hydroxylation of several lysyl residues is incomplete or whether the incomplete hydroxylation is confined to a single lysyl residue.

References

Bornstein, P. (1967a), *J. Biol. Chem. 242*, 2572.
Bornstein, P. (1967b), *Biochemistry 6*, 3082.
Bornstein, P., Kang, A. H., and Piez, K. A. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 417.
Bornstein, P., and Piez, K. A. (1965), *Science 148*, 1353.
Bornstein, P., and Piez, K. A. (1966), *Biochemistry 5*, 3460.
Butler, W. T., Piez, K. A., and Bornstein, P. (1967), *Biochemistry 6*, 3771.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Francois, C. J., and Glimcher, M. J. (1966), *Biochim. Biophys. Acta 133*, 91.
Kang, A. H., Bornstein, P., and Piez, K. A. (1967), *Biochemistry 6*, 788.
Kang, A. H., Nagai, Y., Piez, K. A., and Gross, J. (1966), *Biochemistry 5*, 509.
Kostka, V., and Carpenter, F. H. (1964), *J. Biol. Chem. 239*, 1799.
Lewis, M. S., and Piez, K. A. (1964), *J. Biol. Chem. 239*, 3336.
Miller, E. J., Martin, G. R., Piez, K. A., and Powers, M. J. (1967), *J. Biol. Chem. 242*, 5481.
Miller, E. J., and Piez, K. A. (1966), *Anal. Biochem. 16*, 320.
Nordwig, A., and Pfab, F. K. (1968), *Biochim. Biophys. Acta 154*, 603.
Piez, K. A. (1968), *Anal. Biochem* (in press).
Piez, K. A., Bladen, H. A., Bornstein, P., Butler, W. T., Kang, A. H., Lane, J. M., and Miller, E. J. (1968), *Brookhaven Symp. Biol.* (in press).
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Piez, K. A., Weiss, E., and Lewis, M. S. (1960), *J. Biol. Chem. 235*, 1987.

Comparative Studies on Myosins from Breast and Leg Muscles of Chicken*

Chuen-Shang Chung Wu

ABSTRACT: Comparative studies on chicken myosins from breast and leg muscles revealed several differences in physicochemical characteristics. Myosin from leg muscles (myosin-R) had adenosine triphosphatase activity about 82–86% of that from breast muscle (myosin-W). The former was also less extractable than the latter with the short-time extraction methods employed in this study. There was no difference in substrate specificity; both myosins hydrolyzed adenosine triphosphate and uridine triphosphate the fastest, inosine triphosphate the next, cytidine triphosphate and guanosine triphosphate the slowest. Chromatographic patterns on a DEAE-Sephadex column and sedimentation velocity in an ultracentrifuge did not reveal any differences between these two proteins; the enzyme activities of both increased somewhat after chromatography. Spectrophotometric studies showed a slightly higher extinction coefficient and tyrosine content for myosin-R; however, the absorption spectra at neutral and alkaline pH were very similar. The solubilities, when studied in 5 mM Tris-maleate buffer with various KCl concentrations and pH values, also revealed dissimilarities. The most striking differences were observed in the rate and pattern of tryptic digestion. Myosin-R was digested at a much slower rate; the course of hydrolysis was also different from that of myosin-W. The study of optical rotatory parameters, however, did not reveal any differences in conformation between these myosins.

Since the observation by Cooper and Eccles (1930) that the speed of contraction of red muscle was slower than that of white muscle, numerous physiological and biochemical studies have been conducted on these two types of muscles. However, investigations on the contractile proteins from these muscles were performed only recently. Barany *et al.* (1965) reported that ATPase activity of myosin from red muscle of rabbit was considerably lower than that from white muscle, and that the pH–ATPase profiles of the two myosins were different in the alkaline pH range. Gergely *et al.* (1965) and Maddox and Perry (1966) also found lower enzyme activity in red muscle myosin from rabbit and pigeon, respectively, but Sreter *et al.* (1966) observed no significant differences in the pH–ATPase profile or in the effect of ionic strength on the ATPase activity of red and white

* Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California 94710. *Received August 22, 1968.* The research was conducted while the author was pursuing work on a National Research Council Postdoctoral Resident Research Associateship.

muscle myosins. These authors further noted that the rate of tryptic digestion of red muscle myosin was lower than that of white muscle myosin. More recently, Barany (1967) showed that the speed of shortening of muscle is positively correlated with the level of ATPase activity of myosin.

In view of the above differences, it is of great interest to study the structure and function of both types of myosin from the muscle of other species. This may shed some light on the mechanism of muscular contraction, as well as of the changes that occur in muscle during and following rigor. The objective of the present investigation, therefore, was to obtain myosins from breast (white) and leg (red) muscles of chicken in order to compare their structural and enzymatic properties.

The results indicate that although the difference in ATPase activity of myosins from breast and leg muscles of chicken is not as pronounced as that in those from rabbit and pigeon, the solubility, the mode of tryptic digestion, and several other properties are different.

Materials and Methods[1]

White chicken fryers (meat-type hybrid) about 10 weeks old were obtained commercially and sacrificed by electric stunning and severing the carotid arteries. After the chicken was bled for 3 min, the pectoralis major muscles (white) and the whole legs were removed and chilled immediately on ice. The legs were further dissected in a 3° cold room to obtain adductor longus, quadriceps femoris, and flexor digitus muscles (red). Myosin was prepared by the method of Tonomura *et al.* (1966). Preparations were also made by the procedure of Perry (1955) and that of Hasselbach and Schneider (1951). The time between the death of the chicken and extraction of myosin was usually less than 1 hr. Three precipitation cycles were carried out, followed by chromatography on a DEAE-Sephadex column unless otherwise specified.

Rabbit myosins were prepared from soleus, semitendinosus, and vastus intermedius (red); vastus lateralis and adductor magnus (white) by the method of Hasselbach and Schneider. The preparations were used without chromatography.

Trypsin (twice crystallized, salt free) and soybean trypsin inhibitor were obtained from the Worthington Biochemical Corp. The sodium salts of ATP, CTP, GTP, ITP, and UTP, L-histidine hydrochloride, maleic acid, and Tris were from Sigma. DEAE-Sephadex A-50 was purchased from Pharmacia. All other chemicals were reagent grade.

