TABLE II
CALCULATION OF KINETIC CONSTANT K_0 BY MEANS OF EQUATIONS (9) OR (10)

Salt	K_0 (obs)	K_0 (calcd)	K_0 (calcd)
$(CH_3)_4NBr$	−0.08	−0.07	−0.07
KCH_3COO	−0.08	−0.13	−0.13
KCl	−0.42	−0.24	−0.25
NaCl	−0.41	−0.27	−0.28
CsCl	−0.52	−0.30	−0.32
LiCl	−0.66	−0.69	−0.78
$CaCl_2$	−1.82	−1.48	−1.98
KSCN	−2.85	−1.68	−2.35

column is calculated from the approximate relation, equation (10), while the last column is calculated from equation (9) using 0.5 mole/liter for the salt concentration. It is clear from the data in Table II that rather good agreement is obtained between the calculated and observed results. The agreement is perhaps more significant in that the parameter α was determined for another type collagen, and the constant describing the rate was calculated solely from melting point data.

We can conclude, therefore, that a consistent quantitative analysis of the effect of the neutral salts is obtained for both the equilibrium melting temperature and the reversion kinetics by invoking a direct binding process for melting and the Flory-Weaver (1960) mechanism for the reversion kinetics.

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Studies on the Interaction of Actin with Myosin A*

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The interaction of myosin A and actin was investigated in the presence and absence of various chemical modifiers and with ultraviolet-irradiated myosin A. These studies suggested that the occupancy of an inhibitory group by actin is necessary for the enzymatic interaction between these two proteins. The results obtained with the ultraviolet-irradiated myosin A substantiated the participation of "more than one" group on the active center of the myosin A in the enzymatic interaction of this protein with various modifiers. Physical interaction between myosin A and actin was found to occur also in the absence of any enzymatic interaction between these two proteins.

The association of actin and myosin A at low ionic strength was found to induce a complex change in the enzymatic behavior of the latter (Banga and Szent-Gyorgyi, 1943; Hasselbach, 1952, 1957). Since there was much experimental evidence to show that if ATP and ITP were present together they competed for the same enzyme sites of myosin A (Gergely, 1953; Levy *et al.*, 1962), it was interesting to observe that the association of actin to myosin influenced the splitting of these two substrates rather differently (Kaldor and Gitlin, 1963). A number of compounds were found to accelerate the ATPase activity of myosin A in the presence of Ca^{2+} or Mg^{2+} while ITPase activity was inhibited. Limited similarity in the enzymatic effect of actin and these compounds was thereby suggested. It was shown that DNP competed with actin for the same enzyme sites of myosin A (Chappel and Perry, 1955) and that *p*-mercuribenzoate and DNP did not compete for the same myosin A site, while they stimulated the ATPase in the presence of Mg^{2+} (Levy and Ryan, 1961). We have therefore felt that a systematic study of actin-myosin interaction in the presence and absence of certain chemical modifiers, before and after ultraviolet irradiation–induced molecular changes, may be useful in shedding more light on the nature and function of the enzyme sites involved in the splitting of ATP and ITP.

* Supported by a grant (B-3600) from the National Institutes of Health, U. S. Public Health Service.

EXPERIMENTAL PROCEDURES

Myosin A and actin were prepared as described by Mommaerts (1958). Myosin A was purified three times by reprecipitation and actin was purified twice by polymerization and centrifugation. Only polymerized actin (F actin) was used in this study.

Myofibrillar suspensions were prepared as previously described (Gergely *et al.*, 1959; Kaldor, 1960).

ATP and ITP were purchased from Sigma Chemical Co., St. Louis, Mo. In this work no attempt was

made to establish a Ca^{2+}-free system. Neither the tightly bound Ca^{2+} (intrinsic Ca^{2+}) from the muscle models nor the Ca^{2+} contamination of the commercially available ATP preparations as found by Seidel and Gergely (1963) were removed. As far as the relative changes in our ATP-splitting experiments were concerned, different ATP batches gave identical results.

All other reagents used were of reagent grade. All splitting experiments were carried out at 37°. Buffers used were 0.05 M histidine at *p*H 6.0, and 0.05 M Tris at *p*H 7.5 and 9.0. The total volume of incubation mixtures was 1.0 ml in all experiments. Reaction time varied between 2 and 5 minutes. In each case splitting was linear with time and the amount of substrate split was below 40%. The technique of ATPase assay was described previously (Gergely *et al.*, 1959). Phosphate was determined by the Fiske-Subarrow (1925) method except for those experiments where V_m and K_m values were calculated. For the determination of these kinetic constants at least seven different substrate concentrations were used (5×10^{-5}–5×10^{-3} moles/liter). Phosphate was assayed with the Berenblum-Chain (1938) method and V_m and K_m values were calculated from a Lineweaver-Burk (1934) plot.

Viscosity measurements were performed at 27° in an Ostwald-type viscometer with an outflow time of 80 seconds, in a total volume of 5.0 ml at 1.5 mg/ml myosin A, 0.5 mg/ml actin concentration in a *p*H 7.5, 0.6 M KCl solution to which 5 μmoles/ml Ca^{2+} was added. The actomyosin-forming ability was calculated from the equation of Bailey and Perry (1947), i.e., actomyosin-forming ability (%) $= 1 - [(C - D)/(A - B)]100$, where A = relative viscosity of myosin A plus actin, B = relative viscosity of myosin A plus actin plus ATP, C = relative viscosity of myosin A, D = relative viscosity of myosin A plus ATP.

Superprecipitation was measured in a 0.05 M, *p*H 7.5 Tris buffer at 1.0 mg/ml myosin and 0.05 mg/ml actin concentrations in 2.0 ml total volume to which 0.75 μmole/ml ATP was added. The performance and evaluation of these experiments were published earlier (Kaldor and Gergely, 1959).

Ultraviolet irradiation was performed in a cold room (2–4°) on a 3.2-mm thick, 10-mg/ml myosin A layer under constant slow mechanical stirring as described in an earlier paper (Kaldor *et al.*, 1962). Myosin A was dissolved in a solution containing 0.5 M KCl and 0.1 M Tris buffer at *p*H 7.5. The output of the G15T8 General Electric germicidal lamp was measured by a dose-rate meter (Jagger, 1961) and was found to be 8.54×10^{14} quanta/cm^2 sec.$^{-1}$ All samples were spun after irradiation in a preparative ultracentrifuge at 20,000 rpm for 45 minutes.

