THE EFFECT OF ADENOSINETRIPHOSPHATE UPON ACTOMYOSIN SOLUTIONS, STUDIED WITH A RECORDING DUAL BEAM LIGHT-SCATTERING PHOTOMETER*

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On several previous occasions (13–16), the author has described the effect of adenosinetriphosphate[1] upon dissolved actomyosin. In the earlier studies, this effect was detected by measurable alterations in the turbidity or in the viscosity (12, 13); in later work, only the viscosimetric method was used (15, 16). In order to permit a more rapid study of these transient effects, the light-scattering method has now been redesigned. Other authors have recently also made use of this procedure (4, 5, 26–28) or of rapid viscosimetry (1–3), and the results so obtained overlap in part with those now presented. As the first of a series of communications on the behavior of actomyosin, singly or as a part of a multiple enzyme system, this paper will describe in full the method designed for the purpose of recording turbidimetry, together with a few illustrative results.

Recording Light-Scattering Photometer

General Remarks.—In a properly designed optical system (Brice *et al.* (6)), measurement of the light scattered by a solution at an angle of 90° to the transmitted beam gives direct information about the absolute turbidity of the solution, if this turbidity behaves in accordance with Rayleigh's law. In case of interference within the solution, due to the presence of large molecules or otherwise, the value so determined differs from the true turbidity by dissymmetry and depolarization factors, and has been called the apparent turbidity (Mommaerts (18)). Changes in the apparent turbidity of a solution,

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[1] The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate.

although less unequivocally related to the predominant particle size than is the true turbidity, can equally well be used as a quantitative index of molecular alterations (compare Blum and Morales (5)). In this study, relative measurements of the apparent turbidity are obtained directly from the graphic records. If not converted into apparent turbidity values by comparison with an absolute scattering measurement according to Brice *et al.* (6) in the same run, these recorded relative scattering values are here indicated as "recorded

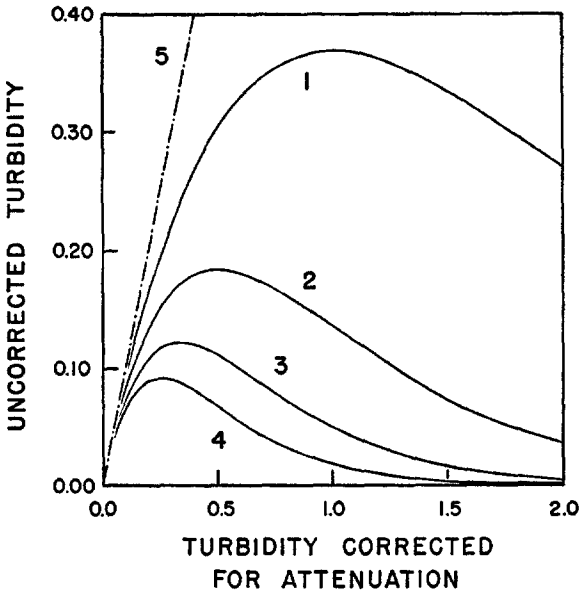

FIG. 1. Comparison between the true turbidity of a solution (abscissa) and the turbidity which would be deduced from the scattered light without regard to its attenuation upon passing through the turbid solution (ordinate). The straight line (5) shows the linear relation for an infinitely thin optical path. Curves 1 to 4 apply to cells in which the optical pathway measures 1, 2, 3, and 4 cm., respectively.

turbidity value," and appear on the ordinate axis of all graphs. In addition, changes in the light-scattering dissymmetry can also be recorded with this method.

The method of Brice *et al.* (6) for the absolute determination of light-scattering intensities is based upon the fact that in a cuvet of square horizontal cross-section the scattered light, while passing through the turbid medium, is attenuated to the same degree as the transmitted light. Indeed, turbidities are calculated from the ratio of the scattered to the transmitted light. If only the scattered light is measured or recorded, the paradoxical situation might be obtained that an increased turbidity is associated with a lesser quantity of

light emerging from the cuvet. The relation between turbidity and emerging light intensity has been calculated, by simple application of Lambert's law for conservative absorption for a square cuvet of different path lengths (Fig. 1). It is seen that, with a 2 cm. cuvet as employed in this work, the mentioned paradox appears at turbidity levels $\tau' > 0.5$, and that even for $\tau' \leq 0.1$, as encountered with actomyosin solutions, marked deviations occur from the proportionality between scattered intensity and turbidity. It follows, therefore, that measurement or recording merely of the intensity changes of the scattered light does not give a true indication of changes of either the absolute or the apparent turbidity.