Chromatography was performed on DEAE-Sephadex A-50 according to the method described previously (Richards *et al.*, 1967). Protein concentration was measured either spectrophotometrically assuming extinction coefficients, $E^{1\%}_{280\,m\mu}$, of 5.33 and 5.05, respectively for chicken myosin-R[2] and myosin-W, or by the biuret method standardized by micro-Kjeldahl nitrogen assuming 17.1% N (Chung *et al.*, 1967). An extinction coefficient of 5.43 was used for unchromatographed rabbit myosin (Woods *et al.*, 1963).

ATPase activities were determined either in 0.1 M Tris-maleate, 10 mM $CaCl_2$, 4 mM ATP, and 0.05 M KCl (pH 6.5) or in 0.05 M Tris-HCl, 10 mM $CaCl_2$, 5 mM ATP, and 0.05 M KCl (pH 7.5) at 25°. The reaction was stopped by the addition of 15% trichloroacetic acid and the liberated phosphorus measured by the Fiske–Subbarow method.

Extinction coefficients were determined both at pH 7.0 in 0.5 M KCl and at pH 13 in 0.1 N NaOH. Myosin after chromatography was dialyzed exhaustively against 0.5 M KCl (pH 7.0). To 2 ml of myosin solution containing 2–3 mg/ml of protein was added an equal volume of 0.2 N NaOH. The solution was left 1 hr at room temperature, centrifuged for 1 hr at 27,000*g*, and scanned in a Cary 15 recording spectrophotometer between 240 and 360 mμ. An aliquot was analyzed for nitrogen by micro-Kjeldahl procedure. Turbidity, if any, was corrected by extrapolating the absorption at 340 and 360 mμ linearly to 280 mμ and subtracted from the 280-mμ readings. $E^{1\%}_{280\,m\mu}$ was then calculated and compared with those obtained at neutral pH in 0.5 M KCl. Tyrosin and tryptophan contents were also calculated from the same data according to Beavan and Holiday (1952).

A Beckman DU spectrophotometer was used for routine ultraviolet optical density measurements, a Cary 15 recording spectrophotometer for ultraviolet spectra, and a Bausch & Lomb Spectronic 20 for phosphorus and biuret determinations.

Spectrophotometric titrations were performed with 20 ml of 0.1% myosin in 0.5 M KCl. The pH was adjusted by microquantities of 6 N NaOH with the aid of a magnetic stirrer and measured in a Corning Model 12 pH meter with glass electrode. $\Delta E^{1\%}_{295\,m\mu}$ was determined by subtracting optical density at neutral pH from that at the pH in question and was plotted against pH. Change of volume after the addition of NaOH was ignored.

Solubility was studied at 0° in 5 mM Tris-maleate buffer with varied KCl concentration and pH. The myosin solutions were dialyzed overnight against a large volume of 0.4 M KCl (pH 7.0). Various combinations of pH and ionic strength were obtained in 1.5 × 12 cm Servall Pyrex centrifuge tubes by adding, in order, 0.5 ml of 20 mM Tris-maleate, 1 M KCl, water, and protein to a final volume of 2.0 ml and a protein concentration of 0.8 mg/ml. The mixture was incubated on ice for 90 min and centrifuged 20 min at 4080*g*. The protein concentration in the supernatant was then determined spectrophotometrically at 280 mμ. With more concentrated solutions (higher than 2 mg/ml), the biuret method was employed for the measurement of protein concentration.

Tryptic digestion was performed in 0.5 M KCl–0.05 M Tris-HCl (pH 7.5) at 25° with 250:1 ratio of myosin to

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

[2] Myosin-R, myosin from red or slow muscle; myosin-W, myosin from white or fast muscle (Sreter *et al.*, 1966).

trypsin. Myosin solution (20 ml) at 5 mg/ml was equilibrated in a water bath at 25°. To start the reaction, 0.4 ml of 1-mg/ml solution of trypsin in 0.001 N HCl was added and stirred gently. At appropriate time intervals, 2 ml of reaction mixture was withdrawn to a test tube containing 0.04 ml of 3 mg/ml of soybean trypsin inhibitor in 0.5 M KCl Tris-HCl (pH 7.5). Each of the solutions was examined in an analytical ultracentrifuge and assayed for ATPase activity. For the determination of nonprotein nitrogen liberated by tryptic digestion, a similar experiment was performed in 0.5 M KCl–0.02 M phosphate (pH 7.5). The reaction was stopped by adding trichloroacetic acid to 6% final concentration. The solution was centrifuged, and the supernatant was analyzed for nonprotein nitrogen by the micro-Kjeldahl procedure.

Sedimentation velocity studies were carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren analyzer, RTIC temperature control and measuring unit, electronic speed control unit, and photoelectric scanning system.

Optical rotations were measured with a Perkin-Elmer Model 141 photoelectric polarimeter. The chromatographed proteins were dialyzed against 0.5 M KCl–0.01 M phosphate (pH 7.5) and used at concentrations between 1 and 2 mg/ml. The measurements were made at 313, 365, 436, 546, and 587 mμ and the data were analyzed according to the Drude equation

$$[\alpha]_\lambda = \frac{k}{\lambda_0^2 - \lambda_c^2}$$

and by the Moffitt–Yang equation (Moffitt and Yang, 1956)

$$[\alpha]_\lambda = \left(\frac{100}{M_0}\right)\left(\frac{n^2+2}{3}\right)\left[\frac{a_0\lambda_0^2}{(\lambda^2-\lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right]$$

where λ_0 is assumed to be 210 mμ and M_0, the average residue weight, 115 (Lowey and Cohen, 1962).

Results

At the beginning of this study, the whole thigh and leg muscles of the chicken were used for the preparation of myosin-R even though they contain muscles as white as the breast muscle. The results showed that the ATPase activity of myosin from this source was not much different from that of chicken myosin-W prepared at the same time. It was also considerably higher than that of rabbit myosin-R prepared later in the present study and those reported by other workers. This high value might have resulted from the mixed type of muscles. Therefore, a simple experiment was carried out to determine which of the leg muscles were the relatively red ones. Although the color of the muscle may not be an accurate indicator for the determination of the type of muscle (George and Berger, 1966), it can serve as a comparative means within an individual bird.