Sedimentation coefficients were determined with the Spinco Model E analytical ultracentrifuge at 50,740 rpm at 20° in a buffer containing 0.5 M KCl and 0.1 M Tris at *p*H 7.5. Since the irradiated myosin A samples, unlike native myosin A, were inhomogeneous the sedimentation coefficients were determined from photographs taken within the first 24 minutes. During this period all samples investigated showed one peak. The sedimentation coefficients thus obtained were extrapolated to zero concentration. These values do not permit us to draw absolute quantitative conclusions but it was thought that they could perhaps be used to describe the ultraviolet irradiation–induced physical changes in a semiquantitative manner.

Optical rotation measurements were carried out with a Rudolph Type 80 polarimeter at 546 mμ wavelength. Myosin A solutions (7.5 mg/ml) were used for these measurements, which were performed in a 200-mm polarimeter tube.

Electrophoresis and diffusion experiments were carried out with a Beckman Model H electrophoresis instrument. A 0.5 g/100 ml myosin solution (11 ml), dissolved in a buffer containing 0.5 M KCl and 0.1 M Tris at *p*H 7.5, was used for these experiments. The duration of the electrophoresis experiments was 6 hours at 20 milliamps and the diffusion experiments were run for 24 hours. The diffusion coefficients were calculated by a plot of the diffusion time against the corrected ratio of the area to the maximum height of the diffusion curve from the equation of Lundgren and Ward (1951). This was corrected for the temperature. Diffusion coefficients were determined at various myosin A concentrations and extrapolated to zero concentration.

Molecular weights were calculated from Svedberg (1940) and from an equation described by Mandelkern and Flory (1952) and Scheraga and Mandelkern (1953). This equation is as follows:

$$\beta = \frac{s_{20}[\eta]^{1/3}N_0\eta}{M^{2/3}(1 - \bar{V}\rho)}$$

where s_{20} = sedimentation coefficient, $[\eta]$ = intrinsic viscosity, N_0 = Avogadro's number, η = viscosity of the solvent, $\bar{V}$ = partial specific volume of the solute, ρ = density of the solvent, $\beta = 3.14 \times 10^6$ (Scheraga and Mandelkern, 1953).

The determination of the free reacting SH-groups of myosin A was done with the method of Boyer (1954). The procedure of Beaven and Holiday (1952) was used for the determination of tyrosine and tryptophane. The method of Weiss-Sobolew (1913–14) was employed for the histidine assay. This method is based on the reaction of the diazotized-sulphanilic acid with the histidine and tyrosine groups of the protein in the presence of 10% sodium carbonate, in which the color was read at 530 mμ. The contribution of tyrosine groups was calculated from a tyrosine standard curve and the amounts of histidine groups was calculated from a histidine standard curve. The number of tyrosine groups was determined separately (see above). The protein concentrations were determined with the procedure of Lowry *et al.* (1951).

Results

Fluharty and Sanadi (1962) found that AsBAL[1] in the 0.1 mM concentration range enhanced the Ca^{2+}-stimulated ATPase activity of myosin A and myosin B in the presence of 0.3 M KCl, while the ITPase activity of the same enzyme was inhibited. Using myofibrillar suspensions as the enzyme, we studied the AsBAL effect at various KCl concentrations (Fig. 1). At low salt concentration AsBAL caused only inhibition. Its activating effect on the Ca^{2+}- and Mg^{2+}-stimulated ATPase appeared only at high salt concentration where myosin A and actin are dissociated in the presence of ATP. These findings suggested that AsBAL stimulated the ATPase activity of the myosin A enzyme and inhibited the ATPase of the actomyosin enzyme. To obtain more information about the activation the effect of AsBAL on myosin A was studied at three different *p*H values (Fig. 2). The results were similar to those obtained by Stracher and Chan (1961) with *p*-mercuribenzoate. The activation was present only at *p*H 7.5, not at *p*H 6.0 or 9.0. Under similar conditions the ITPase activity of myosin A was inhibited at all *p*H values investigated (Fig. 3). We have found previously

[1] Abbreviations used in this work: AsBAL, arsenite and British antilewisite, always in an equal concentration, as indicated.

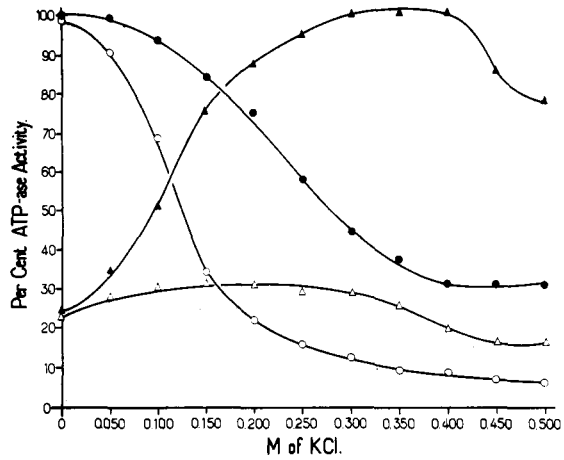

FIG. 1.—Effect of AsBAL on the ATPase activity of myofibrills at various salt concentrations; 37°, *p*H 7.5. Each incubation mixture contained 5 μM ATP, about 1 mg myofibrillar protein, and 50 μM Tris in 1.0 ml total volume to which the following additions were given: ●, 5 μM Ca^{2+}; ○, 5 μM Mg^{2+}; ▲, 5 μM Ca^{2+} and 0.25 μM AsBAL; △, 5 μM Mg^{2+} and 0.25 μM AsBAL.

