The Circular Dichroism Spectra of the Complexes of 1-Anilino-8-naphthalenesulfonate with Bovine Serum Albumin. Evidence for Heterogeneity of Binding*

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ABSTRACT: The adsorption of 1-anilino-8-naphthalenesulfonate by bovine serum albumin induces asymmetry in the ligand molecule. The sign and magnitude of the resulting circular dichroic bands vary with the average degree of saturation of the protein with ligand. Matrix rank analysis of the data demonstrates two spectrally distinguishable components within the set of five primary binding sites. The accurate differential measurements obtained in circular dichroism reflect heterogeneity undetectable in direct determinations of protein–ligand equilibria.

In neutral solutions the bovine serum albumin molecule has five ligand binding sites capable of enhancing the fluorescence of 1-anilino-8-naphthalenesulfonate (Weber and Laurence, 1954; Weber and Young, 1964). These sites have either identical or closely overlapping affinities for the dye since the order of the binding reaction, j, is close to one (Daniel and Weber, 1966). Daniel and Weber could not distinguish between these possibilities since an uncertainty of a few per cent in j prevents resolution of independent binding processes (Weber, 1965).

Observation of the dependence of some distinctive property, such as the circular dichroism spectrum of the bound dye, on the average degree of saturation of the protein with ligand may reveal heterogeneity undetected in the equilibrium determinations. The free 1-anilino-8-naphthalenesulfonate molecule is symmetric and therefore has no optically active absorption bands. Binding, however, induces asymmetry.

The circular dichroism spectra of the complexes of 1-anilino-8-naphthalenesulfonate with bovine serum albumin are important because they reflect heterogeneity in the adsorbing population. I shall discuss these spectra and present an analysis of the heterogeneity required to explain the results.

Materials and Methods

Crystalline bovine plasma albumin was purchased from Armour Pharmaceutical Co. The magnesium salt of 1-anilino-8-naphthalenesulfonate acid was prepared by Weber and Young (1964). All other chemicals were reagent grade.

The circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. The scan speeds used were 1.8 and 3.6 nm per min. The direct contribution of the protein was eliminated by measurement of the difference between the circular dichroism spectra of the albumin–dye mixture and the equivalent solution of albumin alone. This correction is significant at wavelengths below 320 nm. The total optical density of the sample was generally less than one. The concentration of bound dye determined by equilibrium dialysis was used to calculate $(\epsilon_l - \epsilon_r)$ (Pasby and Weber, 1970).

Absorption spectra were determined on the Cary 15 spectrophotometer.

Results

Binding at pH 7. At pH 7, the circular dichroism spectra of the complexes of bovine serum albumin with 1-anilino-8-naphthalenesulfonate vary with the average number of ligand molecules bound (Figure 1). When $\bar{n}$[1] is small, the circular dichroism spectrum contains two bands of opposite sign corresponding to the two bands composing the 350-nm absorption peak (Anderson and Weber, 1969). The circular dichroic band at 337 nm increases monotonically with $\bar{n}$ while the 380-nm band undergoes a smaller biphasic change. Thus the circular dichroism spectrum contains overlapping positive bands between 310 and 420 nm when $\bar{n}$ equals 5. Repeated experiments confirm the absence of common isodichroic points. The strong negative band at 277 nm does not vary significantly with $\bar{n}$ ($\pm 10\%$).

Two unrelated phenomena could explain a change in circular dichroism with $\bar{n}$. The first, dipole–dipole interaction between dye molecules, is excluded by several observations. The bound 1-anilino-8-naphthalenesulfonate molecules are preferentially oriented in planes perpendicular to the long axis of the hydrodynamic ellipsoid (Anderson and Weber, 1969). Interaction would result in the splitting of each circular dichroic band into two bands of opposite sign (Tinoco *et al.*, 1963; Tinoco, 1964). Thus the change from two bands of opposite sign to a set of positive bands is not explained by coupling. The average distance between bound dye molecules is 21 Å (Weber and Daniel, 1966; Anderson and Weber, 1969). In the exciton model, the rate of transfer is inversely proportional to the cube of the distance between interacting centers. Thus intermolecular interaction at this distance is unlikely to affect the absorption properties of the bound dye (Forster,

* From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon. *Received July 29, 1969.* Supported by a grant from the Oregon Heart Association.

