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ISOLEUCYL-tRNA SYNTHETASE OF E.COLI B: EFFECTS OF MAGNESIUM AND SPERMINE ON THE AMINO ACID ACTIVATION REACTION

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Isoleucyl-tRNA Synthetase of E. coli B. Effects of Magnesium and Spermine on the Amino Acid Activation Reaction*

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Running Title: Effect of Magnesium and Spermine on Isoleucine Activation

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ABSTRACT: We have investigated the interaction of magnesium and spermine with Ile-tRNA synthetase under conditions in which catalysis of L-isoleucine activation is observed. We used a fluorimetric method and titration and stopped-flow techniques. The results support the previous findings by Cole and Schimmel (1970, Biochemistry 9, 3143) that ATP reacts as the magnesium salt. At concentrations higher than 1 mM, magnesium becomes inhibitory with an inhibition constant of 3 mM to 5 mM.

Spermine is found to inhibit accumulation of enzyme bound L-isoleucyl adenylate. Inhibition proceeds via two routes; one is binding of spermine to an effector site and the other is the formation of catalytically inert spermine-ATP. Presumably, binding to the effector site is followed by a conformation change of the enzyme, leaving the Michaelis-Menten complex less reactive. Binding of the effector is associated with an enhancement of the fluorescence intensity of the reporter group 2-p-toluidinyl-naphthalene-6-sulfonate, which is complexed with the enzyme. This response is in contrast to the fluorescence quenching observed for binding of substrates and products. Spermine binds to ATP. The strength of the interaction is comparable with that for magnesium. Spermine-ATP appears to bind to the enzyme, however, ability to participate in the catalysis of the amino acid activation reaction is lost.

Introduction

The catlysis of an amino acid specific ATP-[^{32}P]PP$_i$ exchange reaction together with the isolation of an enzyme bound amino acyl adenylate were taken as strong evidence for the formation of an intermediate as part of the specific charging reaction of a cognate tRNA catalyzed by an amino acyl-tRNA synthetase (Berg, 1958; Bergmann, Berg, and Dieckmann, 1961; Berg, Bergmann, Ofengrad, and Dieckmann, 1961; Norris and Berg, 1964). The rate of exchange as well as the preparative accumulation of the intermediate have been found to possess a substantial magnesium dependence. A careful analysis of this dependence has been recently reported by Cole and Schimmel (1970). That the requirement is not universal has been shown for the Tyr-tRNA synthetase from E. coli which catalyzes the formation of tyrosyl adenylate in the absence of divalent cations (Chousterman and Chapeville, 1971). More important, a series of papers report that catalysis of the tRNA-charging reaction is adequately maintained after substitution of magnesium with polyamines, in particular with spermine and spermidine (Takeda and Igarashi, 1969; Igarashi, Matsuzaki, and Takeda, 1971; Steinmetz Kayne and Cohn, 1972). Surprisingly, the ATP-[^{32}P]PP$_i$ exchange is blocked (Igarashi, Matsuzaki, and Takeda, 1971, Steinmetz Kayne and Cohn, 1972), and there is evidence that the amino acyl adenylate intermediate is not accumulated (Igarashi, Matsuzaki, and Takeda, 1971; Pastuszyn and Loftfield, 1972).

We have recently reported a fluorimetric method (Holler, Bennett, and Calvin, 1971), which has been shown suitable for rapid kinetic investigation of the amino acid activation reaction of the L-isoleucine system of E. coli (Holler and Calvin, Biochemistry, in press). We report in

this paper the effect of magnesium on the rate of formation of the enzyme bound amino acyl adenylate and the inhibition by spermine. We have collected information about the sites and the strength of interactions.

Materials and Methods

300- to 350-fold-purified Ile-tRNA synthetase was obtained from E. coli B cells (Miles Laboratories) following the method of Baldwin and Berg (1966). TNS was purchased from Sigma as Lot 60C-5270. Care was taken that the dye preparation consisted of material fluorescing between 480 nm and 500 nm (excitation 366 nm). Spermine tetrahydrochloride was obtained from Nutritional Biochemical Corporation, and 8-hydroxyquinoline-5-sulfonic acid as dihydrate from Aldrich. All other chemicals were reagent grade (Baker). Deionized and distilled water was used which had been boiled and cooled under nitrogen to remove oxygen and carbon dioxide.

Fluorescence measurements. Methods and fluorimeter have been described previously (Holler, Bennett, and Calvin, 1971; Holler and Calvin, 1972, submitted to Biochemistry). Kinetics were followed with use of a modified Durrum-Gibson stopped-flow spectrophotometer as has been described (Holler and Calvin, 1972). In a typical experiment, a solution containing 0.15 μM Ile-tRNA synthetase, 43 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol was rapidly mixed with a solution containing 0.88 μM L-isoleucine, 11 μM ATP, 43 μM TNS, 0.05 M Tris-HCl, 0.01 M 2-mercaptoethanol and $MgCl_2$ plus spermine at various concentrations. When the effect of ionic strength was studied, NaCl was added to the substrate

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[1]Abbreviation used is: TNS, 2-p-toluidinylnaphthalene-6-sulfonate.

mixture. Procedures for evaluation are generally based on linear plots for saturation functions as has been described by Eadie (1942).