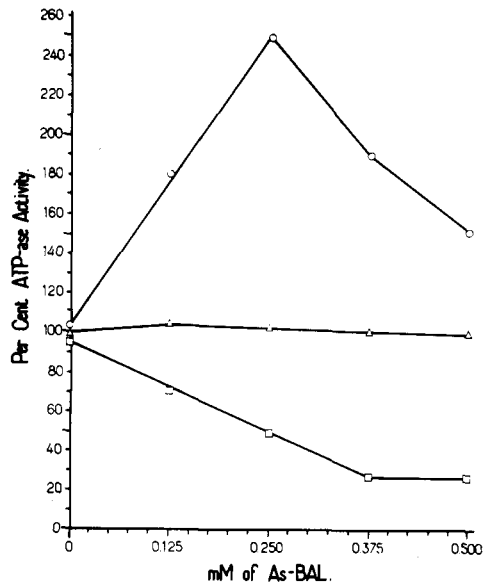

FIG. 2.—Effect of AsBAL on the ATPase of myosin A at various *p*H values. Every sample contained 5 μM ATP, 5 μM Ca^{2+}, and 0.15 mg myosin A in 1.0 ml total volume to which the following additions were made: ○, 50 μM Tris (*p*H 7.5); △, 50 μM Tris (*p*H 9.0); □, 50 μM histidine (*p*H 6.0).

that actin depressed the ITPase and accelerated the ATPase activity of myosin A in the presence of Ca^{2+} (Kaldor and Gitlin, 1963). Since AsBAL acted similarly in this respect, we decided to study the actin effects at the same *p*H values where the AsBAL effect has been investigated. Results are summarized in Figures 4 and 5. It may be seen that, while Ca^{2+}-activated ATPase was stimulated only at *p*H 7.5, the Ca^{2+}-activated ITPase was depressed equally well at *p*H 7.5 and 9.0 and to some extent also at *p*H 6.0 by actin. The Mg^{2+}-stimulated ATPase and ITPase were accelerated best at *p*H 7.5 and only to a much smaller extent at the other two *p*H values. The similar *p*H characteristic of actin and AsBAL effects on Ca^{2+}-activated ATPase and ITPase of myosin A suggested that perhaps the same group on the active site of the myosin A molecule is involved in both cases. We therefore simultaneously investigated the effects of actin and AsBAL on the ATPase and ITPase activity of myosin A using Ca^{2+} and Mg^{2+} as the activators. Results are summarized in Table I. As can be seen, the stimulating effect of AsBAL and actin on the Ca^{2+}-moderated ATPase activity and their inhibiting effect on the Ca^{2+}-stimulated ITPase activity were additive. Comparing the simultaneous effects of these modifiers, if Mg^{2+} was used as activator, with those of the Ca^{2+}-activated

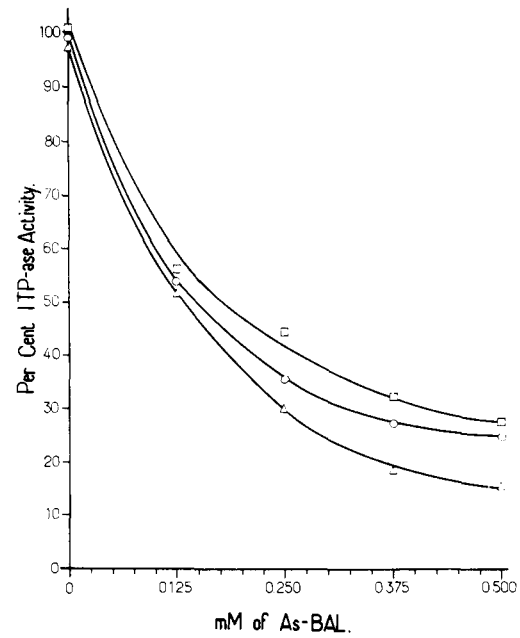

FIG. 3.—Effects of AsBAL on the ITPase activity of myosin A at various *p*H values at 37°. Every sample contained 5 μM ITP, 5 μM Ca^{2+}, and 0.15 mg myosin A in 1.0 ml total volume. Additions similar to Fig. 2.

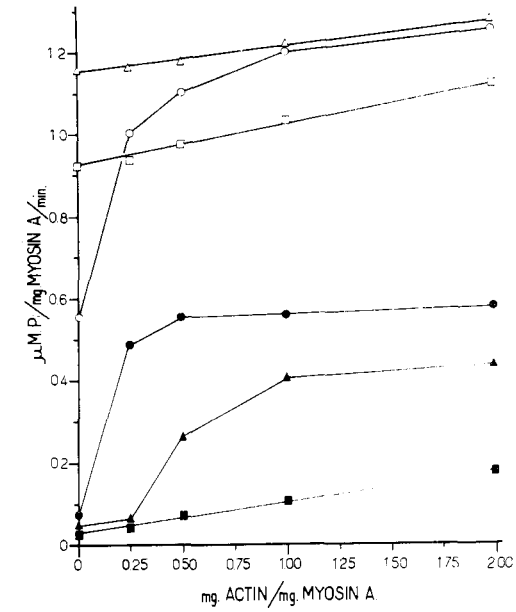

FIG. 4.—Effect of actin on the ATPase activity of myosin A at various *p*H values. Each incubation mixture contained 5 μM ATP and 0.15 mg myosin A in 1.0 ml total volume, to which the following additions were made: □, 5 μM Ca^{2+} and 50 μM histidine (*p*H 6.0); ○, 5 μM Ca^{2+} and 50 μM Tris (*p*H 7.5); △, 5 μM Ca^{2+} and 50 μM Tris (*p*H 9.0); ■, 5 μM Mg^{2+} and 50 μM histidine (*p*H 6.0); ●, 5 μM Mg^{2+} and 50 μM Tris (*p*H 7.5); ▲, 5 μM Mg^{2+} and 50 μM Tris (*p*H 9.0).

TABLE I
THE SIMULTANEOUS EFFECTS OF ACTIN AND AsBAL ON THE ATPASE AND ITPASE ACTIVITIES OF MYOSIN A

Substrate (5 μM)	Activator (5 μM)	Added: Actin (mg)/ Myosin A (mg)	Added: AsBAL (mM)	P_i Split (μM P_i/mg min^{-1})	Relative Activity (%)
ATP	Mg^{2+}	0	0	0.048	100
ATP	Mg^{2+}	0.5	0	0.480	1000
ATP	Mg^{2+}	0	0.25	0.048	100
ATP	Mg^{2+}	0.5	0.25	0.240	500
ITP	Mg^{2+}	0	0	0.070	100
ITP	Mg^{2+}	0.5	0	0.280	400
ITP	Mg^{2+}	0	0.25	0.035	50
ITP	Mg^{2+}	0.5	0.25	0.070	100
ITP	Mg^{2+}	0	0.05	0.070	100
ITP	Mg^{2+}	0.5	0.05	0.147	210
ATP	Ca^{2+}	0	0	0.850	100
ATP	Ca^{2+}	0.15	0	1.104	130
ATP	Ca^{2+}	0	0.05	1.148	135
ATP	Ca^{2+}	0.15	0.05	1.740	205
ATP	Ca^{2+}	0.5	0	1.700	200
ATP	Ca^{2+}	0	0.25	2.030	238
ATP	Ca^{2+}	0.5	0.25	1.910	225
ITP	Ca^{2+}	0	0	1.820	100
ITP	Ca^{2+}	0.15	0	0.545	30
ITP	Ca^{2+}	0	0.05	0.815	45
ITP	Ca^{2+}	0.15	0.05	0.366	21
ITP	Ca^{2+}	0.075	0	1.130	62
ITP	Ca^{2+}	0	0.05	1.000	55
ITP	Ca^{2+}	0.075	0.05	0.545	30

