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SEDIMENTATION AND AGGREGATION OF COD MYOSIN: A RE-APPRAISAL

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SUMMARY

A reexamination of the sedimentation behaviour of cod myosin and its aggregated components has been made. There is no evidence that the sedimentation coefficients of this protein or its aggregated components depend upon either the age of the preparation or its extent of aggregation. Judged from corrected values of the amount of myosin remaining at various times of storage at 0°, the course of the aggregation reaction has been found to be second order at protein concentrations of 0.1–0.4 %. At concentrations of about 1.3 %, departures from second-order kinetics are indicated. It has been found that aggregation during the precipitation of cod myosin by dilution to low ionic strength can be avoided.

INTRODUCTION

In a previous investigation[1], the kinetics of the lateral aggregation of cod-myosin molecules were analysed by measuring the rate at which the area at the photographic plate of the ultracentrifuge component corresponding to monomeric myosin diminished. This diminution in area followed exactly neither first nor second-order kinetics over a range of protein concentrations of from 0.3 to 1.5 % and it was proposed that the overall aggregation reaction involved two consecutive stages, a first-order denaturation stage followed by a second-order aggregation. The measured areas in this investigation were corrected for neither radial dilution nor boundary "pile-up" effects[1]. JOHNSON AND ROWE[2] have analysed the aggregation of rabbit myosin by a similar method but corrected the observed area for both effects. Their corrected results obey second-order kinetics exactly over a range of protein concentrations of from 0.18 to 0.69 % and they conclude, therefore, that the rate-limiting step in the ultracentrifugally-observed aggregation is a dimerisation. In an earlier paper[4] uncorrected data had given the impression that the reaction was exactly first order. It is thus apparent that the nature of the corrections to be applied is of critical importance in efforts to arrive at a correct reaction mechanism.

A reevaluation of the previous results on cod myosin has shown that corrections for the effects just described cannot be "minimised" as earlier stated[1]. However, whilst correction for radial dilution is straightforward that for the "pile-up" effect described by JOHNSTON AND OGSTON[3] is less so. Correction for the latter effect requires (a) an accurate knowledge of the concentration dependence of sedimentation of the

components and (b) that the influence of the components of a mixture on the sedimentation rate of one component in the mixture is the same as the influence of itself on the sedimentation rate of that component. Two features of the sedimentation behaviour of the mixture of components in an aggregated myosin system as presented by JOHNSON AND ROWE[2] apparently do not fulfil these requirements.

The first of these features is that the rate of sedimentation of the components in question decreases substantially during the course of aggregation. The observed decreases occurred in at least one preparation of myosin (*i.e.* VIII (see ref. 2)) at stages of the aggregation reaction used to compute kinetic data and are of sufficient magnitude to materially affect the JOHNSTON–OGSTON correction. In order to correct validly for the JOHNSTON–OGSTON effect at all stages in the aggregation one would require to know, therefore, the concentration dependence of sedimentation of each component in the mixture at all stages. In their calculations, JOHNSON AND ROWE[4] used only sedimentation data obtained from the mixture of components present at an early stage in the aggregation reaction.

A second, related, feature is that the measured concentration dependence of sedimentation of the faster components (*i.e.* Components 2 and 3 in JOHNSON AND ROWE's nomenclature[2]) is much more pronounced than that of myosin Therefore, requirement b may not be fulfilled

In view of these difficulties, a reexamination of the sedimentation and aggregation behaviour of cod myosin has been made.

EXPERIMENTAL

Preparation of cod myosin

The new experimental results described in this paper were obtained on preparations of cod myosin made in the manner described previously[1], *i.e.*, by washing the muscle with dilute buffer and then extracting the residue with buffer of an ionic strength of 0.5–0.6 containing pyrophosphate. In all cases the protein was dissolved in 0.45 M KCl–3.38 mM KH_2PO_4–15.5 mM Na_2HPO_4 (pH 7.5). In determining protein concentrations by the Kjeldahl method, a nitrogen content of 16.4 % has been used[5].

Sedimentation methods

The methods of determining sedimentation coefficients and of measuring areas under peaks in sedimentation photographs have been described previously[1,5].

Fatty acid content of cod myosin

The preparations of cod myosin contained varying amounts of lipid, including free fatty acid. Total lipid and fatty acid were extracted by either of two methods.

In the first, 1 volume myosin solution was added to 3 volumes methanol + 4 volumes chloroform to make a homogeneous mixture, which after vigorous stirring was dropped through water to remove the methanol according to the method of FOLCH *et al.*[6]. The chloroform layer resulting from this treatment was then evaporated at 40° in a rotary evaporator.

The second method of extraction is an adaptation of that used for muscle tissue by BLIGH AND DYER[7]. 80 ml of myosin solution are blended at full speed in an Atomic blendor for 30 sec with 200 ml methanol and 100 ml chloroform. Successive 100-ml

quantities of water and then chloroform are added with blending as before. The mixture is allowed to settle and the bottom chloroform layer removed and evaporated as before.