Results

Magnesium ions. The rate constant for the formation of Ile-tRNA synthetase-L-isoleucyl~AMP complex was measured as a function of magnesium concentration in the range 0.01 mM to 11 mM at fixed concentrations of 0.88 μM L-isoleucine and 11 μM ATP, Figure 1. It can be seen that the plot is biphasic, indicating that more than one molecule of magnesium could bind to the Michaelis-Menten complex. At concentrations below 1 mM the interaction was indicated as an increase of the rate of catalysis whereas at concentrations above 1 mM catalysis was inhibited. We have ruled out the possibility that inhibition is caused by the increase of ionic strength. For instance, 20 mM NaCl in the presence of 1 mM $MgCl_2$ had little effect (only a 10% decrease) on the rate constant. Higher concentrations of NaCl (0.1 and 0.2 M) reduced the rate by 50% and 70%, respectively. The analysis of the rate constants as function of low and high concentrations of Mg^{2+} was processed in the form of linear plots (Eadie, 1942), as shown in Figure 1, inset, and in Figure 3. The evaluation was based on the assumptions of separate 1:1 interactions between magnesium and enzyme. In the case of low concentrations, evaluation was based on the following kinetic scheme:

$$(1)\quad E + Ile + ATP^{4-} + Mg^{2+} \underset{E_1}{\overset{K_{Mg}}{\underset{\longleftarrow}{K_{Ile},\ K_{ATP}}}} E\cdot Ile\cdot Mg^{2+}\ ATP^{4-} \xrightarrow{k_f} E\cdot Ile\sim AMP + MgPP_i^{2-}$$

Magnesium, substrates and enzyme, come together to form the Michaelis-Menten complex, $E\cdot Ile\cdot Mg^{2+}\cdot ATP^{4-}$. The quaternary complex turns over to

enzyme-L-isoleucyl adenylate complex, E·Ile~AMP, associated with the release of magnesium pyrophosphate. The reverse reaction is not considered because dilute solutions of enzyme and substrates were used ($(Ile)_o < K_{Ile}$, $(ATP)_o < K_{ATP}$, $(PP_i)_o << K_{PP_i}$ Holler and Calvin, 1972, submitted to Biochemistry). No distinction is made, whether magnesium combines with free ATP or with the Michaelis-Menten complex. The expression for the rate constant is given by the following equation:

$$(2) \qquad k_{obs} = k_o \frac{(Mg^{2+})_o}{K_{Mg} + (Mg^{2+})_o}$$

The symbol K_{Mg} refers to the dissociation constant for binding of the first magnesium ion. The parameter k_o refers to complete saturation by Mg^{2+} ions and is defined by equation

$$(3) \qquad k_o = k_f \frac{(Ile)_o \cdot (ATP)_o}{K_{Ile} \cdot K_{ATP}} \quad ,$$

as has been previously derived by Holler and Calvin (1972, submitted to Biochemistry). The symbols K_{Ile}, K_{ATP} refer to dissociation constants for binding of L-isoleucine and ATP, respectively. The subscript zero marks initial concentrations of reactants. The value for K_{Mg} was determined from the slope of line a) in Figure 3 to be (19 ± 1) μM.

In the case of high concentrations of magnesium, evaluation is tentatively based on a mechanism in which the second magnesium combines with the Michaelis-Menten complex reacting like a noncompetitive inhibitor. This model is similar to the one which will be used for the inhibition by spermine. The expression for the rate constant is given by the following equation:

$$(4) \qquad k_{obs} = k_o \frac{K_i}{K_i + (Mg^{2+})_o}$$

The symbol K_i refers to the inhibition constant at high magnesium concentration. For evaluation, $k_o - k_{obs}$ was plotted as the function of concentration as shown in Figure 1, inset. With use of a value for k_o obtained from the intercept of line a) in Figure 3, we have determined the value for K_i from the slope in Figure 1 to be 3 mM.

We have further evidence for a specific interaction at high concentrations of magnesium ions from the following titration experiments. When enzyme was titrated with a solution containing ATP and $MgCl_2$ in at 1:1 concentration ratio, fluorescence was quenched to approximately the same extent as for ATP alone (Figure 2a). When a solution of enzyme plus ATP was titrated with magnesium at concentrations in excess of ATP, the intensity of the fluorescence was enhanced as the concentration of magnesium increased, until, finally, the original level of fluorescence (before addition of ATP) was exceeded (Figure 2b). The control experiment where enzyme-TNS was titrated with magnesium up to 10 mM while ATP had been omitted from the solution, indicated that fluorescence enhancement was not due to a direct interaction with the enzyme-TNS system.

