

ACTION OF DINITROPHENYL AMINO ACIDS ON SKELETAL MUSCLE PROTEINS

II.* ABSORPTION OF BIS-DINITROPHENYL-LYSINE BY LIGHT AND HEAVY MEROMYOSINS AND BY LIGHT MEROMYOSIN FRACTION I

By R. W. BURLEY,† W. J. H. JACKSON,† and JEAN P. ROBERTSON †

[Manuscript received August 14, 1967]

Summary

The quantity of bis(2,4-dinitrophenyl)-L-lysine (abbreviation bis-DNP-lysine) absorbed at 5°C by myosin and meromyosins of rabbit skeletal muscle was estimated in phosphate buffer (pH 7, ionic strength 0.5) by two methods, one based on equilibrium dialysis, the other on high-speed centrifugation. According to both methods, heavy meromyosin absorbed more of the reagent than did the same weight of the parent myosin; light meromyosin absorbed less, and light meromyosin fraction I absorbed less still. There were, however, relatively large quantitative differences between the two methods, possibly because of an effect of the slightly different conditions of treatment.

Blocking of the sulphhydryl groups with *p*-chloromercuribenzoate greatly increased absorption of bis-DNP-lysine by heavy meromyosin, had a smaller effect on its absorption by light meromyosin, and had no effect on absorption by light meromyosin fraction I.

At low ionic strength (0.1) aggregates of light meromyosin absorbed less bis-DNP-lysine than did the monomer at high ionic strength (0.5), but absorption was still less than that by aggregated myosin. Absorption by heavy meromyosin was less sensitive to changes in ionic strength but, after treatment with *p*-chloromercuribenzoate, the relationship between ionic strength and absorption suggested the possibility of a change in configuration at an ionic strength of about 0.3. At low ionic strengths monomeric heavy meromyosin absorbed about four times as much bis-DNP-lysine as did aggregated myosin at the same ionic strengths, thus suggesting that a considerable change occurred in the structure of the heavy meromyosin region of myosin as a result of aggregation.

Heavy meromyosin resembled myosin in that part of the absorbed bis-DNP-lysine was not easily desorbed, especially if the protein was first treated with *p*-chloromercuribenzoate. By contrast, light meromyosin or light meromyosin fraction I showed much less of this "strong" absorption and prior treatment with *p*-chloromercuribenzoate did not increase it.

The tryptic digestion of myosin under the mild conditions used for the production of meromyosins had no effect on either the "weak" or the "strong" absorption of bis-DNP-lysine.

It is concluded that strong absorption is confined to the heavy meromyosin region of the myosin molecule, and that weak absorption is an attribute largely associated with certain non-helical regions of the molecular structure of myosin.

* Part I, *Aust. J. biol. Sci.*, 1967, **20**, 983-99.

† Division of Food Preservation, CSIRO, Ryde, N.S.W. 2112.

I. INTRODUCTION

In Part I (Burley, Jackson, and Robertson 1967) it was shown that in solution at neutral pH the myosin of rabbit skeletal muscle absorbs a relatively large amount of bis(2,4-dinitrophenyl)-L-lysine (abbreviation bis-DNP-lysine). It was also shown that, though most of the absorbed reagent is easily removed from the myosin, an independently variable proportion ("strong" absorption) is very firmly bound under certain conditions, namely when sulphhydryl groups of the myosin have been blocked, or when reagent concentrations are high, or when the temperature is above about 25°C. A large difference in "weak" absorption between monomeric myosin (i.e. myosin in solution in high ionic strength buffer) and aggregated myosin (i.e. that thrown out of solution at low ionic strength) was reported; but the small difference in the amount of strong absorption shown by these two forms of myosin suggested that aggregation is not a relevant factor in determining capacity for strong absorption.

Largely on the basis of the results in Part I, deductions were made about the relative distributions of both weak and strong absorption between the two easily recognizable regions of the myosin molecule, namely, the heavy and light meromyosin regions (see Lowey and Holtzer 1959 for early literature on the meromyosins). It was postulated that the capacity for strong absorption might be associated with certain unidentified changes within the heavy meromyosin region (this region being known to contain the actin-binding and adenosinetriphosphatase sites), and that these changes possibly involve the cross-bridges. It was also deduced that the phenomenon of weak absorption occurs at various rates, the fastest being associated with the heavy meromyosin region.

As a check on some of these predictions, the experiments described here were designed to provide data on the absorptive characteristics towards bis-DNP-lysine of the heavy and light meromyosins isolated in the usual way from myosin after mild tryptic digestion. Experiments on the highly helical subfraction of light meromyosin, known as fraction I (Szent-Györgyi, Cohen, and Philpott 1960), are also described.

The results reported here support the conclusion that only the heavy meromyosin region is involved in the phenomenon referred to as strong absorption. In addition to their possible relevance to the interpretation of the action of 2,4-dinitrophenyl amino acids on muscle (Burley 1967), experiments of the type described may eventually help elucidate the nature of the action of related compounds on other proteins.