The fatty acid in the lipid extracts was separated on columns of silicic acid as described by McCARTHY AND DUTHIE[8]. The acid fraction eluted from the column was dissolved in 10 ml hot ethanol and titrated with 0.01 N KOH (phenolphthalein). An equivalent weight of 300 for the fatty acid was assumed.

Using these methods, recovery of fatty acid added to cod myosin solutions was satisfactory.

RESULTS

Protein concentration at the sedimenting boundary

Because of varying amounts of impurities in the preparations of cod myosin so far available it has been found necessary to measure protein concentrations directly from the areas under the peaks of the sedimenting components rather than rely on total nitrogen content of the solution. Myosin solutions in general often seem to contain heavy polydisperse components that do not always form discrete, observable boundaries. Even some cod-myosin solutions prepared by dissociation of precipitated and redissolved actomyosin followed by preparative ultracentrifugation[5] are less pure than was at first thought.

The factor relating cod myosin concentration to area at the photographic plate under constant conditions of cell depth and diaphragm angle was determined with the aid of synthetic boundary cell runs on preparations clarified by centrifugation for 1–2 h at 100000 $\times$ g. The results (in arbitrary units) of determinations on five preparations were 0.72, 0.74, 0.76, 0.79 and 0.80 (mean 0.76). This appreciable variation is presumably the result of variations in refractive index increment arising from contaminating impurities, particularly lipid, nucleic acid and nucleotide[9,10]. (Values ranging from 0.18 to 0.20 for the refractive index increment of purified rabbit myosin at 546 mμ have been noted in the literature.) As a check on these results, bovine serum albumin under the same conditions of cell depth and diaphragm angle gave a mean value of 0.77 for the factor in both sedimentation velocity and synthetic boundary cell runs. Rather than determine the correct factor for each myosin preparation, a mean value of 0.76 has been used routinely. Concentration determinations by this procedure are therefore subject to a possible uncertainty of about $\pm 5\%$.

Concentration dependence of sedimentation of unaggregated cod myosin

The sedimentation coefficients of freshly prepared, unaggregated cod-myosin solutions at concentrations (corrected for radial dilution) determined from area measurements are shown in Fig. 1. This data includes 11 determinations recalculated from previous results[5] obtained on 16 cod-myosin preparations by dissociating actomyosin followed by preparative ultracentrifugation. The previous results were obtained using a bar as diaphragm in the ultracentrifuge optical system and in only 11 out of the 16 determinations could the concentration of the myosin be assessed accurately from area measurements. A further 6 new preparations of ultracentrifugally-prepared myosin measured using a phase-plate diaphragm are included in Fig. 1. The remaining data were obtained on preparations made either as described in the experimental section or by precipitation and resolution as described below.

In about one half of the preparations analysed a small amount of a component sedimenting at a rate slower than that of myosin is present; in these cases the concentration of the slow component is included in the total concentration at the myosin boundary.

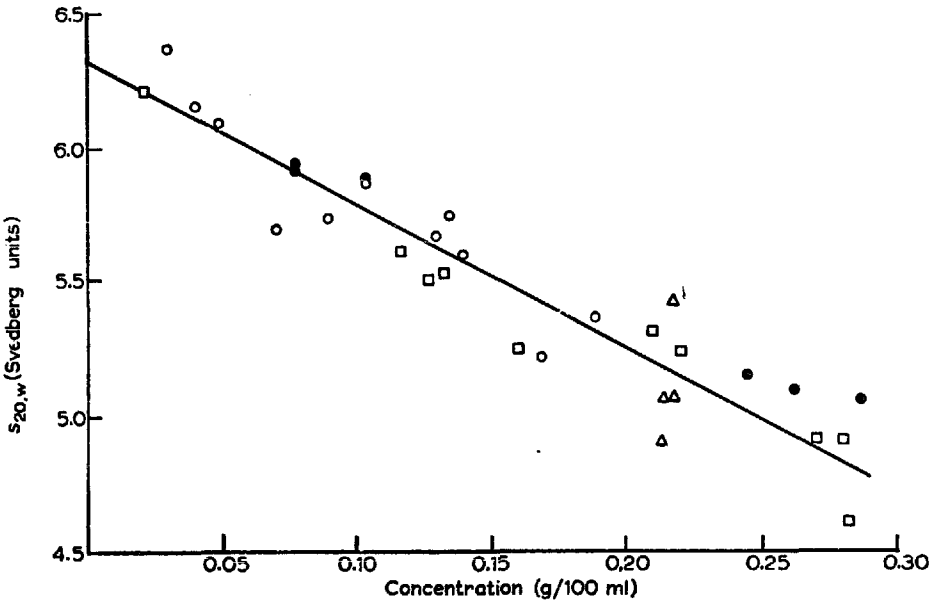

Fig. 1. Concentration dependence of sedimentation of freshly-prepared cod myosin. O, data recalculated from CONNELL[5], □, myosins prepared by extracting washed muscle with pyrophosphate-containing buffer (protein not precipitated); △, myosin prepared as for □ only precipitated by dilution as described in the text, ●, myosins prepared by dissociating cod actomyosin followed by preparative ultracentrifugation.