A dual beam recording scattering-photometer has therefore been conceived which is based upon the presence of two photomultiplier cells, one of which (the numerator cell) views the scattered light, while the other (the denominator cell) views the transmitted beam suitably weakened by inserted filters. This system is connected through a ratio proportionator to a single pen ink-writing recorder, which therefore plots directly any changes of the ratio of the scattered to the transmitted light, hence any changes in the apparent turbidity. Alternatively, with the photocells placed at angles of 45 and 135° to the primary beam, and using a semioctagonal cuvet, one can also record alterations in the light-scattering dissymmetry.

Construction and Operation.—The requirements outlined in the preceding section are fulfilled by additions to the standard Phoenix-Brice light-scattering photometer, which were designed and constructed at the author's request by Mr. Edward J. Fuhrmeister.[2] These consisted of a second photomultiplier cell oriented perpendicularly to the standard photocell; power supply and associated circuitry for this cell; ratio proportionator; and recorder. The recorder is a 5 ma. Brown recorder (Minneapolis-Honeywell Corp.) into which the Fuhrmeister ratio proportionator is built. By means of a multiple switch,[3] the recorder can be made to register the signal from either the numerator or the denominator photocell through its standard mechanism, or the ratio of these two signals through the ratio proportionator. (Additional switch positions permit the measurement of the numerator or denominator signals through a galvanometer without changing wires.) After a cuvet with solution has been placed in the center of the cuvet holder (if absolute measurements are desired, its turbidity can first be measured with the standard procedures), the light intensities impinging upon each of the photocells are approximately equalized by the insertion of neutral filters. As measured by the recorder in single beam operation, the signals from the two photocells are now accurately set at 5 ma. by means of the respective sensitivity dials. The apparatus is next switched to ratio recording, and by means of the sensi-

[2] The complete recording photometer as described in this paper can be obtained from Phoenix Precision Instruments Company, 3803–05 North Fifth Street, Philadelphia. The author expresses his appreciation to Mr. Edward J. Fuhrmeister of that company for his generous cooperation in the development of this instrument.

[3] Designed and constructed by Mr. Carl Miller in this laboratory.

tivity knob, the deflection is set to a predetermined value, which is near maximal deflection in case only decreases in turbidity are expected.

Condition of Linear Response.—In order to obtain true linearity in the response of the ratio circuit, it is not sufficient that the maximal signals be equalized, but it is also necessary that the dark currents of the two photocells be matched more accurately than can be done by adjusting the dark current controls. This condition is tested by inserting a filter of about 5 per cent transmission into the primary beam, which, affecting the transmitted and the scattered light equally, should cause

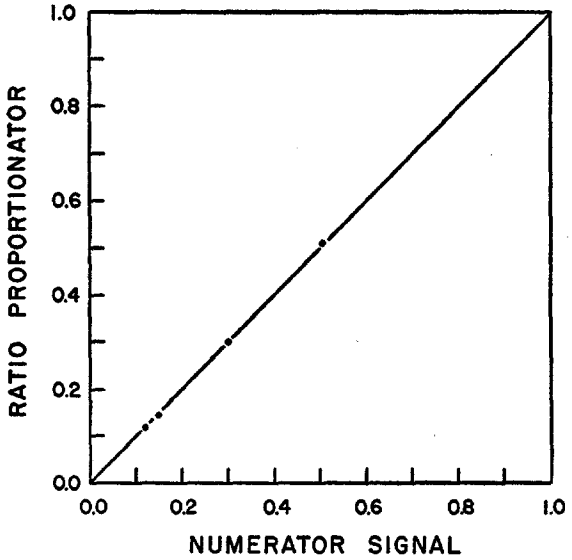

FIG. 2. Experimental test for linearity of the ratio proportionator. The light intensity impinging upon the numerator cell was reduced by various combinations of filters, the effects of which were measured by the signals from the numerator cell (abscissa); the same reductions were also measured through the ratio circuit (ordinate). The 0.0, 0.0 and the 1.0, 1.0 points are fixed by definition; the intermediate points illustrate the satisfactory linearity of response.