II. MATERIALS

(a) *Buffers and Reagents*

These were described in Part I except for trypsin (type I, crystallized twice), and trypsin inhibitor (type 1-S, soybean, crystallized twice), which were from the Sigma Chemical Co., St. Louis, Missouri, and ammonium sulphate "special enzyme grade", from Mann Research Laboratories Inc., New York.

(b) *Preparation of Myosin*

Myosin was prepared from rabbit skeletal muscle as described in Part I. Each complete series of tests described here was performed on samples of myosin and the corresponding

meromyosins from two rabbits. Less complete results were obtained on some preparations derived from several other rabbits. Most of the diagrams are based on results from the two complete series (which, except where stated otherwise, were in good mutual agreement), and these results were augmented occasionally by data from other preparations.

(c) Preparation of Meromyosins

Heavy and light meromyosins were prepared essentially by the method of Lowey and Cohen (1962) as follows: a solution of myosin [2–3% (w/v)] in phosphate buffer (pH 6.5, ionic strength 0.35) containing trypsin [0.005% (w/v)] was shaken at 25°C for 60 min. Enzyme action was stopped by adding trypsin inhibitor equal to twice the weight of trypsin added originally, and the solution was then dialysed overnight at 3°C into 10 volumes 0.007M phosphate buffer, pH 7. The precipitate of light meromyosin was centrifuged off at 20,000 r.p.m. for 10 min. This precipitate was dissolved in a minimum of 2M potassium chloride solution and diluted with phosphate buffer (pH 7, ionic strength 0.5) to give a protein concentration of c. 1.5%. It was purified by reprecipitation and stored at 0°C.

Heavy meromyosin was precipitated from the supernatant liquor (i.e. the solution from which light meromyosin had been removed) by adding ammonium sulphate to 55% of saturation. It was redissolved in phosphate buffer (pH 7, ionic strength 0.1) and purified by gel-filtration on a column (5 cm by 75 cm) of Sephadex G-200.

Light meromyosin fraction I was prepared by a method essentially that of Szent-Györgyi, Cohen, and Philpott (1960). To a 1% (w/v) solution of light meromyosin in phosphate buffer (pH 7, ionic strength 0.5), ethanol (3 vol.) was added, and the mixture allowed to stand at 3°C for 2 hr. After being separated from the mother liquor by centrifuging at 2000 r.p.m. for 10 min, the precipitated protein was suspended in the initial volume of fresh phosphate buffer, dialysed against a large volume of this buffer at 3°C, and then centrifuged for 10 min at 2000 r.p.m. to separate it from fraction I, which remained in solution. Fraction I was precipitated in the form of needle-like paracrystals by dialysing the supernatant solution against 10 volumes of 0.1M phosphate buffer, pH 6.5–7. It was stored at 0°C in phosphate buffer (pH 7, ionic strength 0.5).

The meromyosin preparations were homogeneous, according to ultracentrifuge measurements (see Fig. 1). Compared with that of myosin, the adenosinetriphosphatase activity of light meromyosin was small (less than 7% on a weight basis when determined by the method of Asai and Morales 1962), but heavy meromyosin was more active than myosin from which it was prepared.

III. METHODS

(a) Estimation of Total Absorption of Bis-DNP-lysine by Myosin and Meromyosins

(i) Centrifuge Method

This method, in which the protein is sedimented by high-speed centrifuging after reaction for 48 hr in solution with bis-DNP-lysine, has been described in Part I where its use for determining the absorption of DNP compounds by myosin was illustrated (Burley, Jackson, and Robertson 1967). Exactly the same method was used at high ionic strength for light meromyosin, light meromyosin fraction I, and heavy meromyosin. All of these proteins were sedimented from the top third, at least, of the centrifuge tubes by centrifuging for 12 hr at 360,000 *g* at 5°C. At low ionic strength only heavy meromyosin needed such prolonged centrifuging, owing to its greater solubility; the others, which readily aggregated at ionic strength 0.1 and needed a shorter reaction time (24 hr), sedimented in 10 min or less.

(ii) Equilibrium Dialysis

Because of difficulties inherent in methods involving prolonged centrifuging (Steinberg and Schachman 1966), and in view of the need for comparisons amongst myosin fragments with different sedimentation characteristics, verification by an alternative method was desirable. Accordingly, the equilibrium dialysis method (Rosenberg and Klotz 1960) was also used, although

there are minor disadvantages in using this method with bis-DNP-lysine (Burley, Jackson, and Robertson 1967). The procedure adopted was as follows:

Two-ml aliquots of protein solution (0.1%, in buffer), the protein pretreated with *p*-chloromercuribenzoate (pCMB) if appropriate, were placed in dialysis bags of cellulose-casing (size 8/32, Visking Corporation) and suspended in identical, but protein-free, buffer solutions (14.0 ml) containing bis-DNP-lysine, the mixture being allowed to equilibrate at 5°C. The amount of bis-DNP-lysine absorbed by the protein was calculated from the difference in optical density at 360 m μ between the contents of the bag and the external solution. Before measuring the optical density, the internal solution was diluted at least 10-fold with buffer and left for 24 hr or more to permit desorption of most of the bis-DNP-lysine absorbed by the protein. In calculating the quantity of bis-DNP-lysine absorbed by the protein, account was taken of the final protein concentration of the internal solution to allow for possible precipitation of protein on the dialysis membrane. The quantity absorbed by the protein could not normally be calculated from measurements on the external solution alone because absorption of bis-DNP-lysine by the dialysis tubing was apt to be large and variable. At high reagent concentrations the bis-DNP-lysine complex with myosin or light meromyosin sometimes precipitated in the internal solution. A small amount of solid subtilisin or other proteolytic enzyme was added to such mixtures to dissolve the precipitate before the solution was diluted for measurement of optical density.