It is clear that the sedimentation behaviour of all these types of preparation is the same.

Analysis of the data of Fig. 1 by the method of least squares yields the following equation ($s = s_0 - k \cdot c$); $s = 6.33\ (\pm 0.11) - 5.35\ (\pm 0.63)c$, where s is the sedimentation coefficient in Svedberg units at a concentration c in grams/100 ml. As expected, the new value of s_0 does not differ significantly from that previously obtained[5], but the new value of k is greater. The smaller value of k in the previous experiments results from using concentrations that are overestimated by being obtained from the total nitrogen contents of solutions that were impure. The value of S_0 for cod myosin is in good agreement with recent values for rabbit myosin (*e.g.* JOHNSON AND ROWE[11]) and only barely significantly different from that (6.03 ± 0.12) quoted for mullet and rabbit myosins by HAMOIR, MCKENZIE AND SMITH[12]. The latter authors' data includes concentrations up to 0.6 % over which range there is an evident departure from a linear relationship between s and c. The value of k obtained in the present work is also in reasonable agreement with that found for rabbit myosin[5].

Concentration dependence of sedimentation of cod myosin and its components in aggregated systems

The sedimentation coefficients of cod myosin monomer and the two more rapidly sedimenting components (referred to here as dimer and trimer, corresponding, respectively, to the Components 2 and 3 of JOHNSON AND ROWE[2]) in aggregated systems

of this protein are shown in Fig. 2. In all cases aggregation has been produced by storing the protein at 0° for 1–10 days; after 10 days under these conditions 10–20 % of the myosin remains unaggregated.

The protein concentrations of the dimer and trimer are the total concentrations at the sedimenting boundary taking into account radial dilution.

From Fig. 2, the sedimentation behaviour of cod myosin monomer in a mixture of monomer, dimer and trimer conforms to the equation:

$$s = 6.24\ (\pm 0.08) - 4.83\ (\pm 0.55)c,$$

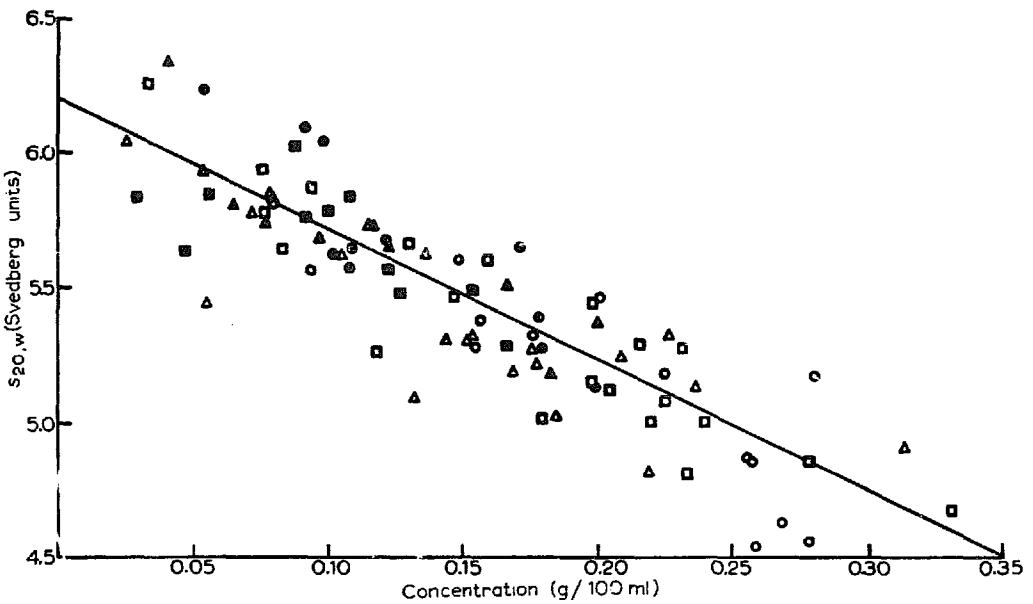

Fig. 2a.