To enhance the red color, one leg was boiled in water for 30 min and dissected, while the other was dissected without cooking. The muscles from both legs were compared visually for the relative myoglobin content and rated as shown in Table I. It was found that quadriceps femoris, adductor longus, and flexor digitus were the most highly colored muscles of the major muscles examined. In some of the muscles (sartorius and tensor fasciae latae), varied shades of color within a single muscle were observed. Only the three most red muscles were used for the preparation of myosin-R.

TABLE I: Relative Darkness of Chicken Leg Muscles.

Muscle	Color[a]
Sartorius	+
Tensor fasciae latae	++
Biceps femoris	−
Semimembranosus	−
Semitendinosus	++
Quadriceps femoris	+++
Gracilis	+
Adductor longus	+++
Pectineus	+
Gastronemeius	++
Flexor digitus	+++
Peroneus longus	+

[a] − = white; + = slightly red; ++ = intermediate; and +++ = most red.

Extractability. Differences in extractability between myosin-R and myosin-W were repeatedly observed during the preparation of these proteins. Table II shows some of the data obtained with different extraction methods and time for both chicken and rabbit proteins. These values were approximate since some losses occurred during the three cycles of precipitation–resolution. Nevertheless, it will be noticed that the yield of myosin-R was generally one-half to one-third less than that of myosin-W even with 45-min extraction periods. Washing of the minced red muscle with dimethyl sulfoxide before the extraction of myosin-R did not increase the yield.

Nucleosidetriphosphatase Activity. Some examples of ATPase activities of unchromatographed chicken breast and leg myosins together with that of rabbit myosin-R and myosin-W are presented in Table III. The activity levels varied from preparation to preparation and depended on the assay medium and conditions used. The calcium-activated ATPase activity of chicken leg myosin was about 82–86% of that of breast myosin (column 5). On the other hand, the ATPase activity of rabbit myosin-R was only about one-half of that of myosin-W. This result is comparable with those reported by other workers (Barany *et al.*, 1965; Sreter *et al.*, 1966). The activity of chicken myosin-W was slightly higher than that of rabbit myosin-W when assayed under the same conditions at the same time (preparations X and XI).

The other nucleoside triphosphatase activities of chromatographed chicken myosin-R and myosin-W are

TABLE II: Extractability of Myosins from Red and White Muscles of Chicken and Rabbit.

Source	Type of Muscle	Method of Extraction[a]	Time of Extraction (min)	Fresh Muscle (g)	g of Myosin/100 g of Fresh Muscle
Chicken	White	A	11	98	0.79
Chicken	Red	A	11	90	0.33
Chicken	White	B	15	100	0.96
Chicken	Red	B	15	100	0.52
Rabbit	White	B	15	92	0.63
Rabbit	Red[b]	B	15	98	0.46
Rabbit	White	C	45	77	1.18
Rabbit	Red	C	45	25	0.67

[a] A = Tonomura *et al.* (1966); B = Perry (1955); C = Hasselbach and Schneider (1951). [b] Mixed red muscles in addition to those three mentioned in the Materials and Methods section were used.

TABLE III: ATPase Activities of Chicken and Rabbit Myosins.

Prepn No.	Source	Buffer and Conditions	ATPase Activity (μmoles of P_i/min per mg)[c] Myosin-R	Myosin-W	Myosin-R/ Myosin-W
I	Chicken	0.1 M Tris-maleate–0.05 M KCl–0.01 M $CaCl_2$–4 mM ATP, pH 6.7, 30°	1.04[a] (1.25)[b]	1.21 (1.48)	0.86 (0.85)
VII	Chicken	0.05 M Tris-HCl–0.2 M KCl–0.01 M $CaCl_2$–5 mM ATP, pH 7.5, 25°	0.51	0.62	0.82
XI	Chicken	0.1 M Tris-maleate–0.05 M KCl–0.01 M $CaCl_2$–5 mM ATP, pH 6.5, 25°	0.84	1.00	0.84
XI	Chicken	0.05 M Tris-HCl–0.05 M KCl–0.01 M $CaCl_2$–5 mM ATP, pH 7.5, 25°	0.69	0.81	0.85
XIII	Chicken	0.05 M Tris-HCl–0.05 M KCl–0.01 M $CaCl_2$, 4 mM ATP, pH 7.5, 25°	1.05 (1.16)	1.26 (1.42)	0.83 (0.82)
X	Rabbit	0.05 M Tris-HCl–0.05 M KCl–0.01 M $CaCl_2$–5 mM ATP, pH 7.5, 25°	0.40	0.71	0.56

[a] Combined leg and thigh muscles were used. [b] Values in parentheses denote those after chromatography. [c] The figures represent average of six to eight determinations; the standard deviations are all less than 3%.

shown in Table IV. In all cases, the enzyme activities of myosin-W were equal to or slightly higher than those of myosin-R. The levels of ATPase activities of myosin-R and myosin-W were very similar to those of UTPase activities, while CTPase and GTPase activities were only one-third of ATPase activities. In contrast to the report of Barany *et al.* (1965) that the ITPase activities of rabbit myosin-R and myosin-W were more than twice as high as the ATPase activities, the present data showed that the ITPase activities of chicken myosins were lower, about two-thirds of ATPase activities. Further experiments indicated that the difference was due to the lower salt molarities used in the present study. When the KCl concentration was increased to 0.55 M, the ITPase activities of chicken myosin-R and myosin-W also became more than twice as high as their ATPase activities. These results indicated that there was no difference in substrate specificity between chicken myosin-R and myosin-W.

Chromatography. The chromatographic patterns of chicken myosin-R and myosin-W on DEAE-Sephadex A-50 were very similar (Figure 1). Both myosin-R and myosin-W were eluted at the same salt concentration. The shape of the myosin peak was also the same, steep at the front and trailing at the back. The amount of contaminating proteins (peaks I and II) as well as the RNA fraction (peak IV) were also very similar. In some preparations, the impurities were less than those shown here.