The equilibrium dialysis and centrifuge methods usually gave closely concordant results for total absorption of bis-DNP-lysine by sulphydryl-blocked myosin and meromyosins; hence, for these, the two sets of results were treated as one. With most of the untreated proteins, however, both sets of results are reported, because differences between them were often large.

When the rates of total absorption by myosin fragments from different preparations, as estimated by means of equilibrium dialysis, were compared, it was often found that they were different, even though the total absorption might ultimately be the same. Absorption by sulphydryl-blocked myosin and sulphydryl-blocked heavy meromyosin was, however, always faster than absorption by the untreated proteins; also, the absorption of bis-DNP-lysine by untreated heavy meromyosin usually occurred at a slightly faster rate than absorption by the corresponding light meromyosin. After periods of reaction longer than 72 hr (the period used for most of these experiments), absorption by some preparations of light meromyosin was still increasing, though at a much slower rate.

(b) *Estimation of Strong Absorption of Bis-DNP-lysine by Myosin and Meromyosins*

Because of the high solubility of some of the myosin fragments, only the dialysis method (Burley and Jackson 1965) could be used to detect strong absorption. This method, with modifications, was used as follows:

After the reaction mixture [containing protein, 0.1% (w/v), and bis-DNP-lysine, in the appropriate buffer] had been allowed to stand for 48 hr, 1- or 2-ml aliquots were dialysed for two periods of 24 hr each into two 50- or 100-ml volumes of buffer. The contents of the dialysis bags were then made up to standard volume. If necessary, either 2M potassium chloride solution or a minimum of solid proteolytic enzyme was added to dissolve protein aggregates before measuring the optical density at 360 m μ .

(c) *Measurement of Optical Rotation*

The optical rotatory dispersion of myosin and the meromyosins was measured on a Perkin-Elmer Model 141 spectropolarimeter, in which filters to give five wavelengths above 365 m μ were used. The percentages of protein in the helical form in solution at 5°C were calculated from the equation of Moffitt and Yang (1956), assuming $b_0 = -630$, $\lambda_0 = 212$ m μ , and $M = 115$ for a 100% α -helical protein.

IV. RESULTS

(a) Sedimentation and Optical Rotatory Dispersion Data

Table 1 gives sedimentation coefficients corrected to zero protein concentration ($S_{20,w}^0$ in Svedberg units) and the calculated helix contents for typical samples of myosin and meromyosin used in these studies. For heavy meromyosin, results for both high and low ionic strengths are given. These data conform with comparable values in the literature (cf. Lowey and Holtzer 1959; Lowey and Cohen 1962; Hotta and Kojima 1964).

TABLE 1
PHYSICAL PROPERTIES OF MYOSIN AND MEROMYOSINS IN PHOSPHATE
BUFFER AT pH 7.1–7.3 AT 20°C

Protein	Ionic Strength	$S_{20,w}^0$	Helix Content (%)
Myosin	0.5	6.0	60
Light meromyosin	0.5	3.0	83
Light meromyosin fraction I	0.5	3.0	98
Heavy meromyosin	0.5	7.1	42
Heavy meromyosin	0.1	7.0	46

Figure 1 shows sedimentation patterns given by the proteins used for determining the data in Table 1.

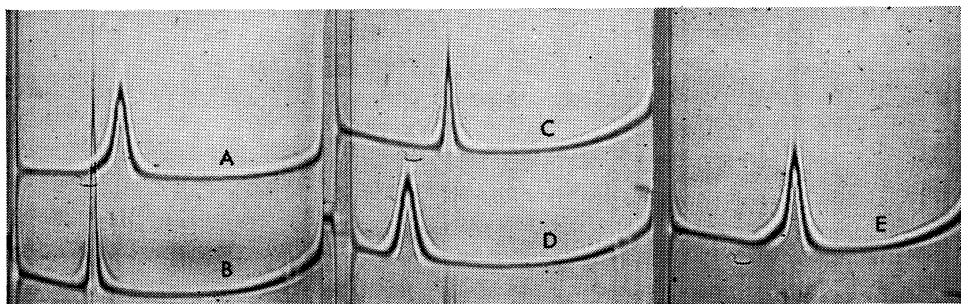


Fig. 1.—Sedimentation patterns of typical myosin and meromyosin preparations used for dye-absorption experiments. Ultracentrifuge speed 59,780 r.p.m., bar angle 60°C, sedimentation from left to right. *A*, heavy meromyosin 0.4%, 50 min after reaching full speed. *B*, myosin 0.25%. *C*, light meromyosin 0.3%, 186 min. *D*, light meromyosin fraction I 0.6%. *E*, heavy meromyosin 0.6%, 64 min. All measurements were in phosphate buffer, pH 7.1–7.3, at 20°C and ionic strength 0.5, except for *A* which was at ionic strength 0.1.

