*COMPETITIVE BINDING OF ATP AND ACRIDINE ORANGE BY MUSCLE**

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Introduction.—The lifetime of electronic singlet excitation in molecules is too short to allow the utilization of these excitational energies in biological systems.[1] Triplet excitations last longer, but the probabilities of their occurrence are small. Circumstances which increase the probability of these transitions may thus have biological importance. It has been shown[1] that dyes and certain other substances which are excited to singlets by ultraviolet light, in their dilute aqueous solution, go into triplets if the solution is frozen. This transition may be due to changes taking place in the solvent or in the solute, or in both. It can be assumed that freezing causes crystallization of the solvent and with it a consequent increased local concentration of the solute. Increasing the concentration of certain dyestuffs in aqueous solution may lead to reversible molecular aggregations in the form of dimers and polymers, which, on excitation, may tend to go into the triplet state. That this is the case for acridine orange has been demonstrated in particular by the work of Zanker.[2]

Acridine orange is especially suited for the study of these relations, since it shows a green fluorescence under ultraviolet light in singlet, and a red phosphorescence in triplet, excitation. Accordingly, a dilute aqueous solution (10^{-3}–10^{-4} M) shows a vivid green color, which turns gradually into red as the concentration of the dye is increased and polymers are formed.

The nature of the bond between two molecules of the acridine orange dimer can be decided by the behavior in organic solvents like alcohol or acetone, which will dissociate complexes held together by dispersion forces,[3] while they leave electropolar bonds unaffected. Accordingly, addition of alcohol or acetone to a concentrated aqueous solution of acridine orange causes a change in color from red to green, as observed under the ultraviolet lamp, dispersion forces being responsible for the bond holding the dimers together.

As is being shown in this laboratory, ATP forms a red phosphorescent complex with acridine orange, as does adenine, indicating that it is the purine part of the molecule which dimerizes with the dye. Thus the dye can also form complexes with molecules of a different substance. Dimers formed by two equal molecules are, in this laboratory, called "homodimers," while dimers formed by two different molecules are called "heterodimers."

If the psoas muscle of the rabbit, extracted with glycerol,[4] is washed out with water, then suspended in a 10^{-3} M acridine orange solution, and placed, after a while, on filter paper, it is found to show an intense red color if illuminated with a high-pressure mercury lamp, the visible light of which is eliminated by an appropriate filter. The dye seeping out into the underlying filter paper is green. This indicates that the dye has been bound by a muscle constituent in the form of a complex, a heterodimer, which goes into the triplet state on excitation. A drop of alcohol placed on the muscle fiber changes the red color into green, showing that dispersion forces underlay the formation of the complex.

It has been suggested[5] that ATP energetizes the muscle by converting the bond energy of its high-energy phosphate into an excitation energy on its purine ring, then transmitting this excitation energy to the contractile protein. How this transmission from the purine to the protein takes place is left open. The experiments with acridine orange suggest the possibility that, like the dye, the ATP molecules also form a complex with a constituent of muscle, probably a group on the protein—a complex capable of triplet excitation. This excitation of the ATP-protein dimer could represent the transmission of the energy of the nucleotide on to the protein.

The formation of complexes between ATP and acridine orange, on the one hand, and the formation of complexes between acridine orange and muscle, and between ATP and muscle, on the other hand, also suggest that dye and nucleotide might be bound by the same atomic groups of the contractile matter. If ATP and the dye react with the same atomic groups, then the dye should inhibit the contraction induced by ATP, and this inhibition should be a competitive one.

Experiments.—A thin bundle of glycerol-extracted psoas muscle fibers,[5] approximately 0.25 mm. in diameter, was cut in two in the middle. One half was allowed to contract in an ATP solution and served as a control for the other half, which contracted in a bath containing an identical ATP concentration but also containing acridine orange. This method eliminated errors due to variations among different fiber bundles. Contraction (shortening) was plotted against time. Acridine orange concentrations from 10^{-2} to 10^{-4} M were studied. By varying the ATP concentration, one set of experiments was performed for each acridine orange concentration employed. Since all experiments showed a uniform pattern, only a few representative examples will be described here.

Figure 1 illustrates the inhibitory effect of 10^{-2} M acridine orange on fibers contracting in a 2-, 4-, 8-, and 16-millimolar ATP solution. It can be seen that with 2 mM ATP the contraction is almost completely inhibited. Rising ATP concentrations diminish this inhibitory effect, and with 16 mM ATP, inhibition is minimal.

