ACTION OF DINITROPHENYL AMINO ACIDS ON SKELETAL MUSCLE PROTEINS

I. WEAK AND STRONG ABSORPTION OF BIS(DINITROPHENYL)LYSINE AND BIS(DINITROPHENYL)ORNITHINE BY MYOSIN

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Summary

Below about 15°C in high ionic strength (0·5) phosphate buffer at neutral pH, the coloured compound bis(2,4-dinitrophenyl)-L-lysine (abbreviation bis-DNP-lysine) was slowly but extensively absorbed from solution by rabbit skeletal muscle myosin. The absorption equations of Scatchard and of Klotz were not obeyed, thus suggesting that absorption did not involve independent sites. At higher temperatures, or if the myosin sulphydryl groups had been blocked with *p*-chloromercuribenzoate, absorption increased, often by as much as fivefold, and, moreover, a part of the absorbed bis-DNP-lysine, probably corresponding to the "precipitation resistant" absorption reported previously, was strongly retained. Total absorption of bis-DNP-lysine by myosin decreased slightly with increasing pH. At low temperatures bis-DNP-lysine treatment induced the formation of small aggregates of myosin.

By contrast with bis-DNP-lysine, absorption of bis-DNP-ornithine by untreated myosin at low temperature was limited and was more nearly described by the Scatchard equation. Absorption increased greatly, however, after blocking sulphydryl groups and the Scatchard equation was no longer applicable. Part of the increase was strong absorption. Absorption of DNP-glycine by myosin was negligible and was unaffected by sulphydryl-group blocking.

At low ionic strength (0·1) at which myosin is normally aggregated, there was less total absorption of bis-DNP-lysine or bis-DNP-ornithine than at high ionic strength, but the rate was faster. Blocking myosin sulphydryl groups increased the total absorption except at high reagent concentration. Strong absorption was a much larger proportion of the total and was approximately the same as at high ionic strength. At low ionic strength bis-DNP-lysine induced partial disaggregation of myosin aggregates.

It is suggested that the extent of weak absorption by myosin of bis-DNP-amino acids is a measure of the flexibility or instability of part of the myosin molecule, and that strong absorption involves only the heavy meromyosin region.

I. INTRODUCTION

In this laboratory the possibility is being examined of using dye absorption as a means of studying changes in the fibrous proteins of skeletal muscle, particularly in the so-called "cross-bridges" between myosin and actin (Huxley 1963). Changes in the amount of absorption of a suitable coloured compound of low molecular weight

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could, in theory, be used as a measure of changes in the configuration of a protein, and such changes should be detectable even when the protein is highly aggregated. So far we have examined as possible reagents only the 2,4-dinitrophenyl (DNP)- amino acids, which were chosen because of evidence that those with two DNP groups are absorbed by glycerinated muscle fibres and that the amount absorbed is responsive to structural changes to the muscle induced by blocking sulphydryl groups (Burley 1967). Absorption of bis-DNP-lysine by glycerinated muscle prevents adenosine-triphosphate-induced contraction, which implies that absorption occurs in a region essential for the contractile mechanism. An unusual fact about absorption of bis-DNP-lysine by muscle is that part of the absorbed reagent is bound very strongly (Burley 1967). It had previously been shown (Burley and Jackson 1965), that myosin at 2°C, after blocking of sulphydryl groups, strongly absorbs a small amount of bis-DNP-lysine. In those experiments absorption was estimated by measuring the amount of DNP compound retained by the myosin after the reaction mixture had been dialysed against buffer; or after the myosin had been precipitated from solution at low ionic strength. Neither procedure would be expected to take into account loosely bound molecules or ions. Because of the evidence (Burley 1967) that loose binding by muscle fibres is extensive and is likely to be of interest, absorption of the bis-DNP-amino acids by myosin has been re-examined with special reference to "weak" absorption* and to the effect of ionic strength and temperature on it; the aim being eventually to interpret changes in the absorption of bis-DNP-lysine by whole muscle.

Preliminary experiments in which weak absorption of DNP-amino acids by myosin was estimated after equilibrium dialysis gave inconsistent results, probably because of variable absorption by the dialysis membranes. The results reported here are those obtained when the absorption was estimated from the concentration of unabsorbed reagent in the reaction mixture after the myosin had been removed from solution by centrifuging.

As expected from the large proportion of myosin in muscle fibres, bis-DNP-lysine is absorbed by isolated myosin. Monomeric myosin absorbs more than a corresponding amount of muscle at the same reagent concentration, but the amount absorbed by aggregated myosin is closer to that absorbed by muscle. Previous conclusions on strong absorption by myosin (Burley and Jackson 1965) are largely unaffected by these observations.

II. Materials

(*a*) *Buffer Solutions*

(i) *pH 7, Ionic Strength 0·5.*—0·019M potassium dihydrogen phosphate, 0·060M disodium hydrogen phosphate, and 0·30M potassium chloride.

(ii) *pH 6·5, Phosphate.*—0·00573M potassium dihydrogen phosphate, 0·00427M dipotassium hydrogen phosphate, and 0·60M potassium chloride.

(iii) *pH 7, Ionic Strength 0·1.*—See Burley (1967).

*In this context the terms "weak" and "strong" refer to the ease or difficulty with which the DNP-amino acids can be removed from myosin after absorption.

(*b*) *DNP-amino Acids*

These were from the Sigma Chemical Co., St. Louis, Missouri. Solutions of bis-DNP-lysine and bis-DNP-ornithine were prepared by dissolving the solid in a minimum volume of 1·0N sodium hydroxide (about 60 μl for 30 mg) and diluting with water to give stock solutions of 0·013M or 0·025M. The 0·013M stock solution was previously and erroneously stated to be 0·13M (Burley 1967). Some batches of bis-DNP-lysine did not dissolve easily in sodium hydroxide solution; for these, warming the mixture or adding a little dimethylformamide was often helpful. Before use, stock solutions of the DNP-amino acids and sometimes those diluted with buffer were clarified by centrifuging for 4 hr at 400,000 *g* or by filtering through a Millipore filter. Solutions of DNP-amino acids were used under artificial light and were kept in the dark.