(b) Total Absorption of Bis-DNP-lysine by Myosin, Light Meromyosin, and Light Meromyosin Fraction I

(i) High Ionic Strength

Total absorption of bis-DNP-lysine by myosin and the light meromyosins at ionic strength 0.5, and the effect on absorption of pretreatment with pCMB are

shown in Figure 2. Both the equilibrium dialysis and the centrifuge procedures (see Section III) were used in collecting data for all these curves, excepting for those of untreated myosin (curve *A*) and untreated light meromyosin (curve *D*) for which only equilibrium dialysis was used. Corresponding data derived from the centrifuge method, and which were consistently lower, are indicated by the curves *B* and *E*. One preparation of light meromyosin showed higher absorption after pCMB treatment (curve *F*) in contrast to other preparations which did not respond to such treatment and followed curve *D*.

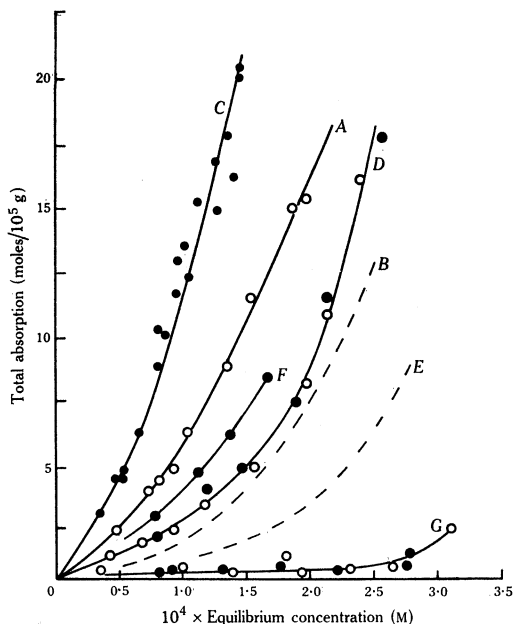


Fig. 2.—Total absorption, at ionic strength 0.5, of bis-DNP-lysine by myosin and light meromyosins in phosphate buffer, pH 7.0–7.2, at 5°C as a function of bis-DNP-lysine concentration.

- A*, myosin untreated, equilibrium dialysis method.
- B*, myosin untreated, centrifuge method.
- C*, myosin pCMB-treated both methods.
- D*, light meromyosin untreated (○) and pCMB-treated (●), equilibrium dialysis method.
- E*, light meromyosin untreated, centrifuge method.
- F*, light meromyosin (from another preparation) pCMB-treated, equilibrium method.
- G*, light meromyosin fraction I untreated (○) and pCMB-treated (●), both methods.

Figure 2 clearly shows that light meromyosin, whether untreated (curves *D* and *E*) or pCMB-treated (curves *D* and *F*), absorbs less bis-DNP-lysine under comparable conditions than does the myosin from which it was produced (curves *A*, *B*, and *C*), whereas light meromyosin fraction I absorbs very little (curve *G*).

(ii) Low Ionic Strength

Figure 3 shows the total absorption, at ionic strength 0.1, of bis-DNP-lysine by myosin, light meromyosin, and light meromyosin fraction I measured after brief centrifuging. The samples used were of about the same post-mortem age, to minimize the effect of aging noted already (Burley, Jackson, and Robertson 1967). Although each protein dissolved slightly during treatment with bis-DNP-lysine, only myosin itself dissolved to a greater extent with increased bis-DNP-lysine concentration (a phenomenon also reported previously).

According to Figure 3, at low ionic strength light meromyosin and fraction I absorb less bis-DNP-lysine than myosin, and pCMB treatment does not affect this absorption. It has previously been shown that at ionic strength 0.1 absorption by aggregates of myosin is less than that by monomeric myosin at ionic strength 0.5 (Burley, Jackson, and Robertson 1967). Aggregates of light meromyosin resemble those of myosin in absorbing less than the monomers do. At an equilibrium concentration of $2.5 \times 10^{-4} \text{M}$, for example, aggregated light meromyosin absorbed a total amount of 3.5 moles per 10^5g , compared with at least 5.5 moles per 10^5g of the monomer. Absorption by light meromyosin fraction I, either monomeric or aggregated, was too small under these conditions for a reliable comparison.