Figure 2c presents the titration data in form of a linear plot (Eadie, 1942), where the concentration of Mg^{2+} ions has been corrected for the amounts bound to ATP. The value for the dissociation constant which is supposed to describe the binding of the second magnesium to the enzyme-$MgATP^{2-}$ complex, is determined from the slope of the line to be 5 mM. This value is in fair agreement with the value determined kinetically.

Spermine. When spermine (3 mM) was added to a substrate mixture containing 1 mM magnesium, formation of L-isoleucyl-AMP was strongly inhibited. We have measured the rate constant of amino acid activation as a function of magnesium concentration at various fixed levels of spermine, as already

described. The titration curves are presented as linear plots in Figure 3. It is seen that spermine inhibited catalysis via both parameters, first by decreasing the maximum velocity, and second by increasing the Michaelis-Menten constant. We have replotted the difference between the observed maximum rate constant and the maximum rate constant in the absence of inhibitor as a function of spermine concentration in Figure 4a. The plot follows a linear relation, indicating a 1:1 interaction which is characterized by the inhibition constant K_I = 0.22 mM. It is seen that catalysis as reflected by the maximum rate constant, is not abolished at infinite concentration of spermine. We believe that this inhibition is induced by binding of spermine to an enzyme effector site, similar as we have discussed for the second magnesium ion. In order to test this hypothesis, we titrated a mixture of enzyme plus TNS in absence of substrates and Mg^{2+} ions, Figure 5. We obtained a typical titration curve associated with an enhancement of fluorescence intensity. Control titration of a solution containing only TNS indicated no significant change. In a second control we titrated a solution of 0.012 mM lysosyme and 27 μM TNS with up to 1 mM spermine without discovering any effect on the fluorescence properties. TNS is known to interact with lysozyme as indicated by a strong enhancement of the fluorescence intensity (Barel, Turneer, and Léonis, 1971; Holler, unpublished results). We take this observation as evidence that the titration of Ile-tRNA synthetase is not an artefact caused by direct interaction with TNS.

The dissociation constant for binding of spermine was determined from the slope of the linear plot, Figure 5 inset, to be (0.25±0.03) mM.

This value is in excellent agreement with the value determined from the spermine dependence of the maximum rate constant, Figure 4a. Furthermore, the interaction of spermine does not interfere with binding of L-isoleucine. This is shown by titration of Ile-tRNA synthetase with L-isoleucine in the presence of 1.7 mM spermine, Figure 6. The value for the dissociation constant for L-isoleucine as evaluated from the slope is (9 ± 1) μM in agreement with the value (5.8 ± 0.8) μM reported recently (Holler, Bennett, and Calvin, 1971). Moreover, the maximum effect of quenching, 44%, is close to (37 ± 6)% when determined in absence of the effector. In contrast, the titration experiments with ATP indicated that binding of spermine and ATP was not independent, Figure 7. In particular, the following observations were made:

1. Addition of ATP to the solution containing Ile-tRNA synthetase, TNS, and spermine was associated with fluorescence quenching. The degree of quenching was dependent on concentration of ATP in the form of a saturation function. The concentration necessary for saturation shifted to higher values as the concentration of spermine was increased. 2. Maximum quenching was dependent on the initial level of spermine concentration. The maximum degree of quenching, as indicated at high ATP concentration, was of the same size as the degree of fluorescence enhancement originally obtained upon addition of spermine to the enzyme-TNS mixture.

A coupling for spermine and ATP is expected when, for instance, the formation of the complex spermine-ATP is considered.

We have undertaken measurements to determine whether or not spermine binds to ATP and to evaluate the dissociation constant of the ATP-spermine

complex. We have used the fluorescence technique to follow the binding of Mg^{2+} ions to 8-hydroxyquinoline-5-sulfonic acid (Schachter, 1961) as a function of the partition between ATP and the dye. Experiments and evaluation were processed as described for Figure 8. It is found that spermine binds to ATP with a dissociation constant $K_{I \cdot ATP} = 0.082$ mM.

