ^{14}C-labeled material and 60% of the ^{3}H-labeled material which was applied to the column was recovered.

content of these glycoproteins (2 mole %), and the molecular weight of the polypeptide portion of about 50,000 (Shimada and Nathenson, 1969).

A previously published comparison (Yamane and Nathenson, 1970b) of H-2d class I glycoproteins isolated from spleen cells or from Meth-A tumor cells showed almost complete similarity in chromatographic profile of CNBr cleaved peptides of these two *H-2^d* sources except for one peptide peak from the tumor antigen (tube 25) which eluted slightly later than its corresponding peak from the normal antigen.

Analyses of ^{3}H and ^{14}C Peptides Produced by CNBr Cleavage and TPCK-Trypsin Digestion. Alloantigens radiolabeled in their peptide moieties were chosen for further study because the antigens could be purified using a final antibody–antigen complex formation step and, further, could be examined by the comparative double-label technique. The radiolabeled antigen was isolated from tumor cells of specific *H-2* genotype since such cells could be highly labeled in tissue culture.

Alloantigens of both *H-2^d* and *H-2^b* genotype was prepared, radiolabeled with either [^{14}C]- or [^{3}H]amino acids using three essential amino acids, leucine, valine, and arginine. The elution profiles for the peptides produced by a combination of CNBr cleavage and trypsin digestion of the H-2 alloantigens differing in genotype and type of label (either ^{14}C or ^{3}H) are shown in Figures 2 and 3 and a control experiment comparing peptides from alloantigen of the same source but differing only in the label used, *e.g.*, ^{14}C or ^{3}H, is shown in Figure 4.

The double-label comparative analysis of the peptides from the H-2b and H-2d antigens showed from 12 to 14 major peaks. At least several contained greater than 1 peptide, for example, the peak around tube 50 which contained about 12–15% of the radioactivity recovered from the column. Of the peptide peaks which were resolved by the chromatographic procedure, about 11 were identical between the 2 strains examined. However, the presence of two peptides at least quantitatively specific for the H-2b alloantigen and one peptide specific for the H-2d alloantigen is clearly demonstrated in Figures 2 and 3. The control experiment (Figure 4) showed the presence of the H-2d specific peak at tube 121 for both ^{3}H- and ^{14}C-labeled peptides, as well as similarity in the position of all other major peaks.

Detection of the Carbohydrate-Containing Glycopeptides Produced by CNBr Cleavage and Trypsin Digestion. H-2 alloantigens prepared by papain solubilization are glycoproteins of about 85% protein and 10% carbohydrate. The carbohydrate consists of sialic acid, glucosamine, galactose, mannose, and fucose. These monosaccharides are combined in carbo-

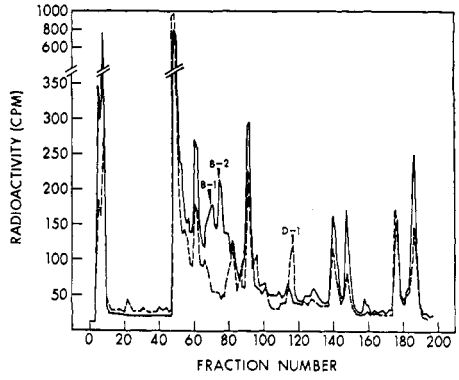

FIGURE 3: Double-label PA-35 peptide column chromatographic comparison of peptides from CNBr-cleaved and trypsin-digested alloantigen (class I) from EL-4 (*H-2*^{b}) tumor cells and ^{14}C-labeled alloantigen (class I) from Meth-A (*H-2*^{d}) tumor cells (converse experiment of Figure 2). Radioactive antigen–antibody-purified H-2b alloantigen (20,000 cpm, labeled with the three [^{3}H]-amino acids) and of H-2d alloantigen (20,000 cpm, labeled with the three [^{14}C]amino acids) were treated and chromatographed as shown in Figure 2. (—) ^{3}H radioactivity in H-2b alloantigen; (– –) ^{14}C radioactivity in H-2d alloantigen. B-1, B-2 peaks were specific for the H-2b alloantigen and the D-1 peak was specific for H-2d alloantigen.