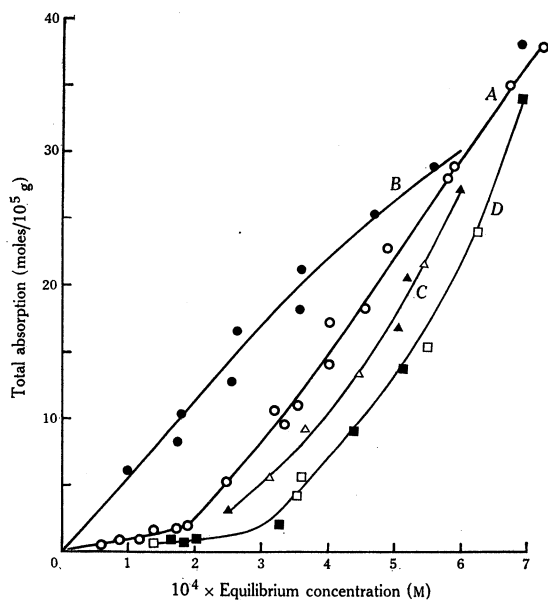


Fig. 3.—Total absorption, at ionic strength 0.1, of bis-DNP-lysine by myosin and light meromyosins in phosphate buffer, pH 7.1, at 5°C , reaction time 24 hr, as a function of bis-DNP-lysine concentration.

- A, myosin untreated.
 B, myosin pCMB-treated.
 C, light meromyosin untreated (Δ) and pCMB-treated (\blacktriangle).
 D, light meromyosin fraction I untreated (\square) and pCMB-treated (\blacksquare).

Figure 3 shows that at relatively high bis-DNP-lysine concentrations even light meromyosin fraction I absorbed large amounts. With these samples it was found that the protein aggregates did not redissolve when the neutral salt concentration was increased, whereas corresponding aggregates of myosin or light meromyosin dissolved easily when the ionic strength was raised in this way. Evidently fraction I may be irreversibly altered after exposure to high concentrations of bis-DNP-lysine.

(c) Total Absorption of Bis-DNP-lysine by Myosin and Heavy Meromyosin

Figure 4 compares the total absorption of bis-DNP-lysine by untreated and pCMB-treated myosin (A, B, C) and heavy meromyosin (D, E, F) in solution at ionic strength 0.5. As before, results for untreated proteins by equilibrium dialysis and high speed centrifuging are given separately (curves A and D, and curves B and E).

Evidently heavy meromyosin absorbs more bis-DNP-lysine than does myosin, and, furthermore, pCMB treatment has the same enhancing effect on absorption by heavy meromyosin as it has on that by myosin.

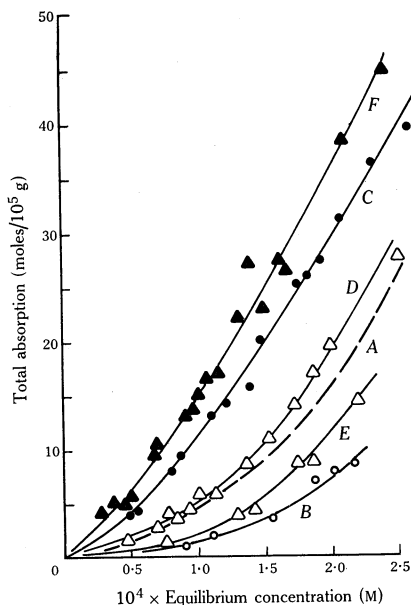


Fig. 4.—Total absorption, at ionic strength 0.5, of bis-DNP-lysine by myosin and heavy meromyosins in phosphate buffer, pH 7.1, at 5°C, as a function of bis-DNP-lysine concentration.

- A, myosin untreated, equilibrium dialysis method.
- B, myosin untreated, centrifuge method.
- C, myosin pCMB-treated, both methods.
- D, heavy meromyosin untreated, equilibrium dialysis method.
- E, heavy meromyosin untreated, centrifuge method.
- F, heavy meromyosin pCMB-treated, both methods.

(d) *Effect of Ionic Strength on Total Absorption of Bis-DNP-lysine by Myosin and Heavy Meromyosin*

Figure 5 indicates the total absorption of bis-DNP-lysine by untreated and pCMB-treated heavy meromyosin at low ionic strength (curves A and B) and at high ionic strength (curves C and D). Evidently absorption increases when the ionic

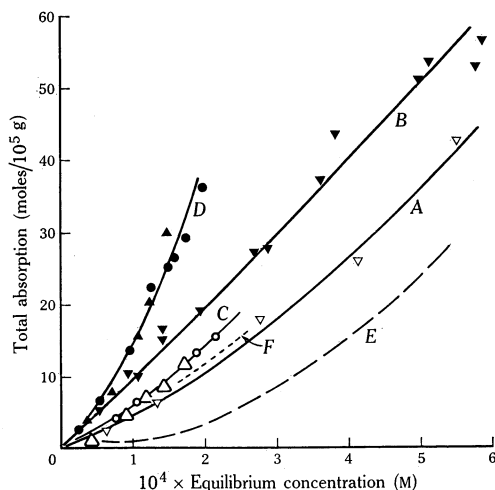


Fig. 5.—Total absorption of bis-DNP-lysine by myosin and heavy meromyosin at various ionic strengths, pH 7.1–7.3, 5°C, as a function of bis-DNP-lysine concentration.