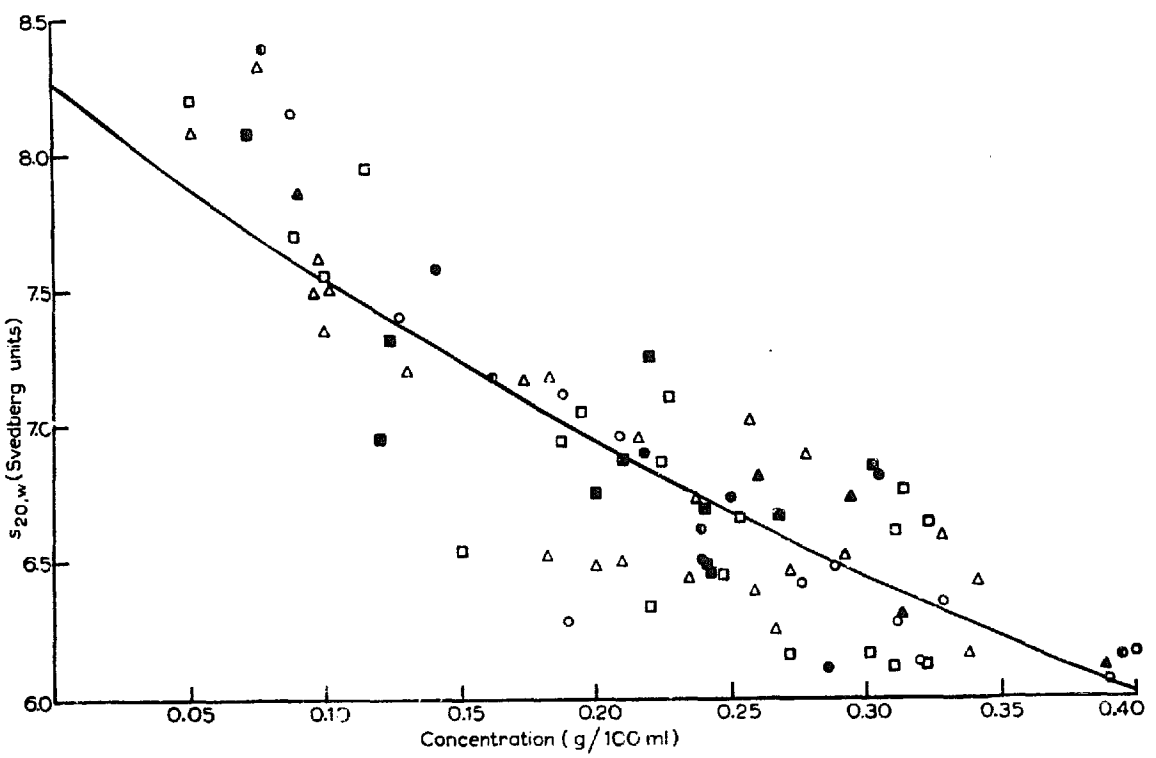

Fig. 2b.

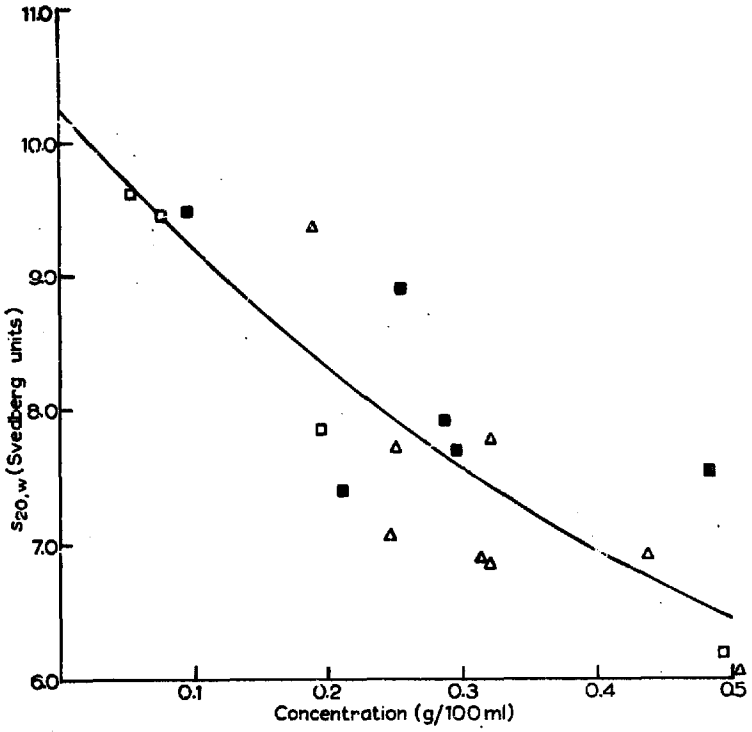

Fig. 2c.