(*c*) p-*Chloromercuribenzoate*

The free acid, from Sigma Chemical Co., was dissolved in a minimum amount of 1·0N sodium hydroxide and diluted with the appropriate buffer to give a 0·008M stock solution of *p*-chloromercuribenzoate (pCMB), pH 10.

(*d*) *Muscle*

White muscles from the back and back legs of either New Zealand White rabbits or Californian rabbits were used.

III. Methods

(*a*) *Centrifuging*

For preparative centrifuging below 20,000 r.p.m., a Servall SS4 centrifuge was used at 3°C. For higher speeds a Beckman L-2 65 preparative ultracentrifuge was used with either the eight-place angle rotor (No. 65) or the swing-bucket rotor (SW65). For analytical ultracentrifuging a Spinco model E ultracentrifuge was used.

(*b*) *Preparation of Myosin*

Slight modifications to existing methods were used as follows (Mommaerts and Parrish 1951; Szent-Györgyi 1951), all operations being done at 1–4°C:

After the excised muscle had been cooled in ice it was minced and left in pH 6·5 buffer (300 ml per 100 g mince) for 15–50 min. The undissolved residue was centrifuged for 10 min at 12,500 r.p.m. and the supernatant solution left for about 1 hr until most of the ATP had been hydrolysed, as indicated by the formation of a precipitate on dilution. Crude myosin was then precipitated by adding water (1000 ml per 100 ml supernatant liquid) and separated by centrifuging for 10 min at 4500 r.p.m. For large volumes, a Servall "continuous flow" system was used at 10,000 r.p.m. The precipitate was then dissolved in a minimum volume of 2M potassium chloride (about 80 ml per 100 g original mince) and dialysed overnight into a 10-fold excess of 0·35 ionic strength buffer, pH 6·5—made by diluting the pH 6·5 phosphate buffer with 0·8 of its volume of water. Contaminating actomyosin was then sedimented by centrifuging for 60 min at 40,000 r.p.m. or for 30 min at 65,000 r.p.m. Lipid flotated

during this centrifuging was removed later. Solutions of myosin were stored in pH 6·5 phosphate buffer at 1°C and, unless otherwise stated, were used within a week post-mortem. Results from myosin stored for more than a few days were checked with fresh myosin if possible.

These myosin preparations were homogeneous according to ultracentrifuge studies, and contained less than 0·2% of lipid according to the Bligh and Dyer (1959) method.

(*c*) *Estimation of Protein Concentration*

Myosin concentration was estimated by the Folin–biuret method (Lowry *et al.* 1951), on 0·25–1·0 ml of the reaction mixture. The method was standardized against myosin solutions whose concentrations were determined as follows: known volumes were dialysed into water, then into volatile buffer (0·5M ammonium formate, pH 7), and into water again before being finally dried at 103°C for weighing.

(*d*) *Measurement of Total and Weak Absorption of DNP Compounds by Myosin*

The buffer solution (15 ml or more) containing myosin (final concentration 0·10%), the DNP compound, and, if necessary, excess pCMB, was allowed to stand at constant temperature for the required length of time. Part of the mixture was then centrifuged in Polyallomer tubes at the same temperature.

With phosphate buffer of ionic strength 0·5 at 5°C, centrifuging was for 12 hr at 360,000 *g* in an angle rotor. In this way 97% of the protein was sedimented from the myosin solutions, though slightly less (95%) was sedimented if the myosin had been treated with pCMB. At higher temperatures a shorter period of centrifuging could be used. Tests on solutions of DNP-amino acids in high ionic strength buffer with no myosin showed a small concentration gradient after centrifuging at 360,000 *g*. This effect, which made a large difference to the results only at low levels of absorption, was allowed for in calculating total absorption. No correction was applied, however, for possible effects due to redistribution of reactants during sedimentation because of evidence (see Section IV) which indicated that absorption and desorption are relatively slow.

With buffers of ionic strength 0·1, in which myosin is highly aggregated, centrifuging for 10 min at 400,000 *g* sedimented all aggregates without redistributing the DNP compound.

After centrifuging, a constant proportion (5 ml or a third) of the supernatant solution was withdrawn from the top of the centrifuge tube and its optical density at 360 mμ determined, usually after appropriate dilution with buffer. The total amount of DNP compound absorbed was calculated from the optical densities of the supernatant solution and the original mixture, correcting if necessary for sedimentation of the DNP compound. For buffers of low ionic strength the original total concentration of DNP-amino acid was usually obtained indirectly from the concentration before adding myosin. When necessary, the total absorption measured in this way was corrected for the "strong" absorption (see below) to give the "weak" absorption.

To inhibit microbial growth in myosin solutions at relatively high temperatures, small quantities of chloramphenicol were sometimes added to the reaction mixtures. Absorption of DNP compounds was not altered by this addition.

(e) *Measurement of Strong Absorption of Bis-DNP-lysine and Bis-DNP-ornithine by Myosin*

The precipitation and dialysis procedures described by Burley and Jackson (1965) were used to estimate "strong" absorption in mixtures of high ionic strength. At low ionic strength, since only dialysis could be used, the procedure was as follows: a small portion (1·0 or 2·0 ml) of the reaction mixture was dialysed for 16 and 8 hr, respectively, against two successive 100-ml volumes of buffer. The small volumes were then made up to known volume with phosphate buffer of ionic strength 0·5 after the myosin aggregates had been dissolved by addition of a minimum amount of 2M potassium chloride; alternatively the aggregates were first dissolved by means of a small amount of solid trypsin. The final solutions were centrifuged if they were not quite clear and the optical density at 360 mμ measured.