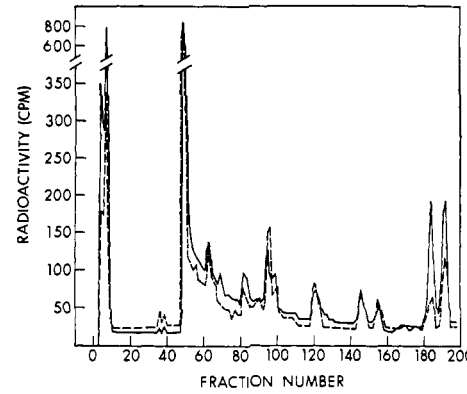

FIGURE 4: Double-label PA-35 resin peptide column chromatographic comparison of peptides from CNBr cleaved and trypsin-digested H-2d alloantigen (class I) from Meth-A tumor cells labeled either with the [^{3}H]amino acid or the [^{14}C]amino acid mixture (control for experiments shown in Figures 2 and 3). The radioactive antigen–antibody-purified H-2d antigen (20,000 cpm for both preparations labeled with the three [^{3}H]amino acids or with the three [^{14}C]amino acids) was treated and chromatographed as shown in Figure 2. (– – –) ^{3}H radioactivity; (—) ^{14}C radioactivity.

hydrate chains of about 3300 molecular weight (including one or more amino acid residues) which appear to be almost identical or at least very similar whether isolated from H-2b or H-2d alloantigens (Muramatsu and Nathenson, 1970b).

To examine the glycopeptides of the alloantigen prepared from *H-2*^{d} tumor cells we mixed [^{14}C]glucosamine (and [^{14}C]-sialic acid) labeled, and [^{3}H]amino acid labeled antigen together, prepared peptides by CNBr and trypsin treatment, and then chromatographed the mixture on the PA-35 resin column. The resulting chromatographic pattern, which is not shown, revealed that only two of the peptides carried the carbohydrate label, and these peptides eluted as the first two peaks from the column. These glycopeptides, further, were common to both H-2b and H-2d antigen. The amount of carbohydrate radiolabel was about equal for these two peaks; however, if the [^{14}C]sialic acid was removed prior to chromatography, the ratio of the first peak to the second peak changed from 1:1 to 1:2.5. This suggests that the difference in the two glycopeptides may be due to sialic acid content.

Discussion

The work described in the present paper continues research on the peptide structure of histocompatibility alloantigens in an attempt to relate the chemical structure to the immunological properties. In studies on alloantigens isolated from mouse splenic tissue, we show that of the resolved peptides formed from cyanogen bromide cleavage, most are shared between the class I alloantigens from the *H-2*^{b} and *H-2*^{d} strains, but at least one or two different peptides are present.

Due to the very small amounts of materials available from mouse spleens, we developed techniques of greater sensitivity and specificity. We utilized radiolabel tracer techniques which not only enhance greatly the sensitivity of detection methods, but also permit the labeling of one protein with [^{14}C]amino acids and of the other protein with [^{3}H]amino acids in order to directly compare the peptide patterns in the same chromatographic run. An additional refinement of our purification procedure included an antibody purification step which assured immunological homogeneity of the final product.

These methods when applied to radiolabeled antigen isolated from the *H-2*^{d} and *H-2*^{b} cell sources showed that about 12–14 radioactive peaks were separated, although several of these peaks obviously contained more than one peptide. Of the resolved peptide peaks approximately one peptide from the H-2d alloantigen and two peptides from the H-2b alloantigen appeared specific (at least quantitatively) to that strain of antigen while the other peptides appeared identical for the two glycoproteins.

The results of these studies confirm our previous studies (Shimada *et al.*, 1970). They are consistent with the serologically detectable antigenic differences, and offer indirect support that the peptide differences reflect antigenic site differences. The observation of 80–90% identity in the peptides from these two purified alloantigen preparations further stresses their similarity—a similarity which would be anticipated since they are products of allelic forms of the same genetic region.