[1] Abbreviation used in this work is: $\bar{n}$, the average number of moles of ligand bound per mole of protein.

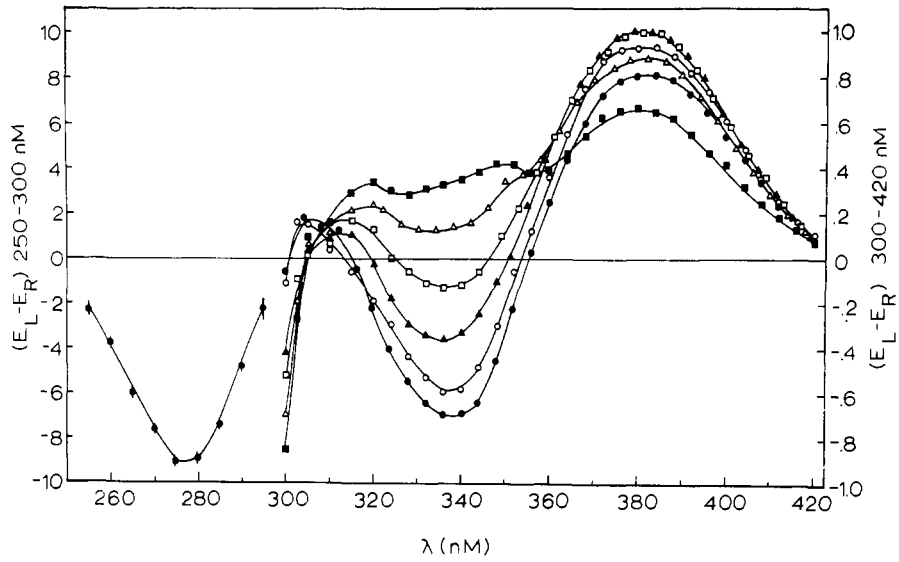

FIGURE 1: The circular dichroism spectra of the complexes of bovine serum albumin with 1-anilino-8-naphthalenesulfonate. Conditions: 0.1 M phosphate (pH 7.0), 25°. (●) $n = 0.5$ (1.5×10^{-4} M dye + 3×10^{-4} M albumin); (⊙) $n = 1$ (1.5×10^{-4} M dye + 1.5×10^{-4} M albumin); (▲) $n = 2$ (3×10^{-4} M dye + 1.5×10^{-4} M albumin); (⊡) $n = 3$ (3×10^{-4} M dye + 10^{-4} M albumin); (△) $n = 4$ (1.5×10^{-3} M dye + 3.8×10^{-4} M albumin); (■) $\bar{n} = 5$ (1.5×10^{-3} M dye + 3×10^{-4} M albumin). The band at 277 nm is corrected for the circular dichroism of the protein and is independent of $\bar{n}$, $(\epsilon_l - \epsilon_r)$ has units of $cm^{-1}\,M^{-1}$, and an uncertainty of ±0.03.

1960). The relative constancy of the absorption and fluorescence spectra at values of $\bar{n}$ ranging from 1 to 5 supports this conclusion (Daniel and Weber, 1966; Anderson and Weber, 1969; Pasby and Weber, 1970).

Matrix Rank Analysis

The alternative explanation is heterogeneity of the binding sites. In order to determine the minimum number of independent spectral components, I have applied the technique of matrix rank analysis (Wallace and Katz, 1964; McMullen *et al.*, 1967). The original 6 × 7 matrix, in which each column represents a different wavelength and each row represents a different value of $\bar{n}$, is given in Table I. I selected the spectrum corresponding to $\bar{n}$ equals 2 as reference (top row). Throughout the reduction, I pivoted the matrix in order to place the largest elements on the diagonal. The resulting reduced matrix (Table I) contains three nonzero rows. Thus the data reflect primarily three spectral components. The estimated error of the individual elements is ±0.03.

Can the existence of three or more distinguishable components be reconciled with current information on the binding of 1-anilino-8-naphthalenesulfonate by albumin? Except for an anomalous inflection at $\bar{n}$ equals one, the binding equilibrium at pH 7 is apparently simple (Daniel and Weber, 1966). The range of j and its standard deviation indicate that the intrinsic dissociation constants differ by less than a factor of five (Weber, 1965). The anomaly makes no obvious contribution to the gradual changes in circular dichroism.