IV. Results

(a) *Experiments at High Ionic Strength*

(i) *Weak Absorption of DNP-amino Acids*

Figure 1, curve *A*, relates the "weak" absorption (*R*) of bis-DNP-lysine by myosin to the equilibrium concentration (*D*); i.e. the concentration of unabsorbed bis-DNP-lysine remaining in solution after the myosin had been treated for 48 hr with this reagent at 5°C. Results were usually reproducible for a given myosin preparation, but some preparations gave values consistently higher or lower than those indicated by curve *A*.

Curve *B* shows that absorption was much greater when the myosin was first treated with a fourfold excess of pCMB over that necessary to block all –SH groups. The results were the same if the pCMB was added at the same time as the bis-DNP-lysine. Curve *B* refers to total absorption and has not been corrected for "strong" absorption, about 10% of the total (see below).

Curves *E* and *F* show the absorption at 5°C of bis-DNP-ornithine by untreated and pCMB-treated myosin from a single preparation. The absorptions were much lower than those for bis-DNP-lysine but variation amongst different myosin preparations was greater. Absorption of DNP-glycine under the same conditions was negligible.

Comparisons between 300 and 500 mμ of spectrophotometric curves of mixtures containing both bis-DNP-lysine (2×10^{-4}M) and pCMB-treated myosin (0·1%) before and after they had been allowed to stand at 5°C for 48 hr (during which period about a third of the DNP compound was absorbed), revealed a small change in the spectrum of bis-DNP-lysine as a result of its absorption by myosin.

Scatchard plots (1949) of *R* versus *R*/*D* for the curves in Figure 1 are shown in Figure 2, and reciprocal plots (Rosenberg and Klotz 1960) in Figure 3. Both of these plots express a relationship implicit in the simple mass action equation:

$$K = R/D(n-R),$$

for the absorption of molecules of equilibrium concentration D by a macromolecule with n binding sites, K being the binding constant and R the average absorption

per mole. The latter equation and its variants are obeyed only if the binding sites are quite independent and have approximately the same binding constants. The

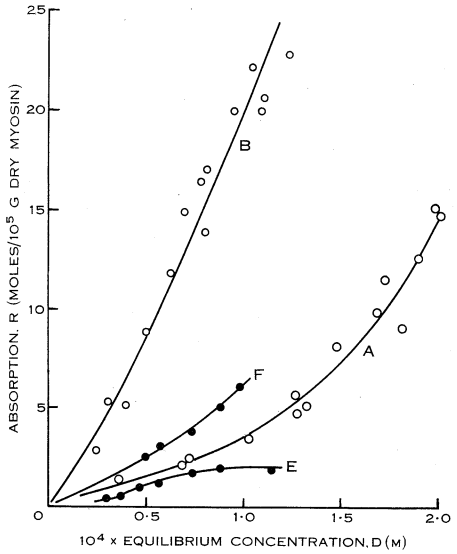

Fig. 1.—"Weak" absorption at 5°C of DNP-amino acids by myosin; ionic strength 0·5, phosphate buffer, pH 7·1–7·3:
A, bis-DNP-lysine; untreated myosin.
B, bis-DNP-lysine; pCMB-treated myosin.
E, bis-DNP-ornithine; untreated myosin.
F, bis-DNP-ornithine; pCMB-treated myosin.

curves for bis-DNP-lysine and bis-DNP-ornithine in Figures 2 and 3 do not show the expected linear relation, though curves *a*, *b*, and *f* in Figure 2 resemble the corresponding curves for absorption of bis-DNP-lysine by whole muscle (Burley 1967).

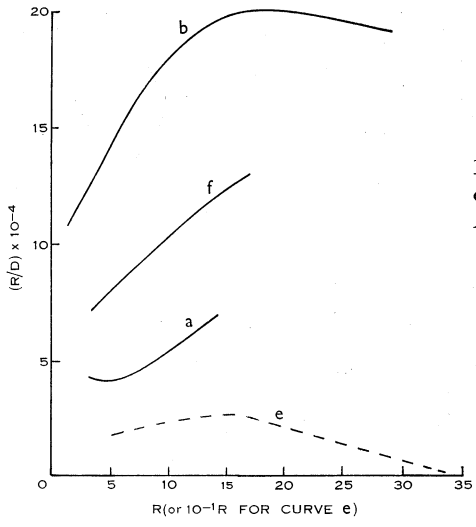

Fig. 2.—Scatchard plots. Curves *a*, *b*, *e*, *f* were derived from the corresponding smoothed curves *A*, *B*, *E*, *F* in Figure 1.

Curve *e* in both Figure 2 and Figure 3, which refers to the absorption of bis-DNP-ornithine by a single preparation of untreated myosin, approaches normal behaviour

at high values of R. Consequently the number of absorption sites at 5°C for this preparation could be calculated, very approximately, as four sites per 10^5 g dry myosin, with a binding constant $1 \cdot 1 \times 10^4$.

Rates of absorption of bis-DNP-lysine by myosin at 5°C are shown in Figure 4 for untreated (curves A, B, and C) and for pCMB-treated myosin (curve X). Each curve refers to mixtures in which the unabsorbed bis-DNP-lysine in solution was at

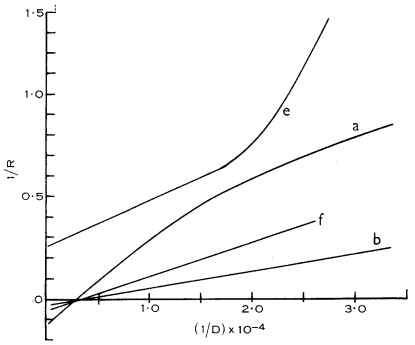

Fig. 3.—Reciprocal plots for the data given in Figure 1. Curve a was derived from the smoothed curve A, and so on.