Although the ATPase activities were approximately 20–50% higher after chromatography, the pH optimum as well as the ATPase–pH-dependence profile was unchanged (Figure 2). However, the alkaline pH optimum increased more than that at pH 6, and myosin-R was

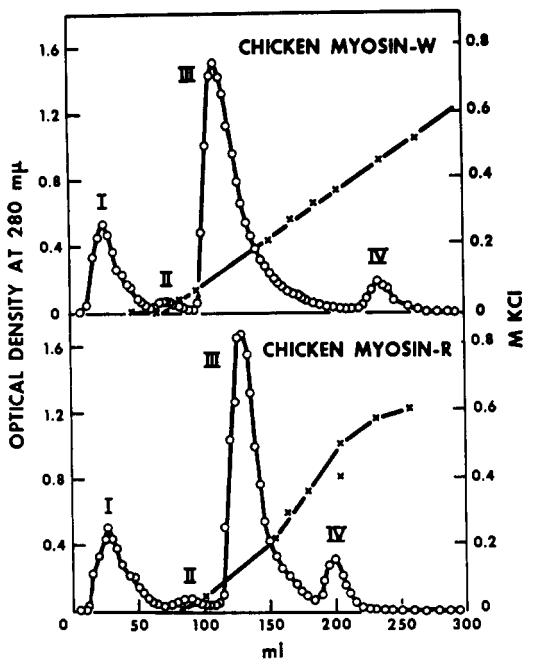

FIGURE 1: Chromatography of chicken myosin-W and myosin-R on DEAE-Sephadex A-50, 1.5 × 30 cm column. Approximately 150 mg of protein was applied on the column and eluted with a linear gradient of KCl containing 0.04 M pyrophosphate at pH 7.5. The flow rate was 15 ml/hr. (O-O-O) Optical density at 280 mμ; (×-×-×) M KCl.

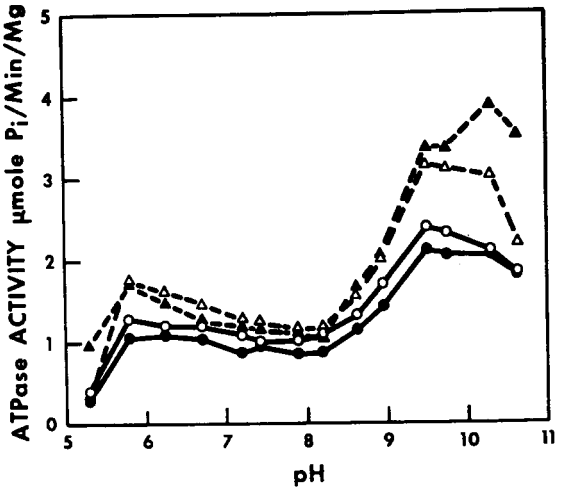

FIGURE 2: pH dependence of Ca^{2+}ATPase activity of chicken myosins. The assay medium contained 0.1 M Tris-maleate, 10 mM $CaCl_2$, 0.05 M KCl, 4 mM ATP, and 1 mg/ml of protein concentration. The mixture was incubated at 30° for 5 min. Open symbols: mysoin-W; closed symbols: myosin-R. Solid lines: before chromatography; and dashed lines: after chromatography.

activated more than myosin-W in the alkaline range after chromatography. This is in contrast to the results of Sreter *et al.* (1966) and Seidel (1967) that rabbit myosin-R was less stable than myosin-W in alkaline pH.

Spectrophotometric Characteristics. In the early stages of this investigation, it was noticed that the extinction coefficient of myosin-R was consistently slightly higher than that of myosin-W at neutral pH. In order to obtain more reproducible results, the extinction coefficients were also measured at pH 13 in 0.1 N NaOH. The results indicated (Table V) that the extinction coefficient of myosin-R at 280 mμ was approximately 6–7% higher than that of myosin-W, regardless of the pH of the solution and the source (rabbit or chicken) of myosin. Tryptophan content was about the same, but tyrosine content was slightly higher in myosin-R. The ultraviolet spectra of myosin-R and myosin-W at neutral and alkaline pH are shown in Figure 3. Here again, the absorption per unit concentration of myosin-R was higher than that of myosin-W through all the wavelength ranges measured. The absorption at near-visible region was negligible, indicating that myosin purified by chromatography was free of turbid material. The wavelength of maximum absorption of myosin-R and myosin-W at neutral and at alkaline pH were the same, 279 and 291 mμ, respectively. The isosbestic point of neutral and alkaline curves was at 281 mμ, and the alkaline spectra had a shoulder at 284 mμ. The ratio of absorption maximum (279 mμ) to minimum (250 mμ) was higher for myosin-W, ranging from 2.62 to 2.70, than for myosin-R, from 2.51 to 2.53.

Stracher (1960), while studying the tyrosyl phenolic groups of rabbit myosin by the spectrophotometric titration method, noticed that there were abnormal tyrosin residues titrated at a pH above 12. He attributed this to the participation of some of the tyrosyl groups in intramolecular hydrogen bonding. A similar study with chicken myosin in 0.5 M KCl, however, revealed no such abnormal tyrosyl residues. Both in myosin-R and myosin-W, all tyrosyl phenolic groups ionized within a normal pH range, with an apparent pK at 10.9 (Figure 4). However, the $E^{1\%}_{295\,m\mu}$ for myosin-R was higher than that for myosin-W. This was in accord with the above finding that myosin-R had higher tyrosine content than myosin-W. As the titration proceeded, the formation of small amounts of fibrous aggregates due to stirring was observed. However, the absorption at 340 and 360 mμ did not increase appreciably. When the pH was

TABLE IV: NTPase Activity of Chicken Myosins.[a]

Substrate	NTPase Activity (μmoles of P_i/min per mg)	
	Myosin-R	Myosin-W
ATP	0.94	1.07
CTP	0.29	0.38
GTP	0.38	0.36
ITP	0.66	0.70
UTP	0.95	1.01

[a] Chromatographed myosin-R (0.22 mg), myosin-W (0.25 mg in 2 ml of 0.05 M Tris-HCl–0.01 M $CaCl_2$–0.05 M KCl), and NTP (1 mM) at pH 7.5 were incubated for 5 min at 25°. The reaction was stopped by 1 ml of 15% trichloroacetic acid and liberated P_i determined by the Fiske–Subbarow method.

TABLE V: Extinction Coefficient and Tyrosine–Tryptophan Content of Chicken and Rabbit Myosins.