Equilibrium dialysis experiments demonstrate that albumin has a group of 15 or more sites giving slight enhancement of the dye fluorescence (S. R. Anderson, unpublished work, 1966; Pasby and Weber, 1970). The affinity of these sites for the dye is more than two orders of magnitude below that of the first five. The circular dichroism spectra obtained at values of $\bar{n}$ beyond 5 show further changes (Figure 2). However, binding at these secondary sites cannot account entirely for the changes in circular dichroism shown in Figure 1. I know that the fraction of bound nonfluorescent dye present does not change appreciably through $\bar{n}$ equals 3 because the fluorescence yield and lifetime are constant (Daniel and Weber, 1966; Anderson and Weber, 1969; Pasby and Weber, 1970). Overlap occurring when $\bar{n}$ is in the range of 4 to 5 probably accounts for the 16% decrease in average fluorescence yield and for the third nonzero row of the reduced matrix.

The set of five primary binding sites contains two spectrally distinguishable components. Determination of the fraction of each present is impossible without identification of the individual spectra. In the following calculations, designed

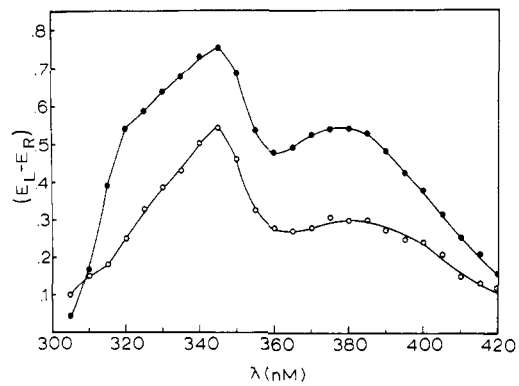

FIGURE 2: The circular dichroism spectra of the complexes of bovine serum albumin with 1-anilino-8-naphthalenesulfonate containing an average of more than 5 moles of ligand bound per mole of protein. Conditions: 0.10 M phosphate (pH 7), 25°. (●) $\bar{n} = 9$ (1.8×10^{-3} M dye + 1.5×10^{-4} M albumin); (⊙) $\bar{n} = 13$ (3×10^{-3} M dye + 1.5×10^{-4} M albumin).

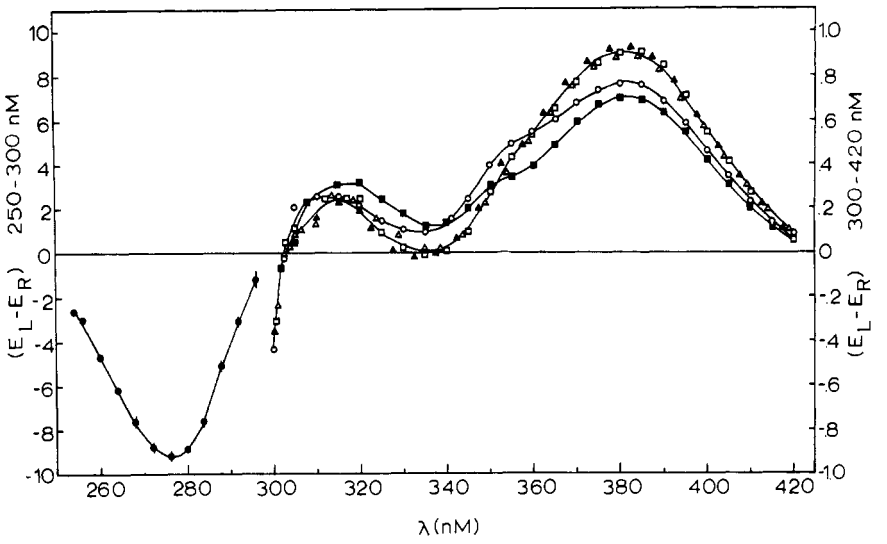

FIGURE 3: The circular dichroism spectra of the complexes of bovine serum albumin with 1-anilino-8-naphthalenesulfonate. Conditions: 0.1 M acetate (pH 5), 25°. Other information is given under Figure 1.

TABLE I: Matrix Rank Analysis of Spectra Given in Figure 1.