a concentration (D) at the time of measurement, this point being obtained from isotherms by interpolation if necessary. The values are only approximate, because of the long centrifuging necessary to separate the reactants; nevertheless, Figure 4 suggests that, with untreated myosin, the reaction time of 48 hr used for most of the experiments at high ionic strength may not have been quite long enough for the

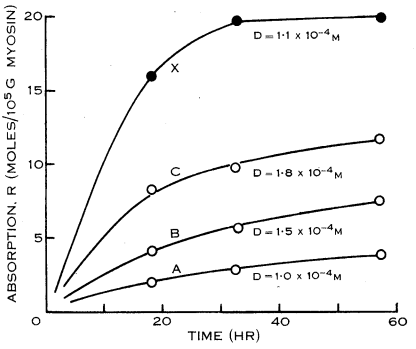

Fig. 4.—Rates of absorption at 5°C of bis-DNP-lysine by myosin at different supernatant concentrations (D) of bis-DNP-lysine, in phosphate buffer, pH 7·2, ionic strength 0·5. Curves A, B, and C refer to untreated myosin, and curve X to pCMB-treated myosin.

attainment of equilibrium, especially at high reagent concentrations. The effect on the slopes of the Scatchard plots if R and D were not true equilibrium values is indicated in Figure 5, where values of R and D corresponding to reaction times of 18, 35, and 57 hr were used. Evidently, deviations due to the non-attainment of equilibrium over this range were not enough to alter the slopes, which are still in the opposite direction to those found with most systems (Scatchard 1949).

In an experiment designed to reveal whether the redistribution of protein during sedimentation affected the absorption measurements, two solutions, each containing myosin (0·1%) and bis-DNP-lysine ($0{\cdot}8\times10^{-4}$M and $1{\cdot}8\times10^{-4}$M) in high ionic strength phosphate buffer, were left at 5°C for 48 hr. Each was then diluted with an equal volume of buffer and centrifuged for 12 hr at 360,000 g as usual. Only

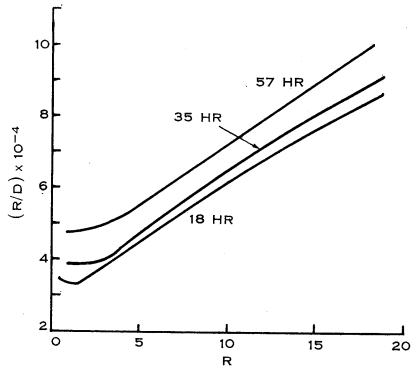

Fig. 5.—Scatchard plots, after three reaction times, of data corresponding to curves A, B, and C of Figure 4.

for the more concentrated bis-DNP-lysine solution was there evidence of desorption of bis-DNP-lysine during the centrifuging and this desorption was relatively slight. As indicated earlier, dialysis into excess buffer causes rapid desorption of most of the bis-DNP-lysine.

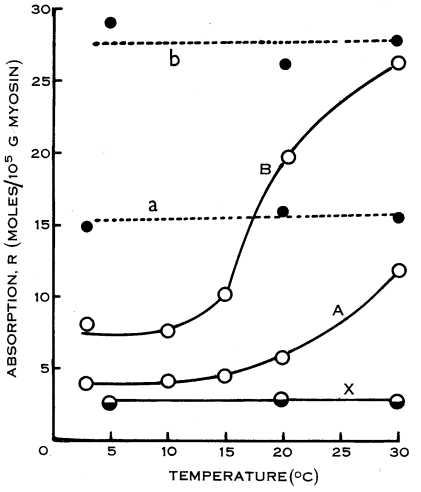

Fig. 6.—Effect of temperature on total absorption of bis-DNP-lysine by myosin, pH 7·1–7·4, for two equilibrium concentrations (D) after reaction for 24 hr in phosphate buffer. Curves A and B represent untreated myosin in buffer of ionic strength 0·5, and $D = 1{\cdot}0\times10^{-4}$M and $1{\cdot}5\times10^{-4}$M, respectively. Curves a and b represent the corresponding pCMB-treated myosin. Curve X represents untreated myosin in phosphate buffer, pH 7·2, ionic strength 0·1, $D = 2{\cdot}0\times10^{-4}$M. Curves A, B, a, and b refer to the same myosin preparation reacted 24 hr, and samples were centrifuged at the appropriate temperature.

Figure 6 shows the effect of temperature on the total absorption of bis-DNP-lysine by myosin at pH 7 and high ionic strength. Curves A and B refer to untreated myosin at two equilibrium concentrations ($D = 1{\cdot}0\times10^{-4}$M and $1{\cdot}5\times10^{-4}$M respectively), and curves a and b refer to pCMB-treated myosin samples at these same D values. These curves show that absorption of the reagent by pCMB-treated

myosin is relatively high, but is not affected by temperature; whereas absorption by untreated myosin increases greatly as the temperature is raised above about 15°C, and by 30°C it is almost equal to that after pCMB treatment. Curve *X* in Figure 6 refers to a low ionic strength solution and is discussed below.

Measurements of the effect of pH on weak absorption of bis-DNP-lysine by untreated and pCMB-treated myosin at high ionic strengths showed that between pH 7 and 9 the absorption decreases about 15% in an approximately linear fashion at either 5 or 20°C. A similar decrease in strong absorption was reported earlier (Burley and Jackson 1965).

Attempts at measuring absorption of bis-DNP-lysine in Tris buffers of the same ionic strength (i.e. 0·5) as used for phosphate were complicated by an unusual difficulty in that after reaction with bis-DNP-lysine in Tris buffer a significant part of the myosin (12–13%) could not be sedimented in 16 hr at 360,000 *g*. Correction for incomplete sedimentation gave values consistently higher than those for the phosphate buffer. Qualitatively, the same conclusions could be reached with either buffer.