Similar experiments performed with lower acridine orange concentrations (10^{-3},

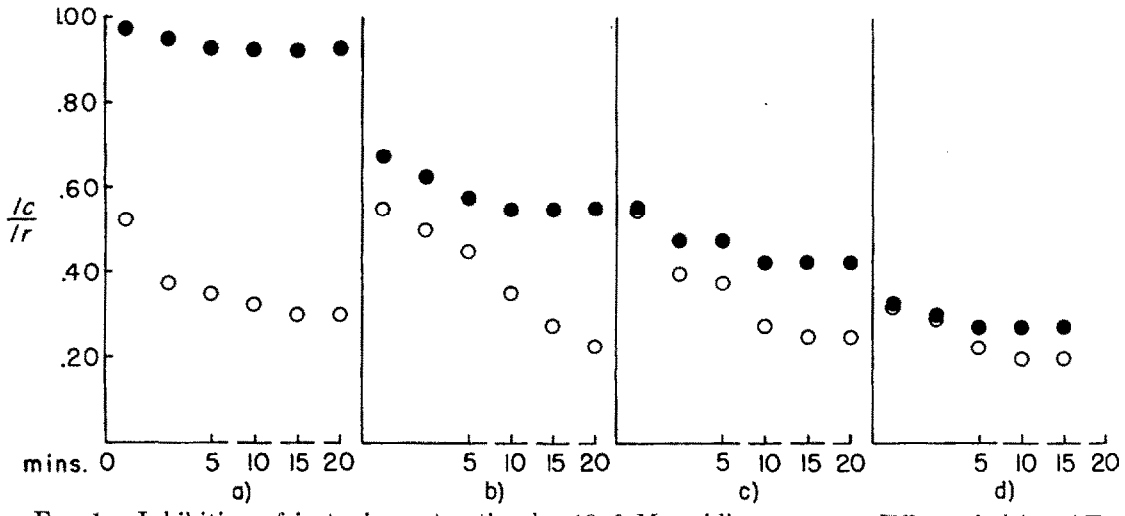

FIG. 1.—Inhibition of isotonic contraction by 10^{-2} M acridine orange. Effect of rising ATP concentrations: *a*, 2 mM ATP; *b*, 4 mM ATP; *c*, 8 mM ATP; *d*, 16 mM ATP. *Full circles:* fiber contracted in presence of 10^{-2} M acridine orange; *open circles:* fiber contracted in absence of acridine orange. l_r: rest length; l_c: contracted length.

5×10^{-4}, 10^{-4} M) showed only quantitative differences: a lesser degree of inhibition, which is further diminished with rising ATP concentration.

The inhibitory effect of a certain acridine orange concentration in the contraction bath was increased by incubating the fiber with the dye prior to contraction. Even short incubations of one minute and less were effective; longer incubations produced more pronounced inhibition. Rising ATP concentrations in the contraction bath diminished this additional inhibition. The pattern illustrated in Figure 1 was observed in all experiments.

With acridine orange concentrations as low as 10^{-4} M, in a contraction bath with 2 mM ATP, inhibitory effects were no longer shown. Even 20 minutes' incubation of the fiber in this dye concentration failed to produce an inhibitory effect for ATP concentrations of 2 mM and more (see Fig. 2, *a*). However, in the weaker and slower contractions obtained with 0.2 mM ATP, the inhibitory effect of 10^{-4} M acridine orange was again apparent (Fig. 2, *b*).