- A, heavy meromyosin untreated, ionic strength 0.1.
- B, heavy meromyosin pCMB-treated, ionic strength 0.1.
- C, heavy meromyosin untreated, ionic strength 0.5 (Δ), ionic strength 1.1 (\circ).
- D, heavy meromyosin pCMB-treated, ionic strength 0.5 (\blacktriangle), ionic strength 1.1 (\bullet).
- E, myosin untreated, ionic strength 0.1.
- F, myosin untreated, ionic strength 0.5.

All measurements, except for aggregated myosin, curve E, were by equilibrium dialysis.

strength is raised but this increase is less than that for myosin, which was discussed in Part I and is indicated for untreated myosin by curves *E* and *F* in Figure 5.

The relationship between total absorption (by myosin and heavy meromyosin) and ionic strength at a constant equilibrium concentration of bis-DNP-lysine ($1.9 \times 10^{-4}M$) is shown in Figure 6, for which the pH was kept constant and the ionic strength varied by means of potassium chloride.

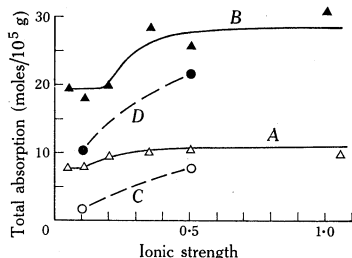


Fig. 6.—Effect of ionic strength on total absorption of bis-DNP-lysine by myosin and heavy meromyosin at constant bis-DNP-lysine concentration of $1.9 \times 10^{-4}M$, pH 7.06–7.08, 5°C, equilibrium dialysis method.

- A*, heavy meromyosin untreated.
- B*, heavy meromyosin pCMB-treated.
- C*, untreated myosin.
- D*, myosin pCMB-treated.

According to Figure 6, pCMB-treated heavy meromyosin increases its total absorption of bis-DNP-lysine by about 35% when the ionic strength is increased from 0.2 to 0.4. There is also an increase of absorption by untreated heavy meromyosin, but this is smaller and the measurements are not accurate enough to be certain of its position and magnitude. As the strong absorption by the same samples decreased slightly with increase in ionic strength, the increases in total absorption shown by curves *A* and *B* were due to increases in weak absorption.

(e) Strong Absorption of Bis-DNP-lysine by Myosin and Meromyosins

Figure 7 shows the relationship between the initial equilibrium concentration of bis-DNP-lysine and its strong absorption by untreated and pCMB-treated samples

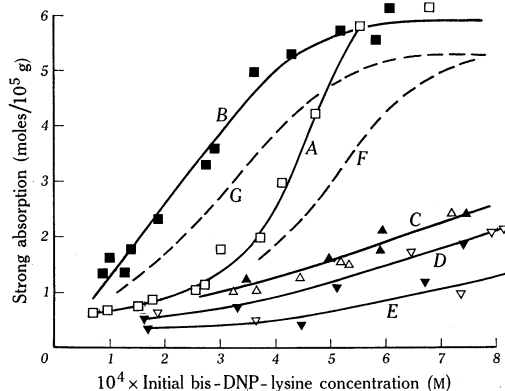


Fig. 7.—Strong absorption, at ionic strength 0.1, of bis-DNP-lysine by myosin and meromyosins in phosphate buffer pH 7.1–7.3 at 5°C as a function of initial bis-DNP-lysine concentration.

- A*, heavy meromyosin untreated.
- B*, heavy meromyosin pCMB-treated.
- C*, light meromyosin untreated (Δ) and pCMB-treated (\blacktriangle).
- D*, light meromyosin fraction I untreated (∇) and pCMB-treated (\blacktriangledown).
- E*, light meromyosin fraction I (from another preparation) untreated (∇) and pCMB-treated (\blacktriangledown).
- F*, myosin untreated.
- G*, myosin pCMB-treated.

of heavy meromyosin, light meromyosin, light meromyosin fraction I, and myosin itself, all prepared from the same rabbit (except for curve *E*), and all in buffer of ionic strength 0.1.

The slopes of the curves for myosin (Fig. 7, curves *F* and *G*) are not as steep as those previously given by myosin obtained from other rabbits (see Part I); another difference is that, for unknown reasons, the curves in Figure 7 cannot easily be extrapolated to the origin. Nevertheless, preparations from several other rabbits for which, in general, less complete data were obtained, confirmed the relative positions of the myosin curves in Figure 7 in relation to each other and to the meromyosin curves. Strong absorption by some preparations of light meromyosin fraction I was even less than that indicated by curve *D* in Figure 7, and the results from one such preparation are indicated by curve *E*.

Measurements for Figure 7 were made at ionic strength 0.1, at which only the heavy meromyosin samples were soluble. Comparable results at ionic strength 0.5, at which all the proteins are soluble, could not be extended beyond an equilibrium concentration of about $3 \times 10^{-4}M$ because of the lower solubility of the reagent; nevertheless they showed that at this ionic strength strong absorption by light meromyosin and its fraction I is unaffected by pCMB treatment, and they tended to confirm the other differences shown in Figure 7 amongst the different meromyosins.