The effect of bis-DNP-lysine on the hydrodynamic behaviour of myosin at pH 7·1, ionic strength 0·5, was examined by measuring the ultracentrifuge patterns at 20°C of equilibrium mixtures containing myosin (0·10% final concentration) and increasing concentrations, *D*, of bis-DNP-lysine. Up to $D = 0{\cdot}6 \times 10^{-4}$M, the sedimentation coefficient and peak shape were unchanged, as compared with a control sample of myosin at the same concentration. At $D = 1{\cdot}1 \times 10^{-4}$M, the monomer peak had decreased by about 40%, and faster peaks—probably representing myosin trimers and higher aggregates—appeared. At $D = 1{\cdot}7 \times 10^{-4}$M, very little monomer remained. Evidently one effect of bis-DNP-lysine absorption is to facilitate the formation of small aggregates.

Little change in the weak absorption of bis-DNP-lysine by myosin at 5°C was detected when the ionic strength of the buffer solutions was increased from 0·5 to 1·1 at pH 7·0.

(ii) *Strong Absorption of Bis-DNP-lysine by Myosin*

Many of the features of strong absorption have already been described (Burley and Jackson 1965). It is now evident that this type of absorption occurs with *untreated* myosin at elevated temperatures. Figure 7, for example, shows that above about 25°C the difference between untreated (curve *A*) and pCMB-treated myosin (curve *B*) disappears. Nevertheless, the *rate* of strong absorption is greater for treated myosin, even at 30°C, as shown in Figure 8, which compares the behaviour of treated and untreated myosin at 20 and 30°C.

Temperature evidently enhances both weak and strong absorption (cf. Figs. 6 and 7), although the increase in strong absorption by untreated myosin occurs at a higher temperature.

If a solution containing bis-DNP-lysine and myosin in equilibrium at 30°C was cooled to 1°C, after 48 hr the same value for strong absorption was found. Apparently strong absorption at 30°C was effectively irreversible in the presence of excess bis-DNP-lysine, but could be largely reversed at 30°C or less by prolonged dialysis against excess buffer.

The above evidence for a relatively large amount of weak absorption of bis-DNP-lysine by myosin implies that the relation between strong absorption and reagent concentration reported previously (Burley and Jackson 1965) is of limited value, for it was assumed that, because of the relatively small amount of strong absorption, the equilibrium reagent concentration was equal to the total concentration. A more reliable relation for both treated and untreated myosin, taking into account weak absorption, is given later (see Fig. 12, curves *X* and *Y*).

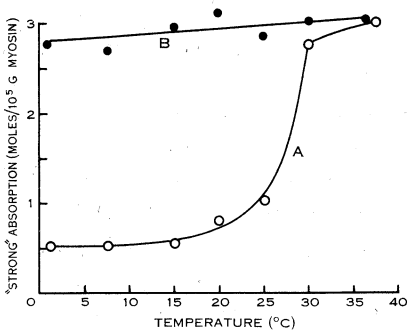

Fig. 7.—Effect of temperature on "strong" absorption of bis-DNP-lysine (3×10^{-4}M) by myosin (final concentration 0·3%) treated at ionic strength 0·5, pH 7·1, for 48 hr: curve *A*, untreated myosin; curve *B*, myosin pretreated with pCMB.

(*b*) *Experiments at Low Ionic Strength*

(i) *Total Absorption of DNP-amino Acids*

To examine absorption under more nearly physiological conditions and closer to those used for muscle fibres (Burley 1967), some of the above experiments were repeated using buffers of lower ionic strength (about 0·1) in which myosin is aggregated

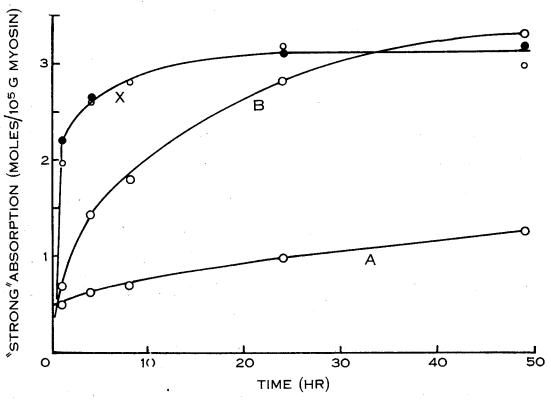

Fig. 8.—Rate of "strong" absorption of bis-DNP-lysine (3×10^{-4}M) myosin at 20 and 30°C: curve *A*, untreated myosin at 20°C; curve *B*, untreated myosin at 30°C; curve *X*, pCMB-treated myosin at 20°C (●) and 30°C (○).

(Huxley 1963; Kaminer and Bell 1966). Higher concentrations of DNP-amino acids were thus possible, but a complication in the presence of bis-DNP-lysine, as mentioned in Section IV, is the disaggregation of the myosin.

Figure 9 shows the total absorption of bis-DNP-lysine by untreated (curve *A*) and pCMB-treated myosin (curve *B*) after 20 hr in phosphate buffer of ionic strength

0·1, pH 7·1–7·3, at 5°C. The points, which refer to preparations from six rabbits, have been selected by the exclusion of a few samples in which more than about 12% of the myosin dissolved under these conditions. Apart from this, no correction was attempted to allow for dissolution, which, as shown in Figure 13, was greater after pCMB treatment and also increased with reagent concentration. Points from freshly prepared myosin have also been excluded from curve *A* in Figure 9, because reproducible results were not usually possible unless the myosin had stood for 2 or 3