no change in the position of the recording pen when the ratio circuit is in operation. If necessary, small adjustments can be made with the control knob of the denominator photocell until this condition obtains, with a tolerance of about 1 per cent of maximal deflection. The linearity of the response can then be checked in two different ways. With a turbid solution in the cuvet,[4] grey filters can be inserted in front of the numerator photocell, and their effect measured with the recorder in both the numerator-recording and the ratio-recording positions (optical test); or, the sensitivity setting of the numerator photocell is reduced stepwise, and its effect upon both the

[4] Colloidal silica solution, Ludox, kindly made available by E. I. Du Pont De Nemours and Co. (Inc.), Grasselli Chemicals Department, Wilmington.

numerator and the ratio signals measured (electrical test). As shown in Fig. 2, the response of the ratio proportionator, so tested, is linear.

Mixing Device.—Reactions are initiated in the system contained in the light-scattering cuvet by the sudden addition of a small amount of ATP or other reagent, in such fashion that the total time of complete mixing in the cuvet is shorter than the time of response of the recorder. Efficient mixing requires the injection of a fair amount of fluid; yet, the addition of such an amount would, by itself, dilute the system so as to cause large changes in turbidity. Hence, fluids are added in small volume only, 0.2 per cent of the total volume, but are propelled into the stationary fluid by a sizable amount of the latter previously withdrawn from the cuvet. These operations are performed by means of a precision syringe of 1 or 2 ml. content with a 3.5 inch 18 gauge spinal needle. First, about 1 ml. of actomyosin solution is drawn into the syringe and is carefully freed of air bubbles. A small amount of solvent, *e.g.* 10 $mm.^3$, is next drawn into the needle to act as a cushion between the enzymatically active protein and the reagent to be added. The ATP or other reagent is now drawn into the needle, *e.g.* in 20 $mm.^3$ volume, by means of a micrometer screw which accurately controls the upward travel of the syringe plunger, and is followed by another cushion of solvent to separate the added reagent from the solution in the cuvet during the waiting period. The syringe is now inserted into the cuvet through a mounting in the cover of the photometer so placed that the needle ends below the meniscus of the fluid in the cuvet, and remains outside the light path in the corner of the cuvet which is remote from both photocells. For mixing, the plunger of the syringe is suddenly lowered, to forcefully eject the contents of the syringe into the cuvet. The efficiency of the mixing operation is tested, on the one hand, by the decolorization of an alkaline phenolphthalein solution with acid or *vice versa*; or, more relevantly, by the addition of a small amount of colloidal silica to a cuvet with water. The main source of error in this operation is the injection of air bubbles, which will reveal itself unmistakably on the record, and which can usually be avoided by exerting care in the filling operations. It is sometimes useful to evacuate the solutions prior to use.

Preparation of Solutions.—Before the measurement, all solutions are freed of dust with the precautions used in ordinary light-scattering work. Solvents are usually purified by positive pressure filtration through UF grade sintered glass filters, actomyosin solutions by ultracentrifugation for 2 hours at 30,000 R.P.M., and solutions added in trace amounts by regular centrifugation. At the turbidity levels encountered in this work, these routine precautions proved perfectly satisfactory.

Observations on Actomyosin Solutions

Preparation of Actomyosin.—Actomyosin was prepared from rabbit skeletal muscle with a procedure which will be described separately, which yields a preparation of high purity seemingly free of adenylate kinase (myokinase) but containing adenylate deaminase. The protein was employed in solutions at final concentrations of the order of 0.5 mg. per ml., in a solvent consisting of 0.4 M KCl and 0.05 M of a mixed succinate and tris (hydroxymethyl) amino-methane buffer of specified pH, mostly 6.9.

Effect of ATP.—As in the author's earlier viscosimetric investigations, the addition of ATP causes first an *initial response* consisting of a reduction in turbidity indicative of the combination of substrate and enzyme causing a dissociation of the actomyosin complex, followed by a *recovery effect* indicative of a reversion of the saturation of the enzyme coincident with the breakdown of the limited amount of substrate (Mommaerts (14, 15)). Subsequent authors