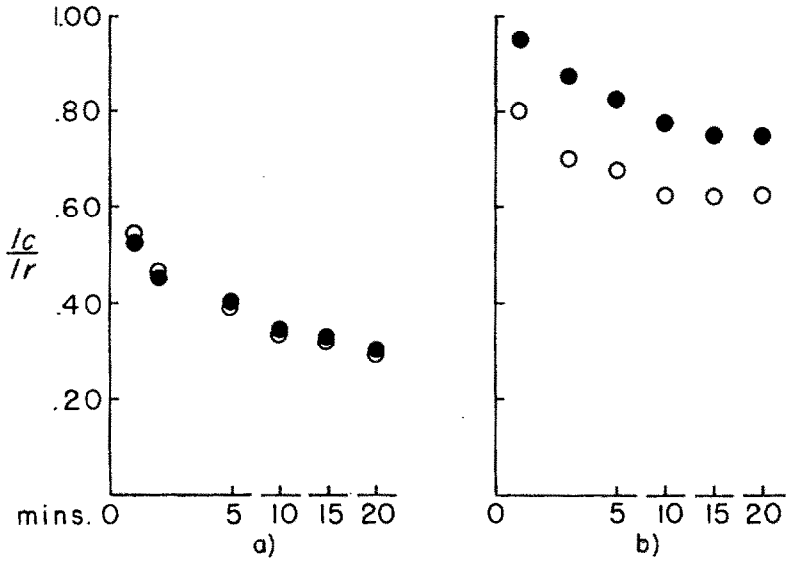

FIG. 2.—Effect of 10^{-4} M acridine orange on isotonic contraction: *a*, with 2 mM ATP; *b*, with 0.2 mM ATP. *Full circles:* fiber contracting in 10^{-4} M acridine orange after 20 minutes' incubation; *open circles:* control fiber in absence of acridine orange. l_r and l_c as in Fig. 1.

These opposite effects of ATP and acridine orange on the rate of shortening and degree of maximal contraction have been observed in all experiments. They seemed to suggest a competitive inhibition, i.e. a competitive adsorption of dye and ATP. Such a process would be analogous to the competitive adsorption of a substrate on its enzyme in the presence of a competitive inhibitor and should show characteristic quantitative relations. The formal and quantitative analysis of our experimental data should clarify this question and is presented below.

The reversibility of the acridine orange action could also be demonstrated by contracting acridine orange–incubated fibers in an ATP solution free of dyestuff. Here the rate of shortening was slowed, but finally the same maximal contraction was developed as by the nonincubated control fibers. Figure 3 illustrates two examples of these experiments. Maximal contraction was reached more rapidly with increasing ATP and more slowly with increasing dye concentration or pro-

longation of the time of incubation. This excludes a denaturation of the contractile proteins by the dyestuff under the experimental conditions employed.

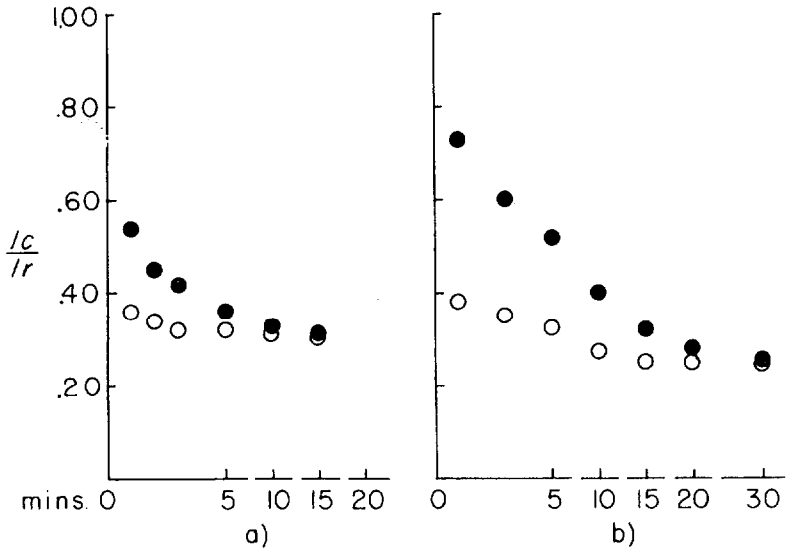

FIG. 3.—Fibers incubated in acridine orange prior to contraction. The rate of shortening is decreased, while the degree of maximal contraction is not impaired. *a*, 2 minutes' incubation in 10^{-3} *M* acridine orange; *b*, 2 minutes' incubation in 10^{-2} *M* acridine orange. *Full circles:* incubated fiber; *open circles:* nonincubated control fiber. l_r and l_c as in Fig. 1.

Discussion.—In the case of competitive inhibition of a reaction, as, for example, that of an enzyme E with a substrate S and that of the same enzyme with an inhibitor I,

$$E + S \rightleftarrows ES,$$

$$E + I \rightleftarrows EI,$$

ES and EI denote the enzyme-substrate and the enzyme-inhibitor complexes, respectively. The velocity v of the enzymic reaction is proportional to the concentration of ES. As is well known, if the reciprocal of the velocity of the enzymic reaction is plotted against the reciprocal of the substrate concentration, we obtain, in the case of competitive inhibition, a straight line which cuts the ordinate $(1/v)$ axis at a point which is independent of the inhibitor concentration, I. However, the slope is dependent on I. Consequently, the straight lines for different values of I cut the $(1/v)$-axis at the *same* point, whereas the slope is larger, the larger the value of I.