Prepn No.	Myosin	$E^{1\%}_{279\,m\mu}$ in 0.5 M KCl	$E^{1\%}_{280\,m\mu}$ in 0.5 M KCl	$E^{1\%}_{280\,m\mu}$ in 0.1 N NaOH	Tyrosine (mole/10^5 g)	Tryptophan (mole/10^5 g)
I	Chicken myosin-R[a]	5.30	5.29	4.97	20.8	3.3
I	Chicken myosin-W	4.98	4.96	4.71	19.6	3.1
IX	Chicken myosin-R		5.37	5.10	20.0	3.8
XI	Chicken myosin-W		5.17	5.00	19.2	3.8
XIV	Chicken myosin-R	5.36	5.33	5.14	19.5	4.1
XIV	Chicken myosin-W	5.14	5.09	4.95	18.7	3.9
X	Rabbit myosin-R[b]			5.45	21.9	3.9
X	Rabbit myosin-W[b]			5.14	19.6	3.8

[a] Combined leg and thigh muscles were used. [b] The rabbit myosins were not chromatographed.

raised quickly from 7 to 13, all the tyrosyl groups ionized immediately, since no change in absorption was detected between 1 and 60 min after the addition of alkali to the myosin-R or myosin-W solution. An experiment with rabbit myosin-W in 0.5 M KCl and another with the same protein in 0.4 M KCl–0.05 M piperidine also showed no abnormal tyrosyl residues. The apparent pK was the same as that of chicken myosin, namely, 10.9.

Solubility. The solubility of chicken myosin was studied in 5 mM Tris-maleate buffer with varied KCl concentration and pH. In order to eliminate any question as to the possible influence of pyrophosphate which was used in chromatography, and to lessen the effect of aging (Johnson and Rowe, 1961), myosin was used immediately after preparation without further purification by chromatography. The results indicated that the solubilities of chicken myosin-R and myosin-W were different. At pH 7 in 5 mM Tris-maleate and ionic strength below 0.2, myosin-W was more than twice as soluble as myosin-R. Above 0.2 μ, both proteins were completely soluble (Figure 5). The solubility of myosin at both low and high ionic strength varied with pH (Figure 6). In 5 mM Tris-maleate (0.015 μ at pH 7.0 and 0.017 μ at pH 8.5) and 0.06 M KCl, both proteins were insoluble from pH 4.5 up to pH 7.0. Beyond pH 7.0, both solubilities increased, with that of myosin-W about two times higher. Above pH 8.5, myosin-W became completely soluble, whereas myosin-R was only 50% soluble. In 5 mM Tris-maleate and 0.56 M KCl, both myosins were soluble above pH 6. Below this pH, both solubilities decreased, with that of myosin-W decreasing before that of myosin-R.

A similar experiment at higher protein concentration and another using the equilibrium dialysis method also showed similar results. Aged (2 weeks old) myosin-R and myosin-W also differed in solubility, although they both became less soluble at low ionic strength.

Tryptic Digestion. It is a well-known phenomenon that limited tryptic digestion of myosin gives rise to two relatively larger molecules, H- and L-meromyosins (Mihalyi and Szent-Gyorgyi, 1953; Gergely, 1953) and a

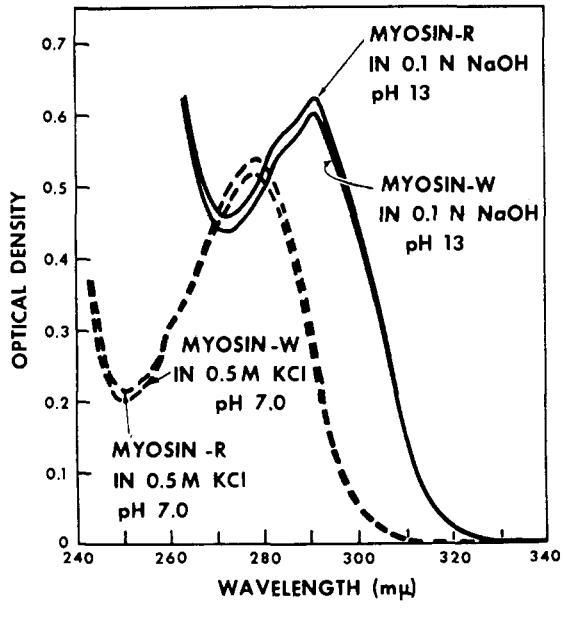

FIGURE 3: Spectra of chicken myosins at neutral and alkaline pH. The curves were corrected to a protein concentration of 1 mg/ml.

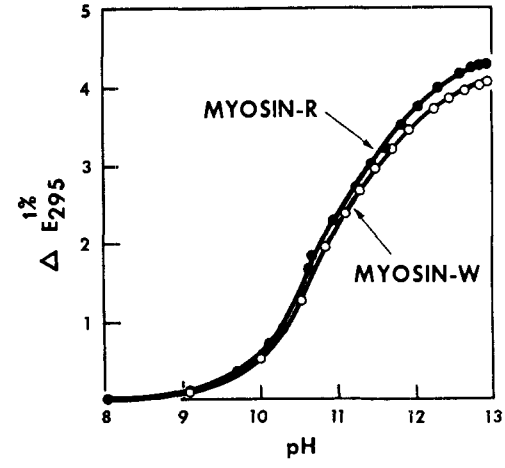

FIGURE 4: Spectrophotometric titration of chicken myosins in 0.5 M KCl.

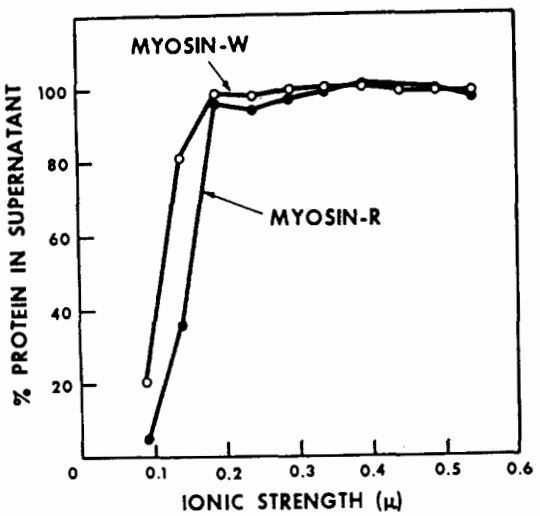

FIGURE 5: Effect of ionic strength on solubility of chicken myosins. Protein concentration, 0.8 mg/ml, in 5 mM Tris-maleate (pH 7.0). Ionic strength was adjusted by KCl.