Fig. 2. Concentration dependence of cod myosin and its components in aggregated systems. a, Mc omer; b, dimer; c, trimer. Days of storage at 0° as follows: ○, 1; □, 2; △, 3; ◐, 4; ●, 5; ■, 6 and 7; ▲, 9, 10 and 11.

which is not significantly different from that for unaggregated myosin alone as given in the previous section. The data for dimer and trimer fit better a reciprocal plot of sedimentation coefficient against concentration. For the dimer:

$1/s = 0.1208\ (\pm 0.0038) - 0.115\ c$, and for the trimer: $1/s = 0.0977\ (\pm 0.0088) - 0.116\ c$, resulting in values of s_0 of 8.28 and 10.23, respectively. These values are appreciably lower than those obtained both from a much smaller group of results on cod myosin[5] and for rabbit myosin[11,13]. However, the present results indicate that the sedimentation coefficient at zero-protein concentration may be higher than that given by reciprocal plots.

It would seem from Fig. 2 that the sedimentation coefficients of the three components do not charge in any regular manner with storage time, but in order to test the point thoroughly, a multiple regression analysis of the data on myosin monomer was carried out. The results shown in Fig. 2a were expressed in the form:

$$s = s_0 + a_1c + a_2P + a_3Pc,$$

where s, s_0 and c have the same significance as before, P is the percentage of the initial amount of myosin remaining unaggregated and a_1, a_2 and a_3 are coefficients. a_1, a_2 and a_3 were evaluated and their significance assessed by the t-test. a_1 was found to be highly significant but a_2 and a_3 were below 5 % significance, showing that there is no evidence that the sedimentation coefficient depends on the amount of myosin remaining unaggregated.

This conclusion has been substantiated in one experiment by adding freshly-prepared monomeric cod myosin to a highly aggregated cod-myosin solution (Fig. 3).

It is evident from this experiment that the sedimentation rates of the myosins in the aggregated and unaggregated solutions are indistinguishable.

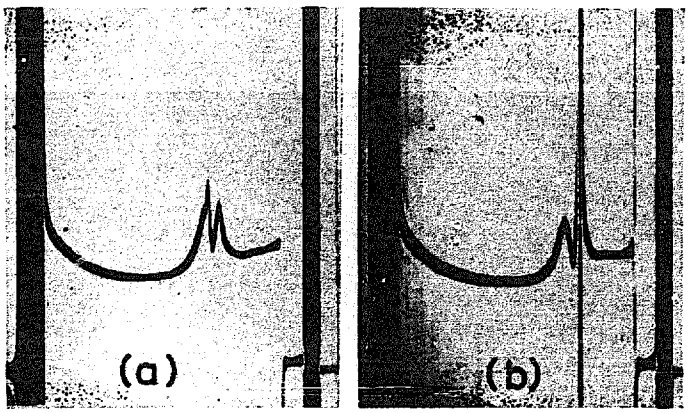

Fig. 3. Ultracentrifuge pictures of: a, cod myosin aggregated by storage of a 0.81 % solution at 0° for 7 days; b, mixture of solution used for (a) and freshly-prepared (unaggregated) cod myosin. The concentration of the aggregated solution is the same in both (a) and (b). Sedimentation from R to L: a, 69 min; b, 69 min after reaching full speed of 59780 rev./min. Temperature: a, 1.2°; b, 4.4°. Protein concentration (Kjeldahl) a, 0.40 %; b, 0.62 %.

Kinetics of the aggregation of cod myosin

The results in the previous section show that the objections to the use of the JOHNSTON–OGSTON method for correcting boundary anomalies raised in the INTRODUCTION in respect of rabbit myosin, are not applicable in the case of cod myosin. The validity of correcting results on an aggregated cod myosin system by this method has been checked by performing ultracentrifuge measurements on the system at various overall protein concentrations. By extrapolating these measurements to zero protein concentration, a correct value for the amount of myosin remaining unaggregated in the uncentrifuged solution can be obtained[14].

A 0.37 % solution of cod myosin was stored for up to 11 days at 0°, diluted at intervals by factors of $^3/_4$, $^1/_2$ and $^1/_4$, and each diluted solution analysed in the ultra-