In the present case of muscle a constituent X corresponds to E, ATP to S, acridine orange (AO) to I. Thus

$$X + \text{ATP} \rightleftarrows X(\text{ATP}),$$

$$X + \text{AO} \rightleftarrows X(\text{AO}).$$

If we call K the equilibrium constant of the reaction of X and ATP, and K' that of the reaction of acridine orange and X, we have

$$\frac{C_0C_1}{C_2} = K \tag{1}$$

and

$$\frac{C_0C_3}{C_4} = K', \tag{2}$$

in which C_0 represents the concentration of the free X, C_1 that of free ATP, C_2 that of X(ATP), C_3 that of AO, and C_4 that of X(AO).

Furthermore, calling X_0 the total concentration of X, which is equal to the sum of that of the free X and that of the bound X in X(ATP) as well as in X(AO), we also have

$$C_0 + C_2 + C_4 = X_0. \tag{3}$$

Simple algebraic manipulation gives, from equation (1),

$$C_0 = K\frac{C_2}{C_1}, \tag{4}$$

and substitution of equation (4) into equation (2) leads to

$$C_4 = \frac{C_0C_3}{K'} = \frac{K}{K'}\frac{C_2C_3}{C_1}. \tag{5}$$

Introduction of equations (4) and (5) into equation (3) yields, after some simple rearrangements,

$$C_2\left(\frac{K}{C_1} + 1 + \frac{K}{K'}\frac{C_3}{C_1}\right) = X_0,$$

from which we obtain

$$C_2 = \frac{X_0}{1 + K/C_1 + (K/K')(C_3/C_1)},$$

or

$$C_2 = \frac{C_1}{C_1 + K(1 + C_3/K')}X_0. \tag{6}$$

Since, experimentally, the length of the muscle fiber was studied as function of time, the natural and most simple assumption which can be made here is that the shortening in the final (equilibrium) state is proportional to the concentration, C_2, of the X(ATP) complex:

$$l_r - l_c = \alpha C_2, \tag{7}$$

in which l_r is the rest length, l_c the length of the muscle fiber in the final (equilibrium) contracted state, and α a proportionality constant.

From equation (6) we see that the concentration C_2 is a (hyperbolically) increasing function of C_1 with saturation value X_0. Therefore, assumption (7) leads to

$$l_r - l_{mc} = \alpha X_0, \tag{8}$$

in which l_{mc} is the length of the maximally contracted fiber in its final (equilibrium) state, as occurs with a (infinitely) large concentration of ATP.

Elimination of α from equations (7) and (8) yields

$$\frac{l_r - l_{mc}}{l_r - l_c} = \frac{X_0}{C_2},$$

or

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} \frac{X_0}{C_2}. \tag{9}$$

Simple rearrangement from equation (6) leads to

$$\frac{X_0}{C_2} = 1 + K\left(1 + \frac{C_3}{K'}\right)\frac{1}{C_1}. \tag{10}$$

Introduction of expression (10) into equation (9) gives

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} + \frac{1}{l_r - l_{mc}} K\left(1 + \frac{C_3}{K'}\right)\frac{1}{C_1}, \tag{11}$$

from which we see that the intercept of the plot of the reciprocal of the shortening, $1/(l_r - l_c)$, versus that of the ATP concentration, $1/C_1$, should yield a straight line with an intercept equal to the reciprocal of the maximal shortening, $1/(l_r - l_{mc})$, which is *independent* of the inhibitor concentration C_3, and a slope $[1/(l_r - l_{mc})]$-$K(1 + C_3/K')$ which is a linear function of the inhibitor concentration C_3, as might have been expected from what has been said before about the competitive inhibition in general and assumption (7).

Without inhibitor, equation (11) leads, with $C_3 = 0$, to

$$\frac{1}{l_r - l_c} = \frac{1}{l_r - l_{mc}} + \frac{1}{l_r - l_{mc}} K\frac{1}{C_1}. \tag{12}$$

The plot of the value of $1/(l_r - l_c)$ versus $1/C_1$ for the experiment without acridine orange indeed yields a straight line, as shown in Figure 4, fitted by the least-squares method. From the intercept we obtain $1/(l_r - l_{mc}) = 1.14$, leading to $l_{mc} = 0.124$ (l_r is taken as unity), so that the maximal contraction with a very large concentration of ATP is 87.6 per cent. For the slope we find 7.37×10^{-4}, from which we obtain with the above-mentioned value of $1/(l_r - l_{mc})$ the value 6.5×10^{-4} for K.