small amount of nonprotein nitrogen. Gergely *et al.* (1965) showed that the myosin prepared from red muscles of rabbit was more resistant to tryptic digestion than that from white muscles. Maddox and Perry (1966) also reported a similar finding for pigeon myosins. The present results further confirmed their findings. In addition to the decreased susceptibility of myosin-R, the modes of action of trypsin toward chicken myosin-R and myosin-W were different (Figure 7). While there was a stage where myosin-W showed two distinct boundaries in the ultracentrifuge, presumably those of L- and H-meromyosins (Figure 7c, lower), no such stage could be found for myosin-R. Undigested myosin-R was present even after 30-min digestion (Figure 7e, second peak of upper pattern). However, beyond this time, the middle peak increased with digestion time, probably due to the simultaneous hydrolysis of the intact myosin molecule

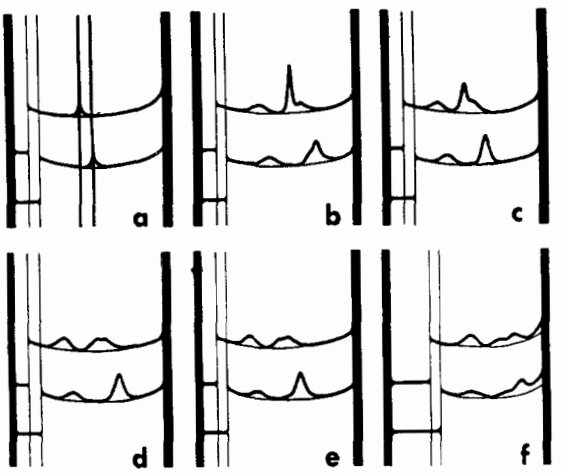

FIGURE 7: Sedimentation patterns of tryptic-digested chicken myosin-R (upper) and myosin-W (lower). Myosin concentration 5.5 mg/ml; rotor speed 60,000 rpm; bar angle 60°; temperature 23.6°. (a) Undigested myosin; (b) 5 min; (c) 10 min; (d) 20 min; (e) 30 min; and (f) 60 min digested. Pictures taken at 135, 134, 131, 134, 132, and 132 min, respectively, after reaching full speed.

and the H-meromyosin. In this respect, myosin-R is very similar to cardiac myosin (Mueller *et al.*, 1964). After 60-min digestion, H-meromyosin of both myosins was degraded further, showing similar patterns. Another difference between myosin-R and myosin-W was that the L-meromyosin peak was larger for the former. This was already apparent after 20-min digestion. Experiments with unchromatographed myosins also showed the same results.

The effect of tryptic digestion on the ATPase activity of chicken myosin-R and myosin-W is shown in Figure 8. Although the specific activities of myosin-R and myosin-W ATPase differ only slightly before the action of trypsin, the treatment with the proteolytic enzyme induced larger differences between them. While the ATPase activity of myosin-R changed only slightly, that

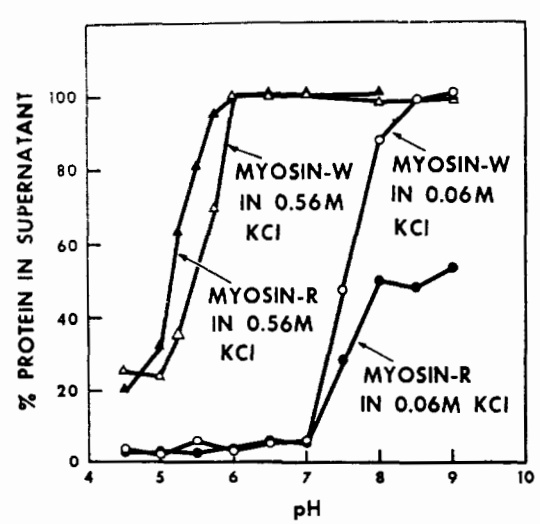

FIGURE 6: Effect of pH on solubility of chicken myosins. Protein concentration, 0.8 mg/ml, in 5 mM Tris-maleate. pH and KCl concentrations as indicated.

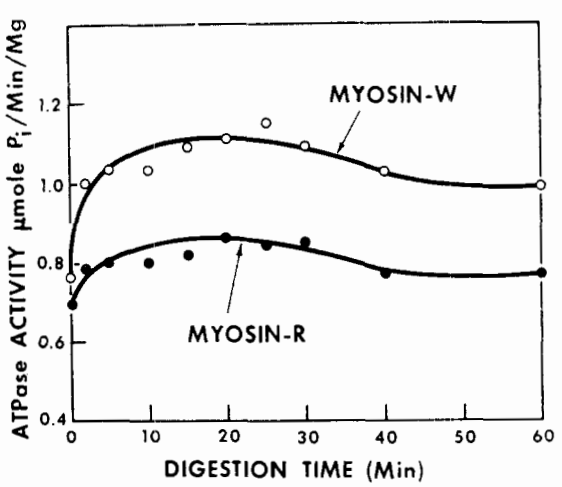

FIGURE 8: Effect of tryptic digestion on ATPase activity of chicken myosins. Assay medium: 0.05 M Tris-HCl, 0.025 M KCl, 10 mM $CaCl_2$, and 4 mM ATP (pH 7.5). Myosin samples same as those in Figure 7.

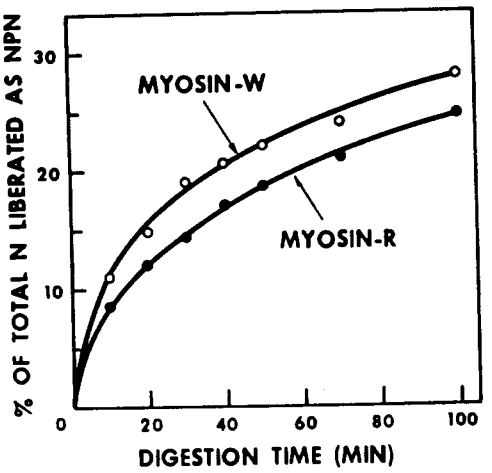

FIGURE 9: Plot of nonprotein nitrogen (NPN) liberated during tryptic digestion of chicken myosins.

of myosin-W increased more than 50% above original in the early stage of enzyme hydrolysis. The ATPase activities of both fell off gradually after 30-min digestion. The reason for the initial activation of myosin-ATPase by trypsin hydrolysis is not known. Gergely *et al.* (1965) suggested that it is due to conformational changes within the myosin molecule.