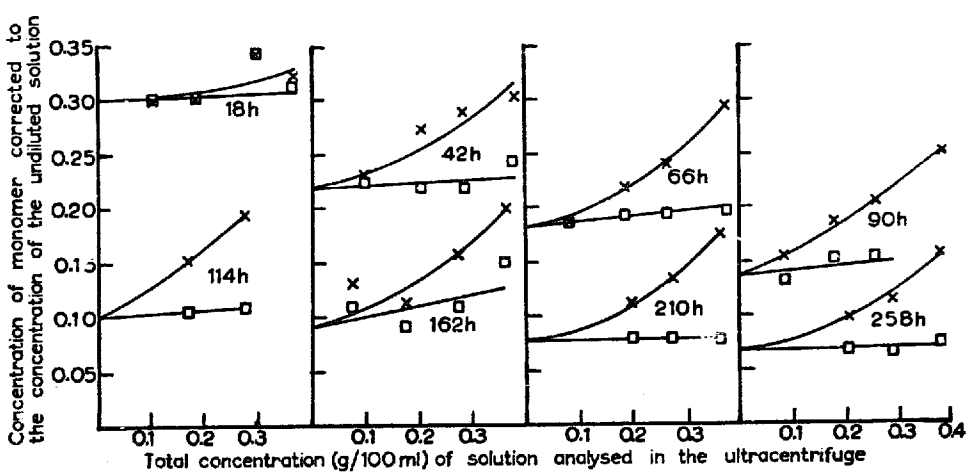

Fig. 4. Concentration of cod-myosin monomer measured in the ultracentrifuge at various dilutions after storage of a 0.37 % solution at 0° for various periods of time. ×—×, observed concentration corrected for radial dilution; □—□, concentration corrected for radial dilution and by method of JOHNSTON AND OGSTON[3]. The times shown on the Figure are the periods of storage at 0°.

centrifuge under as identical conditions as possible immediately after dilution. The amount of myosin remaining unaggregated was measured in each of these runs and corrected for radial dilution. The values so obtained were either left uncorrected or corrected by the method of JOHNSTON AND OGSTON[3], and finally the results for the diluted solutions were multiplied by factors of $^4/_3$, 2 and 4, respectively, in order to adjust them to the total concentration of the undiluted solution.

The results (Fig. 4) show that, as expected, the observed values deviate from those extrapolated to zero concentration by increasing amounts as the concentration at which the run was performed increases. The corrections for the JOHNSTON–OGSTON effect likewise become larger as the concentration increases. At all dilutions and all degrees of aggregation the values corrected by the JOHNSTON–OGSTON method are close to the extrapolated values, though the corrections tend to be less adequate at high protein concentration.

As shown in Fig. 5, the extrapolated values of Fig. 4 conform well to changes expected for a second order reaction, confirming JOHNSON AND ROWE's suggestion[2] that under these conditions the rate determining step in the aggregation is an initial dimerisation. At the longest time of storage 82.4% of the myosin in this experiment had been transformed into aggregates.

Recalculation of earlier results[1] on cod myosin, correcting as above for radial dilution and boundary anomalies, indicates (Fig. 6) that at high protein concentration deviations from second-order kinetics occur. These deviations are in a direction indicative of an underlying first order reaction but it is not possible to say whether the complexity is the result of a series of consecutive or of parallel reactions. Based on changes in partial specific volume, solubility and viscosity, JOHNSON AND ROWE[2]

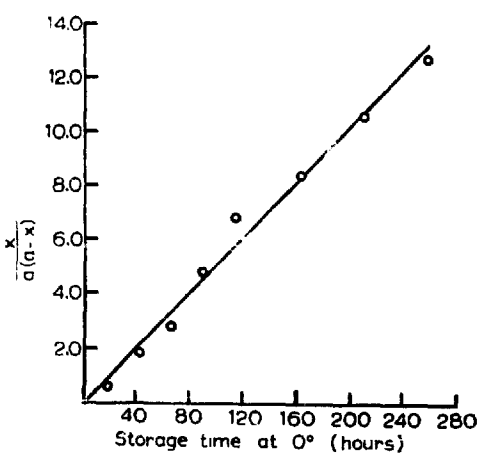

Fig. 5. Second order plot of disappearance of cod-myosin monomer during storage of a 0.37% solution at 0°. a and x are, respectively, the initial concentration of myosin and concentration of myosin transformed at various times of storage. The values of x are calculated from the data of Fig. 4.

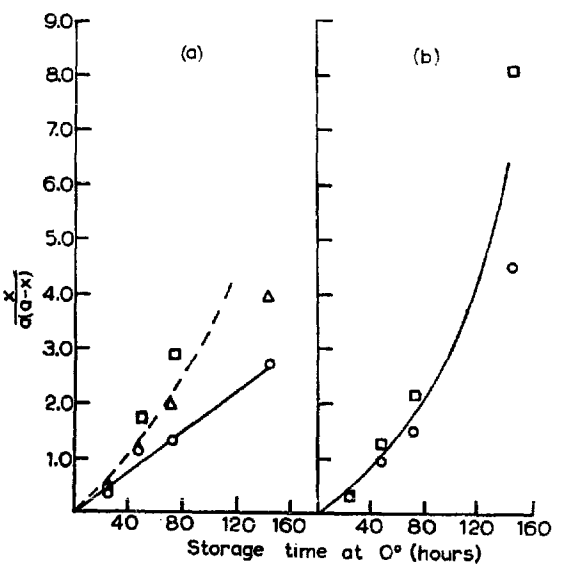

Fig. 6. Second-order plot of disappearance of cod myosin monomer during storage at 0° of concentrated solutions. (a) O, stored at a concentration of 0.26% and examined in the ultracentrifuge at this concentration; △, stored at a concentration of 1.30%, examined at a concentration of 0.43%; □, stored at a concentration of 1.30%, examined at a concentration of 0.26% (b) O and □, two experiments both stored at a concentration of 1.35% and examined at a concentration of 0.44%.

conclude that during storage of rabbit myosin, first order changes analogous to denaturation occur in the molecule independently of the dimerisation reaction. It is possible that the complexity observed with cod myosin at high protein concentrations is the result of participation in the overall reaction by such a first order denaturation under conditions where the dimerisation is not entirely rate-limiting.