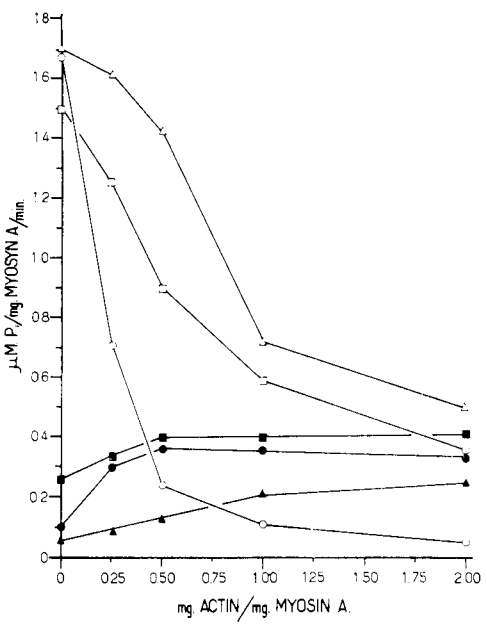

FIG. 5.—Effect of actin on the ITPase activity of myosin A. Each incubation mixture contained 5 μM ITP and 0.15 mg myosin A in 1.0 ml total volume. Additions and symbols as in Fig. 4.

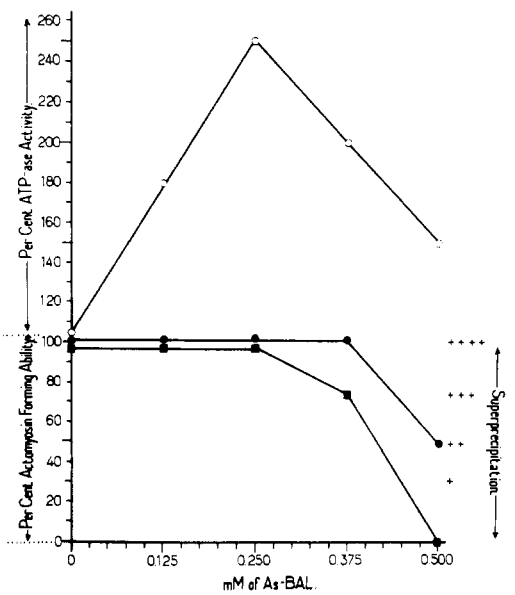

FIG. 6.—Effect of AsBAL on the ATPase of myosin A and on the actomyosin-forming ability and superprecipitation of actomyosin. ○, ATPase activity. Incubation mixture contained 5 μM ATP, 5 μM Ca^{2+}, 500 μM KCl, 50 μM Tris (*p*H 7.5), and 0.15 mg myosin A in 1.0 ml; ●, actomyosin-forming ability (for details see text); ■, superprecipitation.

mixtures, it appears that AsBAL acts with actin in the presence of Ca^{2+} and against actin in the presence of Mg^{2+}. These results supported our assumption that, with regard to their effects on the Ca^{2+}-activated ATPase and ITPase of myosin A, actin and AsBAL were competing for the same enzyme site. Furthermore, the results also suggest that the same group may play an important role in the effect of Mg^{2+} on the enzyme. The effect of this competition on the physical interaction of actin with myosin was then investigated. For this purpose the actomyosin-forming ability of myosin A and actin at high ionic strength was estimated by means of viscometry, and the superprecipitation effect of ATP on actomyosin at low ionic strength was determined in the presence and absence of various amounts of AsBAL. Results (Fig. 6) have shown that in the presence of 0.25 mM AsBAL no change was observed in the actomyosin-forming ability or superprecipitation, although the ATPase activity of myosin A was accelerated by 150%. At 0.50 mM AsBAL concentration, the physical effects of actin appeared to be inhibited, however the precipitation of AsBAL at this concentration introduced some uncertainty in regard to the specificity of these latter findings. Nevertheless, these results proved that the occupancy of the myosin A group by AsBAL, which was shown to be important in the enzymatic interaction of actin and

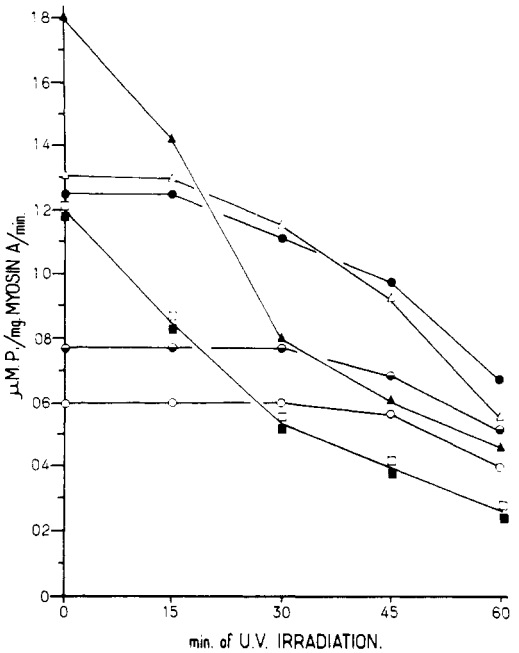

FIG. 7.—Effect of ultraviolet irradiation on the enzymatic activities of myosin A. Each sample contained 0.15 mg myosin A (irradiated as indicated) in 1.0 ml total volume to which the following additions were made: ○, 5 μM ATP + 5 μM Ca^{2+} and 50 μM Tris (*p*H 7.5); ◒, 5 μM ATP + 5 μM Ca^{2+} and 50 μM histidine (*p*H 6.0); ●, 5 μM ATP + 5 μM Ca^{2+} and 50 μM Tris (*p*H 9.0); ▲, 5 μM ITP + 5 μM Ca^{2+} and 50 μM Tris (*p*H 7.5); △, 5 μM ATP + 5 μM Ca^{2+} + 5 μM DNP and 50 μM Tris (*p*H 7.5); □, 5 μM ATP + 5 μM Ca^{2+} + 0.25 μM AsBAL and 50 μM Tris (*p*H 7.5); ■, 5 μM ATP + 5 μM Ca^{2+} + 0.05 μM *p*-mercuribenzoate and 50 μM Tris (*p*H 7.5).