In a further experiment we determined the glycopeptides of the peptide digest. Two glycopeptides were separated from our preparations which eluted in the first two peaks from the column. The positions were identical for both *H-2*^{d} and *H-2*^{b} strains. These facts suggest that the different peptides of each of the two strains are not the result of the differential association of carbohydrate to a peptide shared by both, but are in fact different due to their intrinsic amino acid composition.

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A Proton Magnetic Resonance Study of the Aggregation of Actinomycin D in D_2O†

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ABSTRACT: A detailed 220-MHz proton magnetic resonance study has been made of the concentration, pD, salt, and temperature dependence of the actinomycin D spectrum in D_2O. The results confirm that actinomycin D aggregates to form a dimer at the concentration ranges and temperatures covered in this work. Moreover, the measurements show conclusively that the dimer is formed by an interaction between the actinocyl chromophore groups only. Based upon the direction and relative magnitudes of the shift trends for the actinocyl group protons it is further concluded that the actinocyl groups stack vertically in the dimer with one chromophore inverted with respect to the other. An interpretation of the dimer structure and the resultant shift trends is given in terms of the diamagnetic shielding anisotropy of the actinocyl chromophore group. The chemical shift–concentration curves for the actinocyl signals have been analyzed by a least-squares fitting procedure, to obtain dimerization equilibrium constants of 2.70×10^3 M^{-1} and 1.40×10^3 M^{-1} at 4° and 18° (pD = 7.2), respectively. Finally, the nuclear magnetic resonance (nmr) measurements also show that the structure and stability of the dimer are altered by temperature and by solvent properties (pD, ionic strength).

Actinomycin D, an important inhibitor of mRNA synthesis (Reich and Goldberg, 1964), forms relatively stable aggregates in aqueous solution. Initial discrepancies regarding the stoichiometry of the aggregates (Gellert *et al.*, 1965; Müller and Emme, 1965; Berg, 1965) have been resolved recently by careful equilibrium centrifugation measurements which show conclusively that the dimer form predominates at concentrations $>10^{-4}$ M with no detectable formation of higher aggregates, even at concentrations approaching saturation (Crothers *et al.*, 1968). Although the existence of an actinomycin D dimer is, therefore, well established, the orientation of the two actinomycin D molecules in the dimer is still not clear. It has been suggested, based upon optical (Crothers *et al.*, 1968) and hydrodynamic work (Müller and Emme, 1965), that dimer formation results from interaction between the pentapeptide rings; but the possibility of an involvement of the actinocyl chromophore group cannot be ruled out entirely.

Actinomycin D is in many respects an ideal model compound for studying the types of inter- and intramolecular processes likely to occur in much larger biological molecules. Any additional information relating to the conformation of the dimer and the influence of extrinsic factors such as temperature and pH upon the dimerization process would be of considerable interest. With these points in mind we have reinvestigated the aggregation of actinomycin D in aqueous solution using the proton magnetic resonance (pmr) method.

The pmr method has been widely used in studies of solute–solvent and solute–solute interactions of relatively small molecules, *e.g.*, H bonding, π complexing, donor–acceptor interaction (Jackman and Sternhell, 1969). In favorable cases equilibrium parameters can be determined directly from an analysis of chemical shift–concentration curves. Such an approach can be extended in principle to much larger biological molecules, but in practice it is limited by the complexity and extensive overlapping of signals in proton spectra for these molecules.

Several factors, however, favor the use of pmr measurements for studying the aggregation of actinomycin D. Firstly, a detailed assignment of the proton spectrum has been reported at 60 MHz (Victor *et al.*, 1969) and 100 MHz (Arison and Hoogsteen, 1970) in several nonaqueous solvents and a partial assignment has been made in D_2O. Secondly, the likelihood that actinomycin D aggregates in one of two widely different structural orientations permits a general prediction of expected shift changes. For example, if the dimer is formed by intermolecular interaction between the pentapeptide rings then dimerization-induced chemical shift changes would be expected for key groups (*i.e.*, CH_3, methylene) on these rings but no changes would occur for actinocyl group protons. If

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