Precipitation of cod myosin without aggregation

Early experience with cod myosin[1,15] was that this protein aggregated irreversibly when precipitated by reducing the ionic strength of a solution in the manner usual for preparing myosins. Precipitation in this way, however, did not result in the diminution of the enzyme activity that occurs when myosin is aggregated by storage. HAMOIR, MCKENZIE AND SMITH[12], and HAMOIR[16,17] have also noted that precipitation of carp, though not mullet, myosin results in a similar type of aggregation. This behaviour pointed to the existence of a fundamental difference between some types of myosins and others that might also affect comparisons of their aggregation behaviour during storage in solution. Recent experiments have shown, however, that at least some preparations of cod myosin can be precipitated in the conventional way without aggregating.

For example, a cod myosin extract (concentration 0.9 %) was made as described previously[1] and diluted ten-fold by pouring slowly into water at 0°. The precipitate was removed by centrifuging briefly at 3000 × *g* and after keeping portions of it at 0° for 5 min, 2 h and 24 h, redissolved by the addition of KCl–phosphate buffer (pH 7.5) $I = 0.5$. Immediately after resolution the myosins were analysed in the ultracentrifuge (Fig. 7). It is evident that cod myosin can be precipitated without aggregation if it is not allowed to stand in the precipitated form for too long. The aggregation occurring after storage for 2 and 24 h in the precipitated state at the determined pH of 7.0 is far greater than that occurring in solutions at the same pH. From other experiments the limiting storage time after precipitation at which aggregates are first visible is about 30–60 min. This is longer than the periods of time at which precipitates were kept at 0° in previous experiments[1,15].

As shown in Fig. 8, cod myosin can be precipitated twice by dilution with water

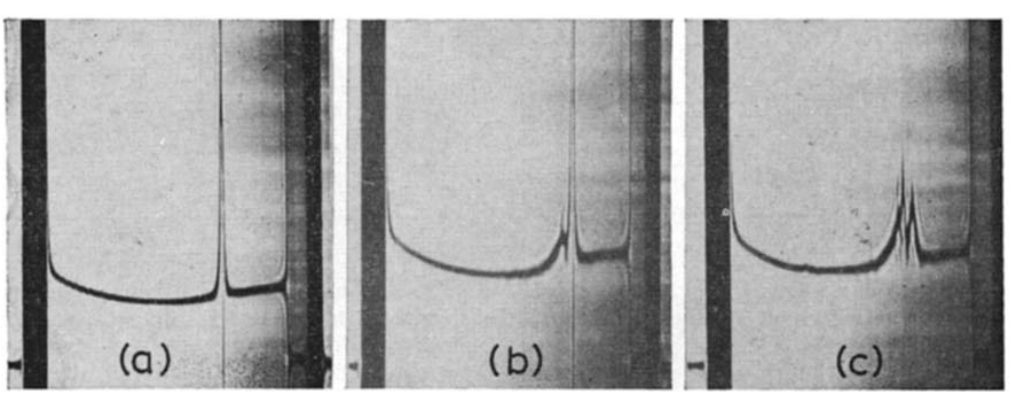

Fig. 7. Ultracentrifuge pictures of cod myosin precipitated once by diluting with water and then redissolving. Precipitates kept at 0° for: a, 5 min; b, 2 h; c, 24 h before resolution. Sedimentation from R to L: a, 71 min; b, 69 min and c, 69 min after reaching full speed of 59780 rev./min. Temperature: a, 5.8°; b, 3.3°; c, 5.5°. Protein concentration (Kjeldahl): a, 0.32 %; b, 0.43 %; c, 0.43 %.

and resolution without it aggregating. In all, six cod-myosin extracts have been precipitated in nine separate experiments without showing more than a trace of aggregates. Inclusion of pyrophosphate in the extract or in the diluting water is not necessary to avoid aggregation during precipitation.