Figure 9 shows the course of liberation of nonprotein nitrogen from chicken myosin-R and myosin-W after tryptic digestion. This clearly indicated a slower action of trypsin on myosin-R than on myosin-W.

Optical Rotatory Dispersion. Because of the striking differences upon tryptic digestion of chicken myosin-R and myosin-W, the optical rotatory dispersion method was employed to study and compare the conformation of these proteins.

The front, middle, and trailing fractions of the myosin peak from DEAE-Sephadex A-50 column chromatography were dialyzed against 0.5 M KCl–0.01 M phosphate (pH 7.5) and centrifuged at 27,000*g* for 1 hr. The optical rotation was measured as described in the experimental section. The results, as analyzed by Drude and Moffitt–Yang equations, are presented in Table VI together with the data obtained for unchromatographed rabbit myosin prepared from back muscle (kindly supplied by Dr. E. G. Richards). The values of $[\alpha]_{578\ m\mu}$ and b_0 for rabbit myosin were essentially the same as those reported by Lowey and Cohen (1962), but λ_c was slightly higher than that found by Young *et al.* (1962), 309 *vs.* 290. These data indicated that there was no significant difference in helical content between chicken myosin-R and myosin-W, although the trailing fraction of myosin-R showed slightly lower values. Furthermore, the amount of helix of chicken and rabbit myosins was very similar.

The calculation of helical content by $[\alpha]_D$ (not shown) assuming +5° for 100% helix and −90° for random coil (Young *et al.*, 1962), and the estimation on the basis of b_0, assuming $b_0 = -630$ for 100% helix, gave a similar value, about 65% for both chicken and rabbit myosins.

TABLE VI: Optical Rotatory Dispersion of Myosins.

Myosin	$[\alpha]^{25}_{578\ m\mu}$	λ_c	a_0	b_0
Rabbit back myosin	−28.9	310	−114	−404
Chicken myosin-R_f[a]	−28.3	309	−111	−408
Chicken myosin-R_m	−28.3	309	−111	−408
Chicken myosin-R_t	−30.4	303	−122	−406
Chicken myosin-W_f	−28.0	309	−108	−419
Chicken myosin-W_m	−28.0	309	−106	−411
Chicken myosin-W_t	−28.0	309	−106	−411

[a] Subscript f denotes fractions from front, m from middle, and t from tail of the myosin peak on DEAE-Sephadex chromatography.

Discussion

The extractability of myosin from breast and leg muscles of chicken or from white and red muscles of rabbit showed significant differences. Barany *et al.* (1965) reported that the myosin content in red and white muscles of rabbit was the same. The present study, however, dealt with the short-time extractable myosin rather than the total myosin content. Connective tissue alone could not account for the low proportional yield of myosin-R since separable connective tissue was removed before extraction. The high fat content in red muscles may have retarded buffer penetration into the site of myosin, but washing with dimethyl sulfoxide (Buttkus, 1966) did not improve the extractability. Briskey *et al.* (1962) reported that the dark section of semitendinosus of pork underwent rigor faster than the light section of the same muscle. Since the extractability of myosin decreases as post mortem time increases (Sayre, 1968), the difference in extractability of myosin-R and myosin-W might be due to the different rates in post mortem changes. This problem is currently under investigation.

Unlike rabbit and pigeon myosins, chicken myosin-R and myosin-W did not show large differences in ATPase, CTPase, ITPase, and UTPase activities. This difference in ATPase activity may reflect the physical activity and genetic difference of the animal. In an active animal or bird which can maintain sustained movement, the activities between fast and slow muscles may differ significantly. In a bird such as the chicken, the muscle activity is limited, so that the difference between fast and slow muscles may have diminished. Perry (1964) suggested that myosin may be an adaptive enzyme which increases in amount and specific activity in response to physical activity. However, it is not known whether this adaptation is at the level of genetic control or merely a modification after it is synthesized. In this connection it is interesting to note that the fiber type of pectoralis muscle of birds depended upon the activity of the bird (George and Berger, 1966). In chickens which have limited flight ability, the white fibers predominate, while in sparrows which are capable of sustained flight, only red fibers are present.

In previous studies (Richards *et al.*, 1967; Chung *et al.*, 1967), it was noticed that the myosin peak on

DEAE-Sephadex column chromatography was asymmetrical, and broader than that expected for a homogeneous protein. However, rechromatography of leading and trailing fractions failed to detect any differences. The present results also showed that myosin-R and myosin-W exhibited the same chromatographic properties. Nevertheless, these two proteins differed in ATPase activity, rate of tryptic hydrolysis, solubility, and ultraviolet absorption properties. Therefore it is possible that the asymmetrical and broad peak of myosin on DEAE-Sephadex column chromatography was due to the presence of multiple forms of myosin. Since different types of fibers may exist in a single muscle, the myosin-R and myosin-W prepared in this study and by other workers may represent mixtures of different forms, with myosin-R predominant in one type of muscle and myosin-W in another. Since myosin is not soluble in the low ionic strength buffers generally used in electrophoresis, it was not possible to detect heterogeneity by this method although it was attempted.

The ratio of ATPase activities of myosin-R and myosin-W did not change after chromatography. This indicated that the lower activity of myosin-R is not due to a larger content of impurities. Maddox and Perry (1966) reported that different characteristics of pigeon breast and leg myosins also persisted after DEAE-cellulose chromatography.

Although the differences in extinction coefficient and tyrosine content between these myosins were very small, it may reflect microheterogeneity of these proteins. Trayer *et al.* (1968) have found that a microconstituent of myofibrillar proteins, 3-methylhistidine, was lower in foetal myosin than that in myosin isolated from white skeletal muscle of rabbit.

The spectrophotometric titration curve in the present study did not agree with that of Stracher (1960). However, it was very similar to that found by Lowey and Kucera (1964) and Lowey (1965). No explanation can be found for this difference at the present time.