Average Number of Moles of Ligand Bound per Mole of Protein ($\bar{n}$)	n^a	277	335	380	320	365	395	350
		A. Original Matrix Wavelength (nm)						
	2	−9.1	−0.36	1.0	−0.01	0.69	0.79	−0.03
	5	−9.1	0.32	0.66	0.34	0.48	0.49	0.42
	0.5	−9.1	−0.69	0.81	−0.23	0.48	0.66	−0.33
	1	−9.1	−0.57	0.93	−0.19	0.59	0.74	−0.21
	4	−9.1	0.13	0.88	0.23	0.68	0.69	0.33
	3	−9.1	−0.13	1.0	0.12	0.70	0.79	0.13
		B. Reduced Matrix Wavelength (nm)						
	n^a	277	335	380	320	365	395	350
	2	−9.1	−0.36	1.0	−0.01	0.69	0.79	−0.03
	5	−0	0.68	−0.34	0.35	−0.21	−0.30	0.45
	0.5	0	0	−0.36	−0.05	−0.31	−0.28	−0.08
	1	0	0	0	−0.05	0	0	0
	4	0	0	0	0	0.04	0	0
	3	0	0	0	0	0	0	0

to depict the range of dissociation constants compatible with the results, I assume that I have an equimolar mixture.

The circular dichroism of a mixture of independent components, $\overline{(\epsilon_l - \epsilon_r)}$, is given by the following equation

$$\overline{(\epsilon_l - \epsilon_r)} = \frac{\sum_{i=1}^{N} f_i \phi_i (\epsilon_l - \epsilon_r)_i}{\sum_{i=1}^{N} f_i \phi_i}$$

where f_i = the fraction of component i and ϕ_i = the average degree of saturation of i with ligand. ϕ_i is related to the independent dissociation constant, K_i, and to the free ligand concentration, [X], by

$$\phi_i = 1/\left(1 + \frac{K_i}{[X]}\right)$$

The sum $\sum_{i=1}^{N} f_i \phi_i$ equals $\bar{\phi}$, the observed average degree of saturation of the protein with ligand. In this case, $\bar{\phi} = \bar{n}/5$.

I calculated the individual values of $(\epsilon_l - \epsilon_r)$ consistent with the changes observed at 337 nm in the range of $\bar{n}$ equals 0.5 to 3 using the assumption that I have an equimolar

TABLE II: Some Values of m, $(\epsilon_l - \epsilon_r)_1$, and $(\epsilon_l - \epsilon_r)_2$ Consistent with the Changes at 337 nm Shown in Figure 1.[a]

m	$(\epsilon_l - \epsilon_r)_1$ cm^{-1} M^{-1}	$(\epsilon_l - \epsilon_r)_2$ cm^{-1} M^{-1}	j
1.1	23.1	−22.5	0.9997
1.2	12.2	−11.6	0.9987
1.5	5.7	−5.1	0.993
2.0	3.5	−2.9	0.980
3.0	2.5	−1.9	0.953
5.0	2.0	−1.3	0.906

[a] These values were calculated for a model consisting of two independent components; $m = K_1/K_2$, the ratio of the two independent dissociation constants; $(\epsilon_l - \epsilon_r)_1$ and $(\epsilon_l - \epsilon_r)_2$ are the values characteristic of each component. The order of the binding reaction, j, was calculated by Weber (1965).