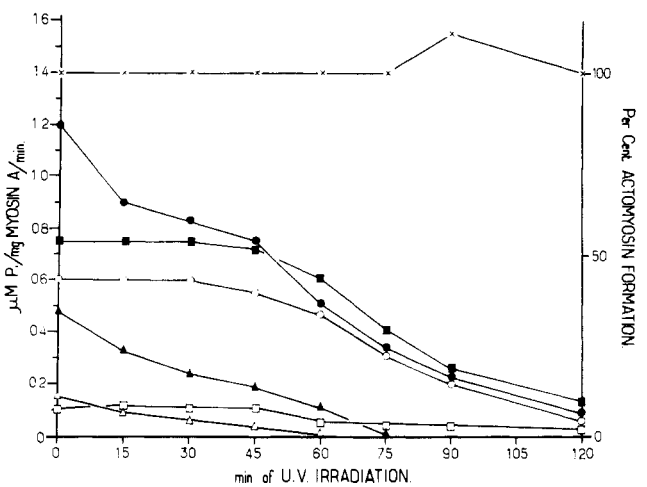

FIG. 8.—Effect of ultraviolet irradiation on the enzymatic activities of myosin A. Each sample contained 0.15 mg myosin A (irradiated as indicated) and 50 μM Tris (*p*H 7.5) in 1.0 ml total volume to which the following additions were made: ○, 5 μM ATP and 5 μM Ca^{2+}; ●, 5 μM ATP + 5 μM Ca^{2+} and 0.05 mg actin; □, 5 μM ATP and 5 μM Mg^{2+}; ■, 5 μM ATP + 5 μM Mg^{2+} and 0.05 mg actin; △, 5 μM ITP and 5 μM Mg^{2+}; ▲, 5 μM ITP + 5 μM Mg^{2+} and 0.05 mg actin; ×, actomyosin-forming ability.

myosin A, was not inhibitory with regard to the physical interaction of the two proteins.

It appears to be well established that the enzyme-modifying effect of *p*-mercuribenzoate, AsBAL, and probably DNP is mediated by –SH groups on or close to the active center of the myosin A molecule (Kielley

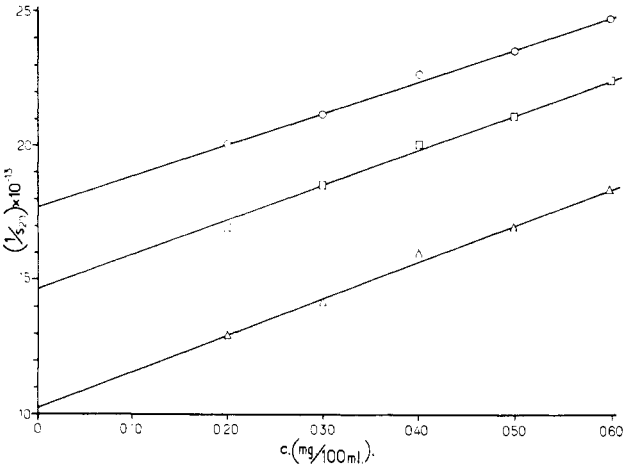

FIG. 9.—Concentration dependence of the reciprocal of the sedimentation coefficient $1/s_{20}$ of native myosin A (○), 30-minute-irradiated myosin A (□), and 60-minute-irradiated myosin A (△).

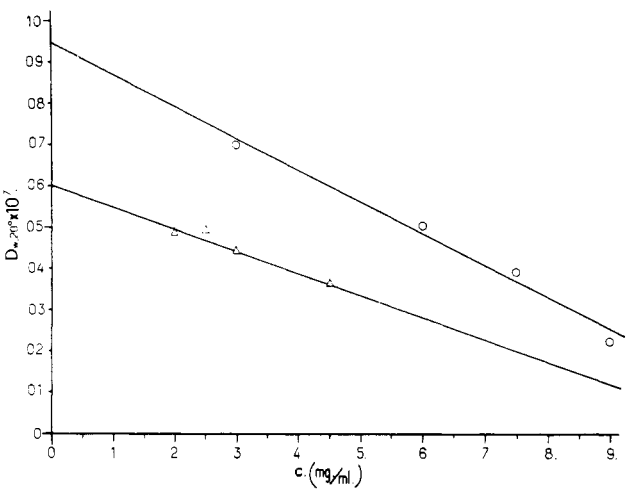

FIG. 10.—Concentration dependence of the reciprocal of the diffusion coefficient $1/D_{20}$ of native myosin A (○) and 60-minute-irradiated myosin A (□).

and Bradley, 1956; Fluharty and Sanadi, 1962; Blum, 1960). However, it is still unknown whether or not the same protein –SH groups are involved in the action of all these compounds. It is also uncertain whether or not these SH groups are directly involved in the ATP splitting. It was felt that further investigation was needed in order to furnish more experimental evidence as to whether one or more groups on the myosin A enzyme are involved in the effects of the various modifiers and in the characteristic *p*H behavior of the myosin A ATPase. Several physical techniques known to induce molecular alterations on the myosin were employed. The behavior of various modifiers and the enzymatic activity of the modified myosin A molecule at various *p*H values were also investigated. For our purpose the use of ultraviolet irradiation proved to be the most fruitful. Since ultraviolet irradiation has not been widely used to modify myosin A, a number of physical and chemical analyses were performed in order to obtain some information on the alterations caused by this treatment. The effects of ultraviolet irradiation on the enzymatic pattern of myosin A are shown in Figures 7 and 8. The s_{20} values were calculated from Figure 9, the D_{20} values from Figure 10, and the $[\eta]$ values from Figure 11. The results of the various physical and chemical analyses are compiled in Table II.