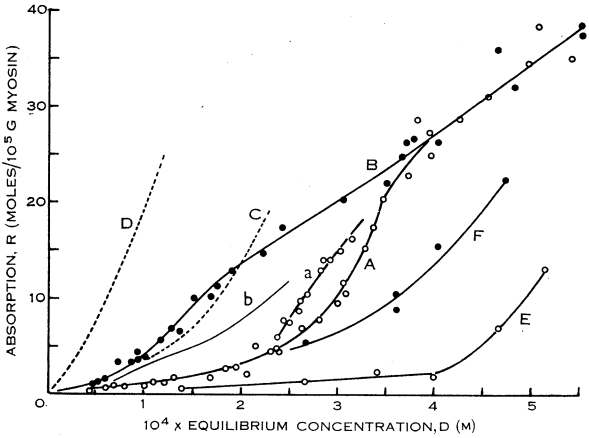

Fig. 9.—Absorption of bis-DNP-amino acids at low ionic strength (0·1 pH 7·1, 5°C): curve *A* total absorption of bis-DNP-lysine by untreated myosin; curve *B* total absorption of bis-DNP-lysine by pCMB-treated myosin; curve *C* total absorption of bis-DNP-lysine at ionic strength 0·5 by untreated myosin; curve *D* total absorption of bis-DNP-lysine at ionic strength 0·5 by pCMB-treated myosin; curve *E* total absorption of bis-DNP-ornithine by untreated myosin; curve *F* total absorption of bis-DNP-ornithine by pCMB-treated myosin. Segment *a* represents absorption of fresh myosin under conditions used for curve *A*. Curve *b*, derived from curve *B*, represents weak absorption of bis-DNP-lysine by pCMB-treated myosin.

days at about 2°C after dialysis into low ionic strength buffer. Such aging was not always necessary if the myosin had been kept for 7 days or more in high ionic strength buffer. In Figure 9, the variable region is indicated by segment *a*, which refers to fresh samples from three rabbits. After 2 days more, these samples followed curve *A* throughout.

Curves *C* and *D* in Figure 9 indicate the total absorption of bis-DNP-lysine by some of the same myosin preparations when the ionic strength was high. Although there may be inaccuracies in the results at low ionic strength, it is quite clear from Figure 9 that total absorption of bis-DNP-lysine by myosin is consistently lower at ionic strength 0·1 than at 0·5. In addition, although at both ionic strengths pCMB treatment greatly enhances the absorptive capacity of the myosin at reagent concentrations less than about 4×10^{-4}M, at higher concentrations than this (attainable only at low ionic strength) the difference is apparently eliminated. It is

shown in Section IV(*b*)(ii) that at low ionic strength strong absorption is often a large proportion of the total. Curve *b* in Figure 9 indicates *weak* absorption by pCMB-treated myosin.

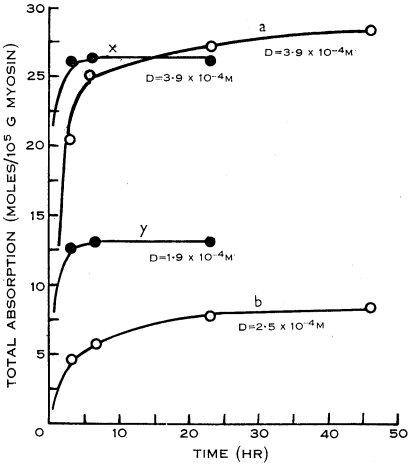

Fig. 10.—Rate of absorption of bis-DNP-lysine by myosin at low ionic strength, pH 7·1, 5°C. Curves *a* and *b* represent untreated myosin and curves *x* and *y* pCMB-treated myosin. The *D* values are concentrations of unabsorbed reagent.

The lower total absorption of bis-DNP-ornithine by untreated and pCMB-treated myosin at low ionic strength is represented in Figure 9 by curves *E* and *F*. No absorption of DNP-glycine could be detected up to a concentration of 6×10^{-4}M at least.

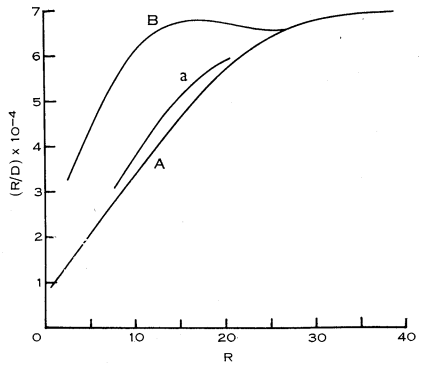

Fig. 11.—Scatchard plots for curves *A*, *B*, and *a* in Figure 9.

Figure 10 compares the *rates* of total absorption of bis-DNP-lysine by treated and untreated myosin at low ionic strength, pH 7·1, 5°C. Absorption is slightly faster for the pCMB-treated samples (curves *X* and *Y*), and for both sets of samples it is much faster than at high ionic strength (Fig. 4). Equilibrium is apparently more readily attainable at low ionic strength, although, as shown by Figure 11, the Scatchard plots derived from curves *A*, *B*, and segment *a* in Figure 9 are still abnormal.

Assessment of the effect of temperature on absorption of bis-DNP-lysine by myosin at low ionic strength was greatly complicated by dissolution of the myosin aggregates, which was apt to be irregular at higher temperatures, for reasons that are not clear. Nevertheless, absorption by untreated myosin did not appear to show the large temperature effect noticed at high ionic strength (e.g. see Fig. 6, curves *A* and *B*). Curve *X* in Figure 6 gives results for some untreated myosin samples having minimum dissolution at ionic strength 0·1, pH 7·2, after 20 hr treatment with bis-DNP-lysine, whose equilibrium concentration was $2{\cdot}0\times10^{-4}$M. Aggregated myosin is evidently more resistant to temperature changes.

Apparently, at low ionic strength it is immaterial whether phosphate or Tris buffers are used.