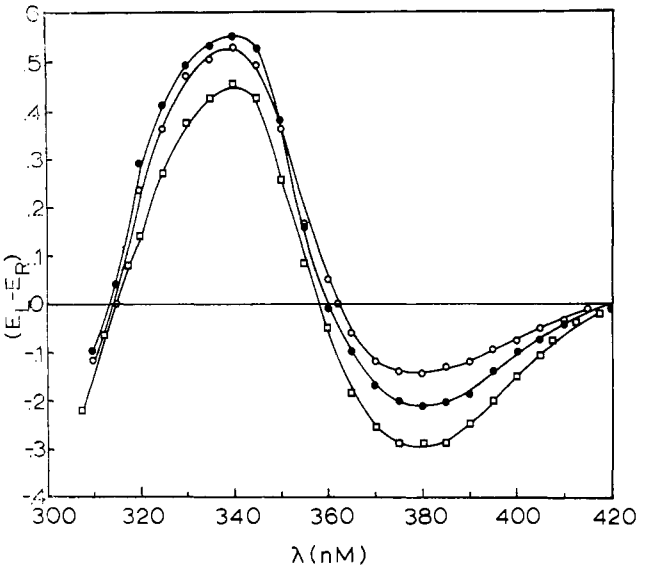

FIGURE 4: The circular dichrosim spectra of the complexes of bovine serum albumin with 1-anilino-8-naphthalenesulfonate. Conditions: 0.10 M phosphate (pH 2.0), 25°. (○) $\bar{n} = 1.8$ (1.5×10^{-4} M dye + 7.5×10^{-5} M albumin); (●) $\bar{n} = 5$ (4×10^{-4} M dye + 7.5×10^{-5} M albumin); (□) $\bar{n} = 19$ (1.5×10^{-3} M dye + 7.5×10^{-5} M albumin).

mixture of two independent components. I varied the ratio of the two independent dissociation constants, m, over the representative range of 1.1 to 5. Table I summarizes the values of m, $(\epsilon_l - \epsilon_r)_1$, $(\epsilon_l - \epsilon_r)_2$, and j. The significance of these calculations is that the circular dichroism spectra directly reveal small differences in K undetectable in precise determinations of the protein–ligand equilibrium. The lower limit of m deduced from the spectra is set by the magnitude of the required values of $(\epsilon_l - \epsilon_r)$. In general, $(\epsilon_l - \epsilon_r)$ is less than 100 (Tinoco, 1965). Alternative treatments of the data lead to the same pertinent conclusions.

Binding at pH 5. The shift to cooperative binding produced by lowering the pH from 7 to 5 should be accompanied by a change in distribution of the bound ligand molecules (Daniel and Weber, 1966). Comparison of the circular dichroism spectra recorded at pH 7 and 5 reveals large differences (Figures 1 and 3).

Binding at pH 2. The acid expansion of albumin is characterized by the appearance of 40 or more binding sites producing enhancement of the fluorescence of 1-anilino-8-naphthalenesulfonate (Daniel and Weber, 1966; Pasby and Weber, 1970). The circular dichroism spectra observed at pH 2 for values of $\bar{n}$ ranging from 2 to 20 are interesting because they suggest that the binding population is comparatively homogeneous (Figure 4). The inversion of the signs of the two overlapping bands results from a change in the polarizability of the binding sites.

Discussion

The circular dichroism spectra of the complexes of 1-anilino-8-naphthalenesulfonate with bovine serum albumin reveal two spectrally distinguishable components within the set of five primary binding sites. The variation in circular dichroism with $\bar{n}$ is consistent with small intrinsic differences in the affinities of the binding sites for the ligand. If tautomeric equilibria exist, their effects are indistinguishable from those predicted for static heterogeneity alone.

The resolution of the properties of a heterogeneous population depends on the method used. The advantage of circular dichroism lies in the accurate measurement of small differences. In this case, $(\epsilon_l - \epsilon_r)$ is *ca.* 1/5000 of the molar absorptivity. Pasby and Weber (1970) have analyzed the fluorescence yields and lifetimes in order to gain information on the heterogeneity of binding. Comparison of these two papers will illustrate how heterogeneity is differently reflected in measurements of unrelated properties.

I hope that the method described here for discerning heterogeneity of binding sites has general utility in the study of protein–ligand equilibria.

Acknowledgment

I thank Professor K. E. Van Holde for the use of the circular dichroism spectrophotometer and Mrs. Alice Clark for her assistance.

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Proteolytic Fragmentation of Bovine Heart Heavy Meromyosin*