TABLE II
SOME PHYSICOCHEMICAL EFFECTS OF ULTRAVIOLET IRRADIATION OF MYOSIN A

	Ultraviolet Irradiation (min)				
	0	15	30	45	60
End groups (μM/10^5 g myosin A)					
Tryptophan	4.3	4.2	4.2	4.1	4.1
Tyrosine	12.8	14.2	14.2	14.1	14.3
Histidine	16.1	15.1	13.2	11.3	11.0
—SH	4	3.8	3.6	3.3	2.8
Sedimentation velocity (Svedberg units)	5.65		6.90		9.70
Diffusion constant ($cm^2\ sec^{-1} \times 10^7$)	0.946				0.609
Molecular weight, sedimentation diffusion	540,000				1,400,000
Intrinsic viscosity (dl/g)	2.14		2.17		3.5
Molecular weight, sedimentation viscosity	400,000		500,000		1,100,000
Electrophoretic mobility ($10^{-5}\ cm^2$/volt sec^{-1})	3.79				3.10
Optical rotation (λ) 5461	28		40		64

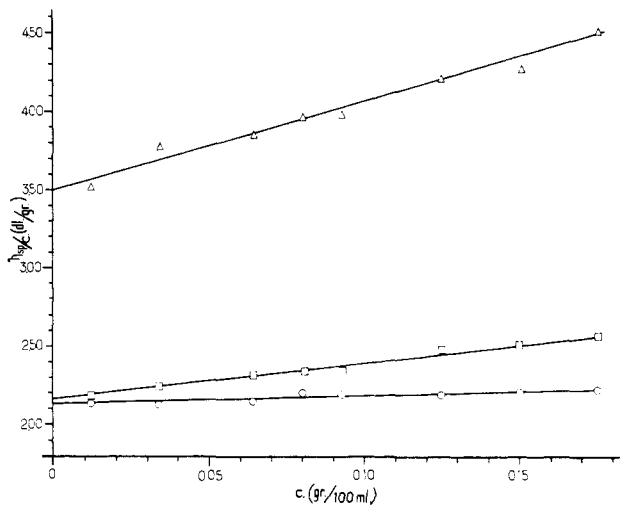

FIG. 11.—Reduced specific viscosity [η] of native myosin A (○), 30-minute-irradiated myosin A (□), and 60-minute-irradiated myosin A (△).

The s_{20} value of 5.65 S for native myosin A was somewhat lower than the average result of 6.5 reported in the literature, and the D_{20} of 0.94 agrees with the average value of previous workers, i.e., of 1.05 as calculated by Kielley and Harrington (1960). The intrinsic viscosity of the native myosin A was in good agreement with the previously reported results of Holtzer and Lowey (1959). While the intrinsic viscosity of the 30-minute irradiated myosin A showed little change except for a somewhat increased concentration dependence, the 60-minute-irradiated sample showed a considerable increase in its intrinsic viscosity value. This latter value of 3.5 dl/g, was slightly higher than the 3.1 dl/g figure obtained by Lowey and Holtzer (1959) with myosin A dimers. The molecular weight of the native myosin A, as calculated by the Svedberg and Pedersen (1940) equation, was within the limits obtained by previous workers; i.e., Holtzer and Lowey (1959), 500,000; Kielley and Harrington (1960) and Woods *et al.* (1963), 600,000, with myosin A monomers. Values of molecular weights calculated on the basis of the viscosity and sedimentation data (Scheraga and Mandelkern, 1953) were lower than those obtained from sedimentation and diffusion measurements. The irradiated myosins showed faster sedimentation and slower diffusion (Fig. 12). The 60-minute-irradiated sample showed shoulder formation and a much wider peak than native myosin in the analytical ultracentrifuge after about 40 minutes of centrifugation at 50,740 rpm (Fig. 12). The quantitative reproducibility of this pattern was not as good as those obtained with native myosin A. All myosin samples showed one peak during 5–6 hours of electrophoresis.

In regard to the enzymatic effects of the ultraviolet irradiation, it was found that after 30 minutes *p*-mercuribenzoate and AsBAL completely lost their stimulatory effect on the Ca^{2+}-activated ATPase at *p*H 7.5, while the activating effect of DNP under similar conditions decreased only to a much smaller extent (Fig. 7). The 30-minute-irradiated specimen, like native myosin A, showed higher ATPase activity at *p*H 9 and 6 than at *p*H 7.5. More than 50% of the Ca^{2+}-activated ITPase was lost while the ATPase did not change (Fig. 7). When Ca^{2+} was the activating cation, actin stimulated the ATPase activity of native myosin A to twice the level of the 30-minute-irradiated myosin A, while with Mg^{2+} ions both the native and irradiated myosin A were activated to the same extent. After 30 minutes of irradiation, surprisingly, no change was found in the number of the free reacting –SH groups. We found 3 μM histidine less per 10^5 g protein, a slight increase in the optical rotation, and a 35% increase in the molecular weight.

After 60 minutes of irradiation the changes in the enzymatic pattern of myosin A were far reaching. The Ca^{2+}-activated ATPase lost about 50% of its original activity at *p*H 9.0 and about 25% at *p*H 7.5 and 6.0. The stimulating effect of DNP was almost completely lost. After 60 minutes of irradiation actin lost all but one of its stimulatory effects on the myosin A enzyme. The Mg^{2+}-activated ATPase was still accelerated, while no stimulation of the Mg^{2+}-activated ITPase or the Ca^{2+}-activated ATPase by actin remained. The actomyosin-forming ability of the 60-minute-irradiated myosin A remained unchanged. It was found that 25% of the free reacting –SH groups disappeared after 60 minutes of ultraviolet treatment and the reduction of histidine groups in-