Fatty acid content of myosin in relation to stability

Addition of fatty acid to solutions of cod actomyosin causes the protein to become insoluble[18], and a similar phenomenon has been noted for cod myosin[19]. It therefore

TABLE I

RATE OF AGGREGATION AND FATTY ACID CONTENT OF COD-MYOSIN PREPARATIONS

Type of muscle used to prepare myosin	*(k^* × 10^2)/ml/g/h*	*Fatty acid**/(%)*
Fresh	6.2	0.12
	6.4	0.07
	6.7	0.04
	8.0	0.03
Stored at −14°	7.9	0.36
	5.6	0.24
	8.6	0.40
	3.7	0.40

* Second order rate of aggregation.
** Grams fatty acid (equivalent weight 300) per 100 g protein.

seemed feasible that the repeatedly observed[1,2,13] variability in the tendency of myosin to aggregate spontaneously is caused by variation in the amount of contaminating fatty acid. However, a comparison of the fatty acid contents and second-order rates of aggregation of several cod-myosin preparations (Table I) shows that no relationship between these quantities exists. Myosins prepared from cod muscle stored for various periods at −14° are included in this comparison because their intrinsic fatty acid contents are appreciably higher than those prepared from fresh muscle. JOHNSON AND ROWE[4] observed that addition of fatty acid was without effect on the rate of aggregation of rabbit myosin.

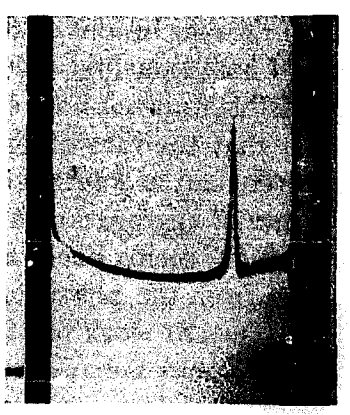

Fig. 8. Ultracentrifuge picture of cod myosin precipitated twice by dilution with water and resolution. Sedimentation from R to L, 53 min after reaching full speed of 59780 rev. per min. Temperature, 9.8°; protein concentration, 0.21 %.

Since different fatty acids vary in their effectiveness in producing insolubility[18,19], one cannot exclude the possibility that variations in the composition of the fatty acids contaminating different myosin preparations are responsible for variations in aggregation rate.

DISCUSSION

Detailed analysis of the sedimentation behaviour of cod myosin and of its aggregated components visible in the ultracentrifuge has failed to show the effect observed on rabbit myosin by JOHNSON AND ROWE[2]. These authors account for the diminution in sedimentation coefficient during aging in terms of the approximately first order opening-up of the tertiary structure of the molecule alluded to above in connection with changes in viscosity, light scattering and partial specific volume. An obvious explanation why a similar diminution is not observed for cod is that the rate of this first order process, if it occurs, is slower than in the case of rabbit. This would be a situation opposite in sense to that of the relative rates of aggregation, for cod myosin aggregates much more quickly than rabbit myosin.

It is perhaps noteworthy that of the two preparations of rabbit myosin for which data are presented[2], the one showing the largest diminution in sedimentation coefficient had been kept at 2–4° for 25 days before aging at 25°; the sedimentation coefficients of the other preparation are only very slightly lower than those of samples examined at "early" stages in aging. Storage of myosin at low temperatures may cause changes in the molecule that are revealed in the ultracentrifuge only on incubation at higher temperatures. Probably such an effect would be impossible to observe with cod myosin because its rate of aggregation is so high even at 0°.

The constancy of the sedimentation behaviour of cod myosins and the similarity between the concentration dependences of cod-myosin monomer and dimer allow one to use with some justification the JOHNSTON–OGSTON correction for boundary anomalies. Within a range of protein concentration of 0.1–0.4%, data so corrected confirm JOHNSON AND ROWE's finding[2] that the rate-determining step in the aggregation is probably a dimerisation. Outside this range, however, the reaction appears to be complex. Thus, at lower concentrations JOHNSON AND ROWE[2] observed anomalous changes in light scattering and viscosity during storage of rabbit myosin, and as the present results show, it is possible that the aggregation of cod myosin departs from a second order course at higher concentrations. It should be pointed out that these findings are unlikely to affect the conclusions on the relative stability of myosins from different species of animals[20] based on first-order rates of aggregation.

Although it has been found possible to precipitate cod myosin by dilution without the protein aggregating it is clear that the rate of aggregation at an ionic strength of about 0.05 is considerably greater than in solution at an ionic strength of 0.3–0.5. Previous data[1] showed that the rate of aggregation is also greater at ionic strengths above the latter figure and it therefore seems likely that cod myosin is most stable at ionic strengths not far removed from the physiological. A comparison of the experimental details of earlier[5,15] and current work has failed to reveal any factor that could account reasonably for the different behaviour observed when cod myosin is precipitated and redissolved. However, where aggregation is very rapid, small and apparently trivial modifications in conditions may alter appreciably the relative rate of aggregation. Also, myosins from the same species of animal undoubtably vary in

their tendency to aggregate even under conditions of maximum stability. Whether these variations are caused by variations in degree of contamination with impurities is a matter for speculation. Certainly the total quantity of intrinsic fatty acid does not influence the stability of cod-myosin preparations.

ACKNOWLEDGEMENTS

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