(f) *Effect of Tryptic Digestion on the Weak and Strong Absorption of Bis-DNP-lysine by Myosin*

Figure 8(a) shows the total absorption and Figure 8(b) the strong absorption, of bis-DNP-lysine by myosin during the course of digestion by trypsin under the

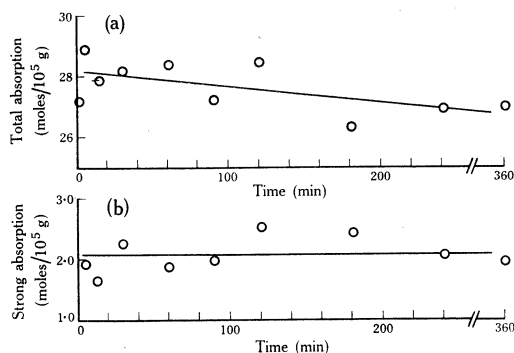


Fig. 8.—Total (a) and strong (b) absorption of bis-DNP-lysine by myosin at intervals during its tryptic digestion. Conditions for digestion: myosin 3.0% (w/v), trypsin 0.005% (w/v), phosphate buffer (pH 6.5, ionic strength 0.35), 25°C. Conditions for absorption: myosin 0.1% (w/v), phosphate buffer (pH 7.0, ionic strength 0.5) at a constant concentration of bis-DNP-lysine of $2.3 \times 10^{-4}M$.

conditions used for the preparation of the meromyosins. Small volumes were withdrawn during digestion, added to trypsin inhibitor, and diluted to an effective myosin concentration of 0.1% with addition of excess pCMB. Part of the diluted solution was then dialysed, first into phosphate buffer (ionic strength 0.5) for 24 hr, and then into a solution of bis-DNP-lysine in buffer for a further 72 hr. No loss of protein through the membrane occurred with any of the samples during dialysis. According to Figure 8, mild tryptic digestion of myosin does not alter its ability to exhibit either weak or strong absorption of bis-DNP-lysine. Other tests confirmed that there was no loss in adenosinetriphosphatase activity of myosin after digestion with trypsin for 2 hr.

V. DISCUSSION

The results cited above provide ample evidence of large differences amongst the meromyosins produced from the myosin of rabbit skeletal muscle, as regards their capacity to absorb bis-DNP-lysine. Some typical values for weak and strong absorption at a single equilibrium concentration are presented in Table 2, ranges being given where one value did not predominate strongly. Evidently, on a weight basis, weak absorption of bis-DNP-lysine by heavy meromyosin is greater than that

TABLE 2

WEAK AND STRONG ABSORPTION OF BIS-DNP-LYSINE BY MYOSIN AND MEROMYOSINS AT 5°C, pH 7.1-7.3, AT CONSTANT EQUILIBRIUM CONCENTRATION

The constant equilibrium concentration for weak absorption was $1.5 \times 10^{-4}M$ and for strong absorption $7.5 \times 10^{-4}M$

Protein	Ionic Strength	pCMB Treatment	Weak Absorption (moles/10 ⁵ g)		Strong Absorption (moles/10 ⁵ g)
			Equilibrium Dialysis Method	Centrifuge Method	
Myosin	0.5	—	9.0	4.5	
	0.5	+	17.5	17.5	
	0.1	—		2.2*	5.3
	0.1	+		5.5*	5.3
Heavy meromyosin	0.5	—	10.5	6.0	
	0.5	+	21.5	21.5	
	0.1	—	6.8	3-4	6.0
	0.1	+	13.5	14.5	6.0
Light meromyosin	0.5	—	4.2	1.5	
	0.5	+	4.2-6.2	2.0-2.5	
	0.1	—		1.2*	2.2
	0.1	+		1.2*	2.2
Light meromyosin fraction I	0.5	—	0.4-1.4	0.4-1.4	
	0.5	+	0.4-1.4	0.4-1.4	
	0.1	—		0.8*	0.5-1.0
	0.1	+		0.8*	0.5-1.0

* Protein insoluble, measured after brief centrifuging.

exhibited by myosin itself; also, weak absorption by light meromyosin is considerably less than that of myosin, while weak absorption by light meromyosin fraction I is almost negligible except at reagent concentrations so high as to cause what is probably extensive structural damage (e.g. Fig. 3). Blocking of the free sulphydryl groups by treatment with pCMB enhances the capacity for weak absorption of bis-DNP-lysine, by heavy meromyosin and myosin to the same extent, but has a much smaller and more variable effect on light meromyosin, and no effect at all on light meromyosin fraction I.

From the shapes of the absorption isotherms in Figures 2-5 it is apparent that the Scatchard equation relating absorption to reagent concentration would not be obeyed for total absorption of bis-DNP-lysine by the meromyosins any more than it is for that of myosin (Burley, Jackson, and Robertson 1967).

The large but occasional discrepancies evident in Table 2 and in Figures 2 and 4, between the two alternative methods of measuring total absorption are difficult to interpret. Such discrepancies could scarcely arise from systematic errors in applying one or both of the methods since all results would then be affected. Instead it is suggested that the different conditions of treatment of proteins with bis-DNP-lysine in the two methods are responsible for variations in protein structure and these lead to differences in the amount of bis-DNP-lysine absorbed. It is probably significant that the largest discrepancies found were with protein samples that had not been pretreated with pCMB and which would be expected to be more sensitive to small changes in conditions.