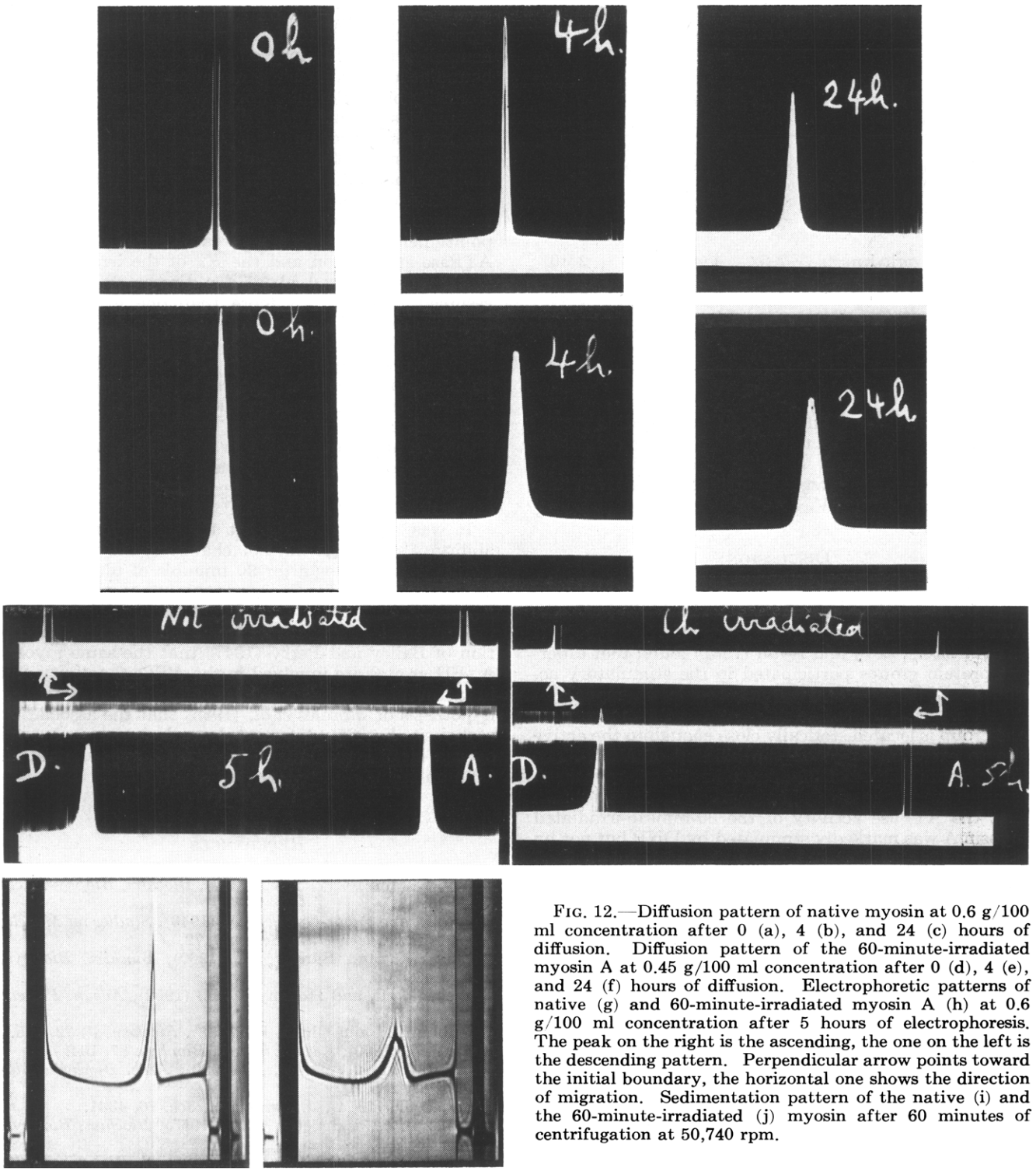

FIG. 12.—Diffusion pattern of native myosin at 0.6 g/100 ml concentration after 0 (a), 4 (b), and 24 (c) hours of diffusion. Diffusion pattern of the 60-minute-irradiated myosin A at 0.45 g/100 ml concentration after 0 (d), 4 (e), and 24 (f) hours of diffusion. Electrophoretic patterns of native (g) and 60-minute-irradiated myosin A (h) at 0.6 g/100 ml concentration after 5 hours of electrophoresis. The peak on the right is the ascending, the one on the left is the descending pattern. Perpendicular arrow points toward the initial boundary, the horizontal one shows the direction of migration. Sedimentation pattern of the native (i) and the 60-minute-irradiated (j) myosin after 60 minutes of centrifugation at 50,740 rpm.

creased to 5μM per 10^5 protein. The apparent molecular weight of the aggregated myosin A doubled, the reduced specific viscosity and the optical rotation increased considerably, while the electrophoretic mobility was somewhat decreased.

Blum and Felauler (1959) and Blum (1960) have found that K_m and V_m of myosin A ATPase were both increased in presence of *p*-mercuribenzoate and DNP. Based upon their findings, the kinetic analysis was extended to actin, AsBAL, and to two high-molecular-weight "interaction inhibitors" (Barany and Barany, 1960; Kaldor and Gitlin, 1964). Similar analyses were also performed with various ultraviolet-irradiated myosin A samples. These experiments were carried out in a 0.05 M Tris buffer at *p*H 7.5 with Ca^{2+} as the activator. Results are summarized in Table III. It may be seen that all modifiers investigated increased both the V_m and K_m of the enzyme. The ultraviolet-irradiated myosin A, however, showed a progressively increased K_m with a gradually decreasing V_m. The results obtained with these chemical modifiers are similar to those of Blum (1960). They thereby suggest a similar mechanism of action. The indicated changes of the kinetic constants of the ultraviolet-irradiated myosin A were most probably caused by a complex alteration of the active site. The observed increase of the K_m might have been a consequence of the disappearance or destruction of an inhibitory group.

TABLE III
KINETIC CONSTANTS OF MYOSIN A ATPASE

Additions	pH	K_m (moles/liter)	V_m (μM P_i/mg min^{-1})
None	7.5	2.0×10^{-4}	1.02
0.5 mg Actin/mg myosin A	7.5	3.8×10^{-4}	2.32
0.25 mM AsBAL	7.5	2.6×10^{-4}	2.58
0.5 mg Heparin/mg myosin A	7.5	2.5×10^{-4}	2.40
0.5 mg Polyethylene/mg myosin A	7.5	1.4×10^{-4}	2.10
30-min Ultraviolet irradiation	7.5	2.2×10^{-4}	0.88
60-min Ultraviolet irradiation	7.5	4.0×10^{-4}	0.70

Whether the observed decrease in the histidine groups had a causal connection with this finding cannot be answered at this time. Similarly, the possible connection between the aggregation of the myosin A molecules and the reduced V_m remains uncertain.