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ABSTRACT: Bovine heart subfragment 1 was isolated from the heavy meromyosin by digestion with papain. This subfragment 1 appeared as a single peak after Sephadex G-200 chromatography and in the analytical ultracentrifuge. Its molecular weight was 110,000 ± 10,000 as determined by the Yphantis method. The Ca^{2+}–adenosine triphosphate phosphohydrolase activity of this subfragment 1 was 1.6-fold higher than the original heavy meromyosin and 2.4-fold higher than the original myosin in the range of pH 6–9. In contrast to the papain digestion of bovine heart heavy meromyosin, no significant quantities of subfragment 1 could be isolated when trypsin or chymotrypsin digestions were performed under various conditions. Trypsin digestion at pH 7.6, 25° for 30 min did not release subfragment 1 and the Ca^{2+}–ATPase activity was lost. Comparative pH-Stat studies showed that bovine heart heavy meromyosin was more resistant to digestion by papain, chymotrypsin, or trypsin than rabbit skeletal heavy meromyosin. Furthermore, bovine heart myosin was also more resistant to trypsin digestion than was rabbit skeletal myosin. Results from sedimentation studies, the Ca^{2+}–ATPase measurements, and quantitation data obtained from chromatography of the papain digests indicated that bovine heart myosin and heavy meromyosin are composed of two subunits of subfragment 1.

In the past few years, the isolation and characterization of subfragment 1, the enzymically active subunit of rabbit skeletal myosin, has been studied extensively (see *e.g.*, Mueller and Perry, 1962; Young *et al.*, 1965; Kominz *et al.*, 1965; Jones and Perry, 1966; Slayter and Lowey, 1967; Lowey *et al.*, 1969). However, little is known about the preparation of subfragment 1 from other myosins. Previous work has revealed that heart myosin differs from skeletal myosin with respect to its ATPase[1] (EC 3.6.1.3, ATP phosphohydrolase) activity and its digestion with trypsin (Gelotte, 1951; Gergely, 1959; Ellenbogen *et al.*, 1960; Brahms and Kay, 1963; Mueller *et al.*, 1964b; Bárány *et al.*, 1964). Thus, studies on subfragment 1 from heart myosin may help in understanding the relationship of the structure of heart myosin to its ATPase activity.

Bovine heart myosin was chosen because it is more available than rabbit heart myosin. It will be shown in this paper that bovine heart subfragment 1 can be prepared from the papain digestion of bovine heart HMM. This is in contrast to the very small quantities of subfragment 1 which can be obtained from the trypsin treatment of bovine heart HMM.

Materials and Methods

Rabbit skeletal myosin was prepared by procedures previously described (Bárány and Oppenheimer, 1967; Gaetjens *et al.*, 1968).

Bovine heart myosin was prepared as follows: Two hearts were obtained from the local slaughterhouse and fat and extraneous tissue were removed; the muscle then was minced in a precooled grinder. The minced muscle (2000 g) was extracted at 4° with 6.0 l. of a solution containing 0.3 M KCl, 0.075 M KH_2PO_4, 0.075 M K_2HPO_4, and 0.0033 M ATP (pH 6.6). Extraction was continued for 30 min with mechanical stirring. The mixture was centrifuged at 13,000*g* for 15 min and then filtered through a paper pad which had been washed with 0.6 M KCl (pH 7.0). The filtrate (5.8 l.) was diluted with 15 volumes of cold distilled water, and the resulting precipitate was allowed to settle overnight. To remove most of the hemoglobin, the supernatant was decanted and the jars were refilled with cold distilled water and 10 ml of 3.0 M KCl/l. of water was added to maintain the precipitated state of myosin. The washing was repeated at least three times or until the supernatant appeared colorless. The precipitate then was collected and dissolved in 0.6 M KCl in a final volume of 1.5 l. An equal volume of cold distilled water was added, and the pH was adjusted to 6.4–6.5; this suspension was centrifuged at 23,000*g* for 30 min. The supernatant (2.2 l.) was diluted with 10 volumes of cold distilled water. The precipitated myosin was allowed to settle overnight. The myosin was collected and dissolved in 0.6 M KCl at 25 mg/ml. The pH of this solution was adjusted to 6.7 and then clarified in a Spinco preparative ultracentrifuge for 2 hr at 275,000*g* (average force in rotor 50.1). The upper two-thirds of the supernatant was removed as bovine heart myosin. About 14 g of myosin was isolated by

* From the Institute for Muscle Disease, Inc., New York, New York 10021. *Received August 13, 1969.* This work was supported by Grant No. AM-4873 from the National Institutes of Health, U. S. Public Health Service, by grants from Muscular Dystrophy Associations of America, Inc., and by a grant from the Muscular Dystrophy Association of Canada.

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[1] Abbreviations used are: ATPase, adenosine triphosphate phosphohydrolase; HMM, heavy meromyosin.