The relevance of the data in Table 2 for testing the predictions in Part I concerning the relative distribution in the myosin molecule of regions characterized by weak or strong absorption, depends on whether changes in the absorption of bis-DNP-lysine are caused by tryptic digestion. It is now thought that trypsin probably hydrolyses a number of susceptible bonds throughout the meromyosins (Mihalyi and Harrington 1959), but that the enzyme does not greatly affect either the adenosinetriphosphatase activity or the actin-binding ability, both of which are retained by heavy meromyosin. Our findings have shown that preliminary mild tryptic digestion does not alter the total capacity of myosin and its digestion products for weak or strong absorption of bis-DNP-lysine (Fig. 8). It is therefore concluded that most of the absorptive capacity of myosin for bis-DNP-lysine is distributed amongst the meromyosins and that the different values in Table 2 represent differences amongst the various regions of myosin. It is also concluded that the differences in absorption between the heavy and light meromyosin regions are qualitatively in the direction predicted in Part I. In view of the profound disagreement in the literature at present about the proportions of the precursors of heavy and light meromyosin that constitute myosin (Laki 1961; Lowey and Cohen 1962; Segal, Himmelfarb, and Harrington 1967), it is not possible to be certain whether the region responsible for the minor fragments produced by trypsin also contributes towards the absorption by myosin. If, as is sometimes assumed, the heavy meromyosin precursor constitutes nearly three-quarters of the weight of myosin, it seems unlikely from the data in Table 2 that there is much absorption by the smaller fragments.

Another deduction drawn in Part I was that the heavy meromyosin region of myosin, or part of it, is able to absorb bis-DNP-lysine more rapidly than the light meromyosin region. This conclusion is not supported by absorption-rate studies on the isolated meromyosins, which suggest that the rate of absorption by heavy meromyosin is only slightly faster than that by light meromyosin. The deduction was, however, based on differences in the rates of absorption between soluble and aggregated myosin. Evidently the isolated heavy meromyosin, which is soluble and monomeric (Fig. 1), is not a suitable basis for absorption-rate measurements that are strictly applicable to the heavy meromyosin region of aggregated myosin. Thus at

low reagent concentrations, at an ionic strength of 0.1, absorption by heavy meromyosin is as much as four times as great as that by myosin at the same ionic strength (Fig. 6). It must be concluded that much of the absorbing part of the heavy meromyosin region is shielded on aggregation and that the exposed part may be altered slightly in structure in a way that leads to faster absorption.

An interesting aspect of the above results is that the α -helix, as it exists in light meromyosin fraction I, and presumably in myosin too, does not contribute much to the absorptive capacity for bis-DNP-lysine. Further attempts to locate the site of action of bis-DNP-amino acids are therefore being concentrated on the non-helical regions of myosin.

VI. ACKNOWLEDGMENTS

We thank Mr. W. C. Osborne for expert technical assistance and Mr. S. J. Rose for operating the analytical ultracentrifuge.

VII. REFERENCES

- ASAI, H., and MORALES, M. (1962).—*Archs Biochem. Biophys.* **99**, 383.
 BURLEY, R. W. (1967).—*J. biol. Chem.* **242**, 543.
 BURLEY, R. W., and JACKSON, W. J. H. (1965).—*Archs Biochem. Biophys.* **110**, 438.
 BURLEY, R. W., JACKSON, W. J. H., and ROBERTSON, J. P. (1967).—*Aust. J. biol. Sci.* **20**, 983.
 HOTTA, K., and KOJIMA, S. (1964).—*Biochemistry* **3**, 1431.
 LAKI, K. (1961).—*Archs Biochem. Biophys.* **92**, 221.
 LOWEY, S., and COHEN, C. (1962).—*J. molec. Biol.* **4**, 293.
 LOWEY, S., and HOLTZER, A. (1959).—*Biochim. biophys. Acta* **34**, 470.
 MIHALYI, E., and HARRINGTON, W. F. (1959).—*Biochim. biophys. Acta* **36**, 447.
 MOFFITT, W., and YANG, J. T. (1956).—*Proc. natn. Acad. Sci. U.S.A.*, **42**, 596.
 ROSENBERG, R. M., and KLOTZ, I. M. (1960).—In "Analytical Methods of Protein Chemistry". (Eds. P. Alexander and R. J. Block.) Vol. 2. p. 133. (Pergamon Press: Oxford.)
 SEGAL, V. M., HIMMELFARB, G., and HARRINGTON, W. F. (1967).—*J. biol. Chem.* **242**, 1241.
 STEINBERG, I. Z., and SCHACHMAN, H. K. (1966).—*Biochemistry* **5**, 3728.
 SZENT-GYÖRGYI, A. G., COHEN, C., and PHILPOTT, D. E. (1960).—*J. molec. Biol.* **2**, 133.