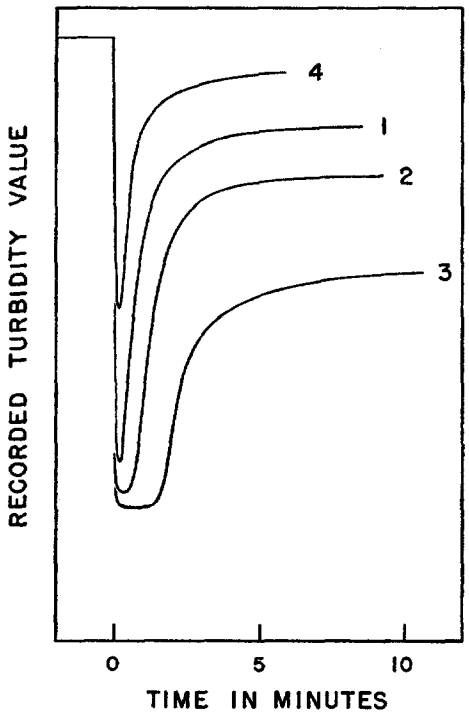

FIG. 3. Turbidimetric response to graded amounts of ATP actomyosin (0.47 mg. per ml.) in 0.4 M KCl, 0.05 M mixed buffer, pH 7.1. Curves 1 to 3, with 0.01 M $MgCl_2$, addition of 0.014, 0.035, and 0.071 μM of ATP, respectively. Curve 4, 0.001 M $MgCl_2$ with 0.014 μM of ATP.

have studied the velocity of the initial response viscosimetrically (Barany *et al.* (1–3)) or turbidimetrically (Watanabe *et al.* (26–28)). The present apparatus, the recorder of which has a full deflection penspeed of 5 seconds, is not suited for the study of very rapid events. However, not more than 1 second is required for 90 to 95 per cent deflection, so that a correct impression is gained of the kinetics of the initial process in those cases in which it takes 5 seconds or more to approach 90 per cent completion. The following descriptions of experiments are given with this reservation in mind.

Examples of the graded responses to ATP are given in Fig. 3. With in-

creasing dosage of the substrate, the initial response becomes larger and faster, and the recovery effect becomes more pronounced. It is obvious that, at low substrate concentrations which might leave the enzyme partly unsaturated, no steady state of enzyme saturation is reached, but that the diminution of ATP by enzymatic splitting overtakes the combination reaction between enzyme and substrate. The experiments in Fig. 3 were done in the

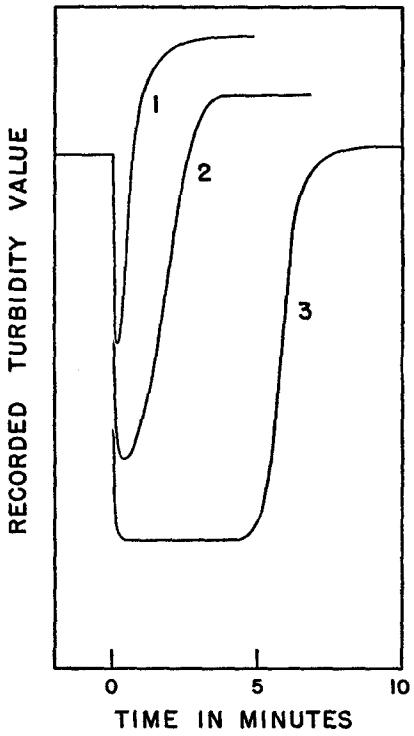

FIG. 4. Effects of Ca and Mg upon the response of actomyosin to ATP. Actomyosin, 0.53 mg. per ml., in the same medium as in Fig. 3, but at pH 6.9. Addition of 0.095 μM of ATP in the presence of (curves 1–3) 0.002 M $CaCl_2$, no bivalent cation, and 0.002 M $MgCl_2$, respectively.

presence of Mg^{++}, but similar families of curves can be obtained without this ion, or in the presence of Ca^{++}.

Effects of Ca^{++} and of Mg^{++}.—When the ATPase activity of actomyosin is activated by Ca^{++} ions (14, 24), the physical responses are correspondingly diminished and shortened (Fig. 4), while the inhibitory ion, Mg^{++}, has the opposite effect (Figs. 3 and 4). These differences may, in addition, be partly caused by effects of Ca^{++} or Mg^{++} upon the affinity of the substrate to actomyosin, or, more likely at this pH, by the effects of these ions upon the Michaelis constant through their influence upon the decomposition constant k_3 (Green

and Mommaerts (8)). It cannot be stated from these measurements whether Ca^{++} and Mg^{++} change the values of k_1, since the apparent rate of combination of enzyme and substrate is continually altered by the diminishing substrate concentration, the kinetics of which have not been investigated.