The solubility of myosin depends upon ionic strength, type of anions and cations, as well as pH of the solvent (Fukui, 1957; Brahms and Brezner, 1961; Johnson and Rowe, 1961). The present study showed that under certain conditions, the solubilities of chicken myosin-R and myosin-W were different, suggesting that these two proteins might be structually distinct. It is unlikely that the differences in solubility were due to contamination with myosin-B, since the magnesium-activated ATPase activity was negligible and the myosin-B test (Rice *et al.*, 1963) was negative for both proteins. Aggregated or aged myosin may also alter the solubility (Johnson and Rowe, 1961), but aggregates could not be detected in the ultracentrifuge. An experiment with aged myosin (2-weeks old) still showed similar differences in solubility.

The results of tryptic digestion further suggested structural dissimilarities between chicken myosin-R and myosin-W. Gergely *et al.* (1965) and Maddox and Perry (1966) also reported differences in tryptic digestion of myosin-R and myosin-W from rabbit and pigeon, respectively. From the fact that several proteolytic enzymes of different specificity induce cleavage in the same region of the myosin molecule in the early stages of hydrolysis (Mihalyi and Szent-Gyorgyi, 1953; Gergely *et al.*, 1955; Kominz *et al.*, 1965), it was hypothesized that the proteolytic sensitive region is an unfolded polypeptide segment of the myosin rod (Mihalyi and Harrington, 1959). More recently, Segal *et al.* (1967) showed that in the early stages of tryptic and chymotryptic digestion, a large amount of proline was liberated, suggesting that this enzyme-sensitive region is a relatively open, nonhelical structure. The results on tryptic digestion of chicken myosin-R and myosin-W, therefore, would suggest that myosin-R might have less unfolded region, thus was less susceptible to the proteolytic enzyme. The optical rotatory dispersion data, however, showed that the helical content of these proteins was essentially the same. Although it is possible that a small difference in conformation may not be detected by this method, it is likely that the primary structure of myosin-R in this region is different from that of myosin-W. This can be studied by cleavage with other proteolytic enzymes of different specificities, or by peptide mapping of the nonprotein fraction of the tryptic digested myosin. Analysis of amino acid composition was not attempted since small differences in this parameter would be difficult to assess for a molecule as large as myosin.

Over-all, myosins isolated from white and red muscles of chicken were structurally different, possibly both at the enzymatically active site and at the region sensitive to proteolytic enzymes. Further fractionation of chicken myosin-R and myosin-W is under way, in the hope that a preparation of more homogeneous protein may reveal more specific information on structural differences.

Acknowledgments

The author is grateful to Dr. H. Lineweaver and Mr. A. A. Klose, in whose laboratory this work was carried out, to Dr. R. N. Sayre for his valuable discussions and help in many aspects, to Drs. H. S. Olcott and E. G. Richards for useful suggestions, and to Mr. D. Rockholt for his assistance in separating rabbit muscles.

References

Barany, M. (1967), *J. Gen. Physiol. 50*, 197.

Barany, M., Barany, K., Reckard, T., and Volpe, A. (1965), *Arch. Biochem. Biophys. 109*, 185.

Beavan, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Brahms, J., and Brezner, J. (1961), *Arch. Biochem. Biophys. 95*, 219.

Briskey, E. J., Sayre, R. N., and Cassens, R. G. (1962), *J. Food Sci. 27*, 560.

Buttkus, H. (1966), *J. Fish Res. Can. 23*, 563.

Chung, C.-S., Richards, E. G., and Olcott, H. S. (1967), *Biochemistry 6*, 3154.

Cooper, S., and Eccles, J. C. (1930), *J. Physiol. 69*, 377.

Fukui, S. (1957), *Sapporo Med. J. 11*, 60.

George, J. C., and Berger, A. J. (1966), Avian Myology, New York, N. Y., Academic.

Gergely, J. (1953), *J. Biol. Chem. 200*, 543.

Gergely, J., Goueva, M. A., and Karibian, D. (1955), *J. Biol. Chem. 212*, 165.
Gergely, J., Pragay, D., Scholz, A. F., and Seidel, J. C. (1965), *in* Molecular Biology of Muscular Contraction, Ebashi, S., Oasawa, F., Sekine, T., and Tonomura, Y., Ed., Tokyo, Igaku Shoin, p 145.
Hasselbach, W., and Schneider, G. (1951), *Biochem. Z. 321*, 462.
Johnson, P., and Rowe, A. J. (1961), *Biochim. Biophys. Acta 53*, 343.
Kominz, D. R., Mitchell, E. R., Nihei, T., and Kay, C. M. (1965), *Biochemistry 4*, 2373.
Lowey, S. (1965), *J. Biol. Chem. 240*, 2421.
Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.
Lowey, S., and Kucera, J. (1964), *in* Biochemistry of Muscle Contraction, Gergely, J., Ed., Boston, Mass., Little, Brown, p 8.
Maddox, C. E. R., and Perry, S. V. (1966), *Biochem. J. 99*, 8P.
Mihalyi, E., and Harrington, W. F. (1959), *Biochim. Biophys. Acta 36*, 447.
Mihalyi, E., and Szent-Gyorgyi, A. G. (1953), *J. Biol. Chem. 201*, 189.
Moffitt, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.
Mueller, H., Theiner, M., and Olson, R. E. (1964), *J. Biol. Chem. 329*, 2153.
Perry, S. V. (1955), *Methods Enzymol. 2*, 582.
Perry, S. V. (1964), *in* Muscle, Paul, W. M., Daniel, E. F., Kay, C. M., and Monekton, C., Ed., Oxford, Pergamon, p 29.
Rice, R. V., Asai, H., and Morales, M. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 549.
Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S. (1967), *Biochemistry 6*, 528.
Sayre, R. N. (1968), *J. Food Sci.* (in press).
Segal, D. M., Himmelfarb, S., and Harrington, W. F. (1967), *J. Biol. Chem. 242*, 1241.
Seidel, J. C. (1967), *J. Biol. Chem. 242*, 5623.
Stracher, A. (1960), *J. Biol. Chem. 235*, 2302.
Sreter, F. A., Seidel, J. C., and Gergely, J. (1966), *J. Biol. Chem. 241*, 5772.
Tonomura, Y., Appel, P., and Morales, M. (1966), *Biochemistry 5*, 515.
Trayer, I. P., Harris, C. I., and Perry, S. V. (1968), *Nature 217*, 452.
Woods, E. F., Himmelfarb, S., and Harrington, W. F. (1963), *J. Biol. Chem. 238*, 2374.
Young, D. M., Harrington, W. F., and Kielley, W. W. (1962), *J. Biol. Chem. 237*, 3116.