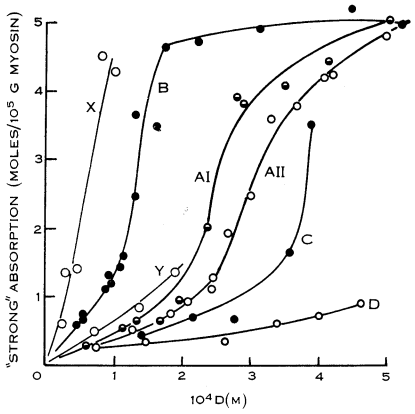

Fig. 12.—"Strong" absorption of bis-DNP-lysine and bis-DNP-ornithine at low ionic strength, 5°C, pH 7. Curves *AI* and *AII*, bis-DNP-lysine absorption by untreated myosin, two rabbits; curve *B*, bis-DNP-lysine absorption by pCMB-treated myosin from the same two rabbits; curves *C* and *D*, bis-DNP-ornithine absorption by pCMB-treated and untreated myosin, respectively; curves *X* and *Y*, bis-DNP-lysine absorption at high ionic strength (0·5), same preparation of pCMB-treated and untreated myosin as used for curves *B* and *AII*.

(ii) *Strong Absorption of DNP-amino Acids*

Figure 12 shows strong absorption, i.e. the absorption not readily reversed by dialysis, of bis-DNP-lysine by pCMB-treated (curve *B*) and untreated myosin (curves *AI* and *AII*) from two rabbits, as a function of the equilibrium concentration of reagent in the initial treatment. The behaviour of bis-DNP-ornithine under the same conditions is indicated by curves *C* and *D* of Figure 12. These five curves refer to low ionic strength. Two of them, curves *AII* and *B*, are directly comparable with curves *X* and *Y* respectively which refer to strong absorption by the same myosin preparation measured by the same procedure, with the difference that the ionic strength was high (0·5). Although they do not coincide, curves *X* and *Y* are sufficiently close to curves *AII* and *B* to suggest that strong absorption is not greatly affected by the initial state of aggregation of the myosin, nor by the change in ionic strength; and that the complete isotherms at high ionic strength would resemble curves *AII* and *B*. Part of the large difference already reported between different preparations (Burley and Jackson 1965) may, it now appears, reside in differences in weak absorption and their effect on the equilibrium reagent concentration. Some differences among preparations are, however, still apparent (e.g. curves *AI* and *AII* in Fig. 12).

(c) *Dissolution of Aggregated Myosin by Bis-DNP-lysine*

As already mentioned, at low ionic strength bis-DNP-lysine partly dissolves the myosin aggregates, especially at high reagent concentrations and in the presence of pCMB. Figure 13 shows the effect of the *initial* concentration of bis-DNP-lysine on the dissolution of myosin, expressed as the percentage of the total protein not sedimented in 15 min at 400,000 *g* after treatment for 20 hr at pH 7·2 and 5°C. Curve *A* refers to untreated myosin from three rabbits, and curve *B* to the corresponding pCMB-treated myosin, all measured 2–7 days post-mortem. One preparation, whose behaviour is indicated by curve *C* (untreated) and curve *D* (pCMB-treated),

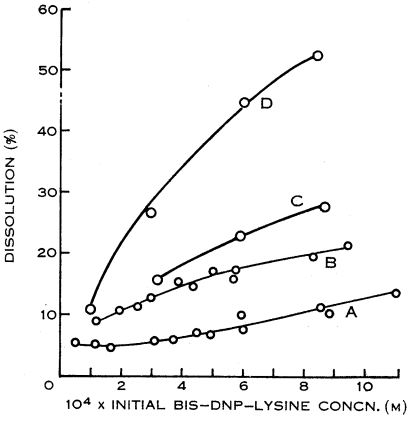

Fig. 13.—Dissolution of aggregated myosin by bis-DNP-lysine at 5°C, ionic strength 0·1, pH 7·1–7·3. Curve *A*, untreated myosin; curve *B*, pCMB-treated myosin; curve *C*, abnormal dissolution of freshly dialysed untreated myosin; curve *D*, abnormal dissolution of freshly dialysed pCMB-treated myosin.

dissolved much more readily when fresh (i.e. 2 days post-mortem) although it behaved similarly to the others when the dissolution was measured after 5 more days at 3°C. Dissolution at the same bis-DNP-lysine concentration was usually slightly higher at 20 and 30°C, but was more variable. Bis-DNP-ornithine had a much smaller effect on aggregated myosin, and DNP-glycine had no measurable effect.

V. Discussion

The above results establish that there are large differences between the similar compounds bis-DNP-lysine and bis-DNP-ornithine in their absorption by skeletal muscle myosin. For each compound absorption is very dependent on whether the myosin sulphydryl groups have been blocked, on the temperature, and on the state of aggregation of the myosin. Furthermore, the total absorption often consists of both "weak" and "strong" absorption which may vary independently. Factors affecting absorption are summarized in Table 1, which includes some previous results for glycerinated muscle. Table 1 emphasizes that aggregated myosin (i.e. at low ionic strength) and muscle fibres need much higher reagent concentrations to attain a given level of absorption than does monomeric myosin (i.e. at high ionic strength).

The molecular basis for the differences shown in Table 1 are not yet known. Some assumptions in line with those previously proposed for muscle fibres (Burley 1967) are possible, however, and are given below together with their implications.

(a) *Weak Absorption*

Since other proteins, including bovine serum albumin, gelatin, and ovalbumin also absorb bis-DNP-lysine weakly (Burley and Robertson, unpublished data) it may be assumed that absorption sites are common. Suitable sites may reasonably be expected to contain positively charged side-chain groups and suitably placed hydrophobic regions, including perhaps aromatic side-chains, but further speculations on their nature do not seem justified. The equations of Scatchard and of Klotz (Figs. 2 and 3) are not obeyed by data for absorption of bis-DNP-lysine on myosin, and rarely by data

TABLE 1

EQUILIBRIUM CONCENTRATIONS (D) FOR BIS-DNP-LYSINE AND BIS-DNP-ORNITHINE NECESSARY FOR WEAK AND STRONG ABSORPTION BY MYOSIN UNDER VARIOUS CONDITIONS

Values given are equilibrium concentrations necessary for the absorption of 10 moles (weak absorption) and 2 moles (strong absorption) of bis-DNP-amino acid per 10^5g myosin