Incomplete Recovery.—It is seen from Figs. 3 and 4 that after complete hydrolysis of the added ATP the original turbidity is usually not regained. At pH 7.1 (Fig. 3), the resulting turbidity is always less than the original, the more so the more ATP is added; Mg^{++} seems to promote this irreversibility while Ca^{++} counteracts it. This incomplete recovery is tentatively ascribed to a partial depolymerization of the F-actin which remains free as long as ATP is present, leading to an actomyosin of smaller particle size upon recombination. Depolymerization of pure F-actin at these high salt concentrations can be induced with ATP to the complete disappearance of flow birefringence. At pH 6.9, this tendency seems much reduced, so that the original turbidity is more nearly regained (Fig. 4); again, Mg^{++} promotes depolymerization while Ca^{++} actually causes the opposite. Viscosimetrically, an exaggeration of the recovery effect has been noted by Laki and Clark (11).

Discussion of the Results Obtained with Actomyosin

The experiments presented were primarily designed to illustrate the applicability of the dual beam recording light-scattering photometer to the study of the molecular changes in actomyosin caused by ATP. The results are in line with those obtained earlier with less rapid techniques and with recent results of Watanabe *et al.* (26–28), Barany *et al.* (1–3), and Blum and Morales (4, 5). Some points, however, deserve additional discussion.

The physical effect exerted by ATP upon actomyosin has been generally ascribed to a dissociation of the complex protein into its constituents (9, 17, 25). This opinion has recently been challenged by Blum and Morales (5; see also Jordan and Oster (10), for a third view) on the basis of light-scattering measurements which will be discussed on another occasion. They pointed, however, to a notable hiatus in the proof for the dissociation theory: when the presumed dissociation of actomyosin in the presence of Mg^{++} and of much ATP is demonstrated in the ultracentrifuge (9, 17, 25; 16, page 161), only the liberated myosin, not the actin, appears as a distinct boundary. The results described herein make it probable that this anomaly is due to far reaching depolymerization of actin, presumably to a heterodispersed mixture of monomers and paucimers, under the conditions prevalent in these experiments. Current work on actomyosin combined with a coupled ATP-synthesizing enzyme (Mommaerts and Hanson, 22) will make it possible to reinvestigate this problem more decisively.

The present work also leads to a reevaluation of early attempts to estimate the molecular ratio in which ATP and actomyosin react. By studying the

responses of a constant amount of actomyosin to graded quantities of ATP, extrapolating the declining effects to zero time, it was estimated that 1 mole of ATP interacts with several hundred thousands grams of actomyosin (Mommaerts (14–16)), and recent work with the light-scattering method seemed to permit a more accurate computation (Watanabe *et al.* (28)). It is now recognized that with small amounts of ATP a marked hydrolysis of the substrate has taken place before the complete response is fully developed (Figs. 3 and 4), so that the earlier approach does only permit crude estimations. Current work with the multienzyme systems (Mommaerts and Hanson (22)) will allow a new approach to this problem also.

Finally, the time course of the responses to ATP (Figs. 3 and 4) provides a graphical illustration of the concept (14) that the physical changes are due to a combination of enzyme and substrate, rather than to a splitting of the latter. This interaction proceeds according to the extended Michaelis theory as follows, M signifying actomyosin:

$$M + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M\text{-}ATP \xrightarrow{k_3} M + ADP + P$$

$$K_s = \frac{k_2 + k_3}{k_1}$$

Since there is evidence that, at least in the pH 6 to 7 region, k_3 predominates over k_2 (Green and Mommaerts (8, 21)), it follows that the use of K_s to estimate the affinity of actomyosin to ATP (15) leads to an underestimation, and lends increased force to the argument that this combination has a high energy effect. However, the theory that this may represent the energy-yielding reaction for contraction, first proposed by the author (15, pp. 54–55; 16, pp. 167–168) and now discussed in several forms by others, cannot be discussed fruitfully until the further development of direct physiological experiments (7, 19, 20, 23).

SUMMARY

A recording dual beam light-scattering photometer is described which permits kinetic studies involving changes in turbidity, and which is not, as are single photocell instruments, affected by the errors due to the attenuation of the scattered light within the turbid medium.

With this method, a renewed study has been made of the physical changes occuring in actomyosin under the influence of ATP.

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