DISCUSSION

Blum and Felauler (1959) and Blum (1960) proposed that DNP and *p*-mercuribenzoate both decrease the ATP-induced conformation change of the myosin A enzyme site. Levy and Ryan (1961) found that different protein groups participated in the stimulatory action of DNP and *p*-mercuribenzoate on myosin A ATPase. It appeared to us therefore that more than one group is located sterically close enough to the active site to exert influence on the binding of ATP. The experiments with the 30-minute-irradiated myosin A samples are in line with this hypothesis. It was shown that the ATPase activity of the 30-minute-irradiated myosin A was markedly stimulated by DNP but not by *p*-mercuribenzoate or AsBAL, and the stimulatory effect of actin was decreased by 50% in the presence of Ca^{2+}. The ultraviolet irradiation–induced aggregation and/or conformation change of the active center may have rendered the –SH group through which the *p*-mercuribenzoate effect was mediated sterically inaccessible. The other group which participated in the DNP stimulation was probably not affected.

We believe that it is reasonable to suppose that the same protein groups (rate-retarding group or inhibitory group) have to be occupied by actin in order to transform the myosin A enzyme to actomyosin enzyme. Competition between actin and DNP for the myosin A enzyme was shown by Chappel and Perry (1955). The experiments designed to study the simultaneous effect of actin and AsBAL on the enzymatic activity of myosin A revealed competition between these two compounds for an "inhibitory" group on the enzyme.

The enzymatic interaction between myosin A and actin was shown to occur best at low ionic strength and at *p*H 7.5. It was characterized by an increase of the ATPase activity in presence of both Ca^{2+} and Mg^{2+} an increase in the ITPase activity with Mg^{2+}, but a decreased splitting of this latter substrate in presence of Ca^{2+}. From the kinetic point of view, the interaction of myosin A with actin, AsBAL, heparin, or polyethylenesulfonate was characterized by an increase in both K_m and V_m of the ATPase.

Gilmour (1960) attributed the characteristic ATPase activity drop of the myosin A enzyme at *p*H 7.5 to the appearance of a single negatively charged inhibitory group other than –SH on the active center of the enzyme. According to him, *p*-mercuribenzoate would neutralize the inhibitory effect of this group by being bound at a site sufficiently close to the active center to sterically block the inhibitor. It was shown that after 30 minutes of ultraviolet irradiation *p*-mercuribenzoate lost its effect, but that the above-mentioned *p*H characteristic of the myosin A ATPase remained essentially unchanged. The *p*H dependence of the enzyme showed a considerable change after 60 minutes of irradiation. It may be interesting to note that at this point neither *p*-mercuribenzoate nor DNP caused ATPase stimulation and the K_m of the irradiated enzyme was increased about ten times. We therefore assumed that perhaps more than one myosin group could be responsible for the well-known *p*H pattern of myosin in A ATPase.

In the presence of Ca^{2+} the *p*H characteristics of AsBAL and actin-induced ITPase inhibition were different from the *p*H characteristics of ATPase activation caused by the same modifiers. The ITPase activity of myosin A also showed a much greater ultraviolet sensitivity than the ATPase. These results indicate that the configuration of the active center of myosin A was different for ATP and for ITP.

It was also observed that the actomyosin-forming ability of myosin A did not change in the presence of 0.25 mM AsBAL or after 90 minutes of ultraviolet irradiation. However the enzymatic interaction between myosin A and actin was very strongly inhibited by these agents. These results do not support the proposition of Bailey and Perry (1947) that the same myosin A –SH groups are involved in the ATPase activity and in actin binding. They are more in line with the hypothesis of Morales *et al.* (1955) that the association of myosin A with actin must depend on charges spread over a sizable region of the protein molecule rather than on the integrity of a few –SH groups.

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Inter- and Intramolecular Interactions of α-Lactalbumin. I. The Apparent Heterogeneity at Acid *p*H*

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Sedimentation velocity and equilibrium measurements carried out at *p*H values acid to the isoelectric zone show that α-lactalbumin exists largely in associated or aggregated form at finite protein concentration. The association process in acid *p*H appears to be a rapidly reversed equilibrium with a negative enthalpy. It can be observed independently of the aggregation reaction by carrying out experiments at sufficiently low protein concentration (below 0.8 g/100 ml). Association persists even as low as *p*H 2, where electrostatic repulsions must be significant. This is in contrast with its behavior at *p*H 8.55, where the weight-average molecular weight shows a slight positive dependence on protein concentration but does not deviate markedly from the value of 16.2×10^3 obtained at infinite dilution. At *p*H values acid to the isoelectric point at protein concentrations in excess of 1 g/100 ml, a "heavy" component is readily seen in the ultracentrifuge. The amount of this component, an aggregate of monomeric α-lactalbumin units, increases with time and with increasing protein concentration and temperature. Association at *p*H values *alkaline* to the isoelectric zone is quite feeble with little or no temperature dependence to the process, as was the case for association at acid *p*H values. The time-dependent aggregation reaction observed at acid *p*H values is essentially absent at *p*H 5.24 and 6.00 even at higher protein concentrations than were employed at *p*H 3.00 and 2.00. It seems probable that the apparent electrophoretic heterogeneity observed previously at acid *p*H values is a consequence of association or aggregation of α-lactalbumin. The feebleness of association at *p*H values alkaline to the isoelectric zone, as well as the absence of a time-dependent aggregation reaction, suggests that the apparent electrophoretic heterogeneity observed in this region may have other origins.

Gordon and Semmett (1953) noted in their paper on the isolation of crystalline α-lactalbumin that the protein was homogeneous electrophoretically in *p*H 2 and 3 glycine buffer, *p*H 6.6 and 7.7 potassium phosphate buffer, and *p*H 8.5 Veronal, but heterogeneous in *p*H 3.3 sodium lactate buffer. At that time they raised the question as to whether the protein were really heterogeneous or if the apparent inhomogeneity might be "an artifact due to some obscure effect of lactate ion." Klostergaard and Pasternak (1957) subsequently confirmed Gordon and Semmett's findings of heterogeneity in *p*H 3.3 lactate buffer and found this also to be the case in *p*H 4.83 lactate. They were unable, however, to demonstrate any such inhomogeneity under comparable conditions in the ultracentrifuge. Both Klostergaard and Pasternak (1957) and Wetlaufer (1961) show electrophoretic evidence for heterogeneity at *p*H values above 7.

It appears from our studies that much of the apparent heterogeneity of α-lactalbumin is the result of association-aggregation processes occurring at acid *p*H. The association process will be described in this paper; the characteristics of the aggregation process will be considered in the accompanying publication (Kronman *et al.*, 1964). Subsequent papers in this series will consider the molecular conformation of α-lactalbumin

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