Sample	pCMB Treatment	Temp. (°C)	Ionic Strength	Weak Absorption		Strong Absorption	
				10^4D for Bis-DNP-lysine (M)	10^4D for Bis-DNP-ornithine (M)	10^4D for Bis-DNP-lysine (M)	10^4D for Bis-DNP-ornithine (M)
Myosin	–	5	0·5	1·75		2·2	
Myosin	–	30	0·5	0·81		0·5	
Myosin	+	5–30	0·5	0·66	1·5	0·5	
Myosin	–	5–30	0·1	3·3	4·9	2·5	7·5
Myosin	+	5–30	0·1	2·2	3·7	1·2	3·6
Muscle fibre*	–	20	0·1	7·0	17	5·5	15
Muscle fibre*	+	20	0·1	4·0		5·5	15

* Glycerinated muscle (see Burley 1967).

for bis-DNP-ornithine, so presumably the absorption sites are not independent. Somewhat similar behaviour has been reported for absorption of certain anionic detergents by other proteins (e.g. Ray *et al.* 1966).

In explanation of the behaviour of myosin and the bis-DNP-amino acids, it is assumed that not many absorption sites are normally available, because of the concealment of most of the hydrophobic regions from the aqueous solution. Further, absorption occurs because bis-DNP-lysine, and to a lesser extent bis-DNP-ornithine, are able to alter the configuration of myosin, or of part of it, so that hidden sites become increasingly available as the reagent concentration is raised, i.e. weak absorption may be regarded as a "cooperative" phenomenon.

In addition to similarities in their absorption characteristics, bis-DNP-lysine and anionic detergents are analogous in other ways; for example, certain anionic detergents also tend to dissolve the fibrillar proteins of muscle (Drabikowski and

Gergely 1962; Connell 1965) and to inhibit muscle contraction (Seidel and Gergely 1963; Burley 1967). On this analogy the smaller affinity of the lower homologue, bis-DNP-ornithine, would not be surprising. Saturated solutions of bis-DNP-lysine in water at high pH occasionally form gels, but extensive formation of micelles under the conditions used for absorption measurements seems to be excluded by the small deviation from Beer's law at 360 mμ (Burley 1967).

(*b*) *Effect of pCMB Treatment and Heat Treatment*

It is known that pCMB treatment alters the structure of myosin (Tonomura, Sekiya, and Imamura 1963; Kominz 1965) presumably in the non-helical regions (Duke, McKay, and Botts 1966). The exact nature of the change has not been elucidated, but it is evidently responsible for greatly increased absorption of bis-DNP-lysine and bis-DNP-ornithine by monomeric myosin (Fig. 1) and by myosin aggregates (Fig. 9). After pCMB treatment the Scatchard equation is still not obeyed (Figs. 2 and 11). In explanation of this it is assumed that pCMB treatment does not itself expose more absorption sites but modifies the protein structure in such a way that the DNP compounds are more readily able to expose these sites.

Bis-DNP-amino acids would thus seem to be sensitive indicators of changes in the flexibility or stability of the myosin structure. For example, the greater effect of high temperatures on absorption of bis-DNP-lysine by monomeric myosin than by aggregated myosin (Fig. 5) would suggest that the aggregates have greater thermal stability.

One disadvantage of bis-DNP-lysine as an indicator for structural changes is that at high reagent concentrations it is able to induce a change in myosin similar to that caused by pCMB treatment (Fig. 9). Another is that it induces partial disaggregation of myosin at low ionic strength (Fig. 13). This behaviour does not necessarily contradict the evidence for its aggregating tendencies at high ionic strength, noted above, because the states of aggregation are undoubtedly different.

Bis-DNP-ornithine seems to be potentially a more sensitive indicator for small structural changes than bis-DNP-lysine (Fig. 1) and it seems to lack its disadvantages, though it has been studied less thoroughly.

(*c*) *Effect of Variations in Ionic Strength*

It is assumed that the greatly decreased absorption of bis-DNP-lysine and bis-DNP-ornithine at low ionic strength, where myosin aggregates to rods that resemble thick filaments (Huxley 1963), may be partly explained by the smaller proportion of exposed surface area per myosin monomer. Similarly, the lower absorption by aged aggregates (Fig. 9) could be due to consolidation of monomers within the rods. Comparison with muscle fibres (Table 1) suggests that the intact fibrillar protein system absorbs bis-DNP-amino acids to a lesser extent than do myosin aggregates. Such a decrease would be expected if the myosin molecules in the thick filaments were less readily available to the reagents than were those in regenerated aggregates.

The greater *rate* of absorption by aggregated myosin and by muscle fibres (Figs. 4 and 10), in spite of the lower absorptive capacity of the aggregates, suggests

that weak absorption may actually occur in several steps, of which only the fastest are detected after aggregation. The postulated fast steps are, it is suggested, associated more with the heavy meromyosin region—part of which projects from the surface of the rods and so is likely to be always accessible to the solution (Huxley 1963)—than with the light meromyosin region.

(*d*) *Strong Absorption*

It is assumed that strong absorption is a different phenomenon from weak absorption. Thus, although strong absorption may vary amongst myosin preparations, it seems to reach a definite limit, unlike weak absorption. Other proteins absorb bis-DNP-lysine weakly, but so far only myosin has been found to exhibit strong absorption. Various attempts have been made to account for this strong absorption (Burley and Jackson 1965; Burley 1967). The above data do not enable any of the tentative explanations to be refuted, but it is clear that although aggregation of myosin greatly decreases weak absorption, strong absorption is not much altered thereby (Figs. 9 and 12). In accordance with our previous postulates, a very accessible region of the myosin molecules is assumed to be involved in strong absorption. As before, the heavy meromyosin region is most likely to be implicated (direct evidence for this will be presented later): the decrease in strong absorption exhibited by glycerinated muscle fibres could then be explained as being a consequence of involvement of part of the heavy meromyosin in cross-bridging.

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