Similarly, the plot of $1/(l_r - l_c)$ versus $1/C_1$, for an experiment containing acridine orange in the concentration 10^{-2} M, yields a straight line, as shown in Figure 4. For this line we find, by the method of least squares, the value 1.11 for the intercept $1/(l_r - l_{mc})$, which value is the same within the experimental accuracy as that found in the experiment without dye. The latter result proves that there is competitive inhibition of ATP and acridine orange for the same site of muscle constituent X.

Also from the slope 5.28×10^{-3} of the last straight line, from the above values of $1/(l_r - l_{mc})$ and K, and from the concentration $C_3 = 10^{-2}$ M of acridine orange, we find the value 15×10^{-4} for K', which shows that the dye is bound by the same site as ATP but is bound somewhat more than twice as strongly.

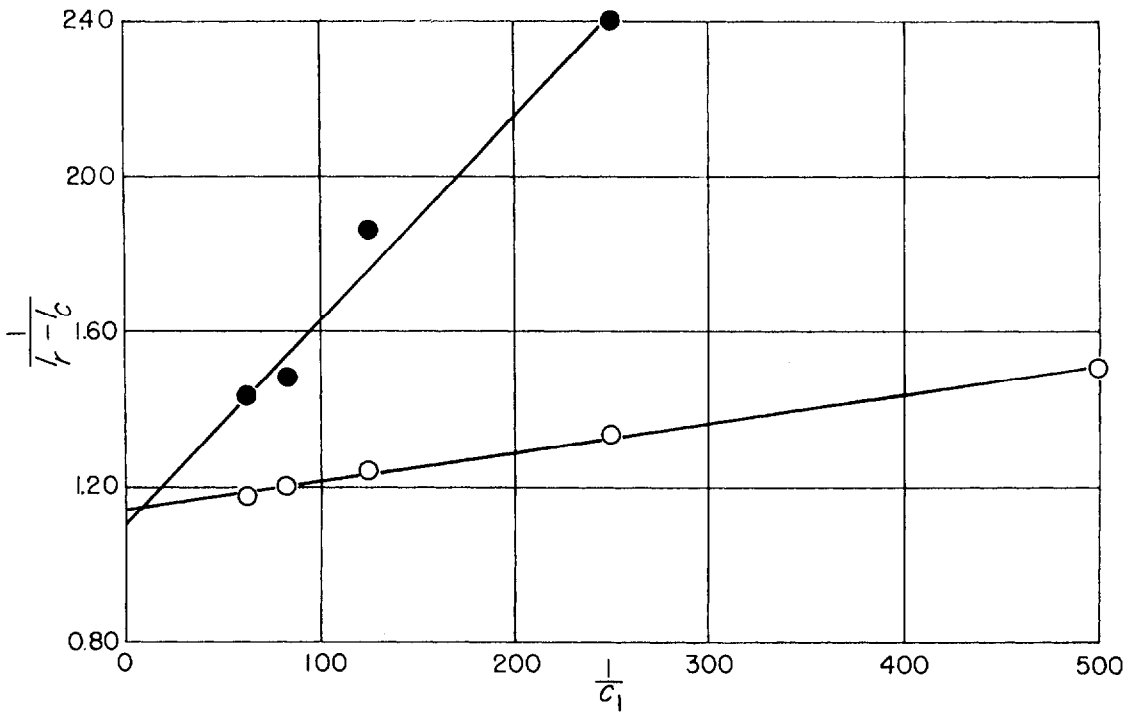

FIG. 4.—Reciprocal value of the final shortening plotted against the reciprocal value of the molar concentration of ATP. *Open circles:* fiber in absence of acridine orange; *full circles:* fiber in presence of 10^{-2} *M* acridine orange. l_c and l_r as in Fig. 1.

Summary.—Acridine orange and ATP form complexes with the same muscle constituents. The formation of the ATP complex is an essential step in the energetization of muscle. Acridine orange inhibits contraction, and this inhibition has the properties of a competitive absorption. Dye and ATP molecules compete for the same site.

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