The titration curves in Figure 7 are consistent with the formation of spermine-ATP; however, we have to assume that spermine-ATP, when bound to Ile-tRNA synthetase, does not induce a change of the fluorescence intensity. This follows from the following consideration: The titration curves in Figure 7 return to the same level of fluorescence intensity upon increasing concentrations of ATP as observed for the absence of ATP and spermine. We have calculated the relative amount of spermine which is bound to ATP at a condition when this level of fluorescence has been attained using the following equation:

$$(I\text{-}ATP) = 1/2[(I)_o + (ATP)_o + K_{I \cdot ATP}] - 1/2 \left\{[(I)_o + (ATP)_o + K_{I \cdot ATP}]^2 - 4(I)_o(ATP)_o\right\}^{1/2}$$

The symbol I refers to spermine. We have calculated the following relative amounts of ATP-bound spermine: a) $(I \cdot ATP)/(I)_o = 93\%$ at $(ATP)_o = 1.5$ mM, $(I)_o = 0.415$ mM, b) 91% at $(ATP)_o = 1.1$ mM, $(I)_o = 0.248$ mM, c) 99% at $(ATP)_o = 0.8$ mM, $(I)_o = 0.155$ mM. We see that according to $(ATP)_o - (I)_o > K_{ATP}$ the concentration of ATP should in all cases be high enough to form substantial amounts of enzyme-ATP complex. Formation of the complex should have been associated with a further decrease of the fluorescence intensity (Holler, Bennett, and Calvin, 1971). This is not observed in Figure 7, indicating that spermine-ATP must be competing with ATP for Ile-tRNA synthetase and that spermine-ATP does not induce a change in fluorescence intensity upon binding to the enzyme.

The kinetic data in Figure 4b were also evaluated on the basis of competition between magnesium and spermine for ATP. The values for the apparent Michaelis-Menten constants as measured upon variation of the concentration of magnesium were replotted as a function of the concentration of spermine and were evaluated as described in Figure 4b.

The value for the inhibition constant was found to be 0.15 mM, in fair agreement with the value determined for the dissociation constant for spermine-ATP.

In conclusion, we have shown that two molecules of spermine can react with the Michaelis-Menten complex of the L-isoleucine activation reaction. One binds to an effector site, decreasing the maximum rate of catalysis, the other combining with ATP to form an unproductive spermine-ATP complex. The expression for the rate constant in the presence of spermine is then given by the following equation:

$$(5)\quad k_{obs} = k_f \frac{(Ile)_o \cdot (ATP)_o}{K_{Ile} \cdot K_{ATP}} \quad \frac{(Mg^{2+})_o}{[K_{Mg}(1+\frac{(I)_o}{K_{I \cdot ATP}})+(Mg^{2+})_o]} \quad \frac{K_I}{[(I)_o+K_I]}$$

for $(Mg^{2+})_o \lesssim 1$ mM

The nature of the effector site. Binding of spermine to the effector site is associated with an enhancement of fluorescence. Upon saturation, the wavelength for maximum emission is shifted to 432 nm (excitation at 366 nm, spectra not corrected for instrument properties). Comparison is made with the maxima 443 nm for enzyme-TNS, and 438 nm for enzyme-TNS-substrate complexes (excitation at 340 nm).

We have observed that the maximum rate constant (at infinite concentration of magnesium) of the amino acid activation reaction decreased when spermine was binding to the effector site of Ile-tRNA synthetase. We interpret this by assuming that spermine induces a conformation change

at the active site, rendering a less active enzyme. A slow process (k_{obs} = 12 sec^{-1}) which is characterized as a fluorescence enhancement has been observed when a solution containing 6.4 mM spermine plus 43 μM TNS was rapidly mixed in a stopped-flow apparatus with a solution containing 0.15 μM enzyme, 2.82 μM L-isoleucine, 39.6 μM ATP, 43 μM TNS, and 20 mM EDTA (pH 8.0, 25°C). The same process was observed when 1.34 mM $MgCl_2$ had been added instead of EDTA. The slowness of the process, although at saturation concentration of spermine, is in accord with the suggestion that the effector induces a conformation change at the active site, when the interconversion step is rate-limiting.

It was of interest to test whether or not spermine and Mg^{2+} ions compete for the same effector site. In a competition experiment where an enzyme solution (0.19 μM enzyme, 12 μM TNS, pH 8.0, 25°C) containing 2 mM $MgCl_2$ was titrated with spermine, a value for the apparent dissociation constant was measured which was twice the value in the absence of magnesium. This finding suggests that both ions might compete for the same site. Furthermore, titration of an enzyme solution containing 0.4 mM and 0.8 mM spermine with a solution of $MgCl_2$ indicated an initial quenching of the fluorescence. However, since effects from increasing ionic strength interfere with the measurement, the problem cannot be definitely solved at present.

Discussion

Cole and Schimmel (1970) have shown in a careful analysis that magnesium interacts with ATP and pyrophosphate and that only the monomagnesium salts are the reactive species. Our results support their findings. At low concentration of Mg^{2+} ions (<1 mM) the rate of formation of enzyme

bound L-isoleucyl adenylate depends strictly on the concentration of $MgATP^{2-}$. The reaction rate versus magnesium concentration profile follows the value 19 μM for a dissociation constant, in agreement with the value 25 μM reported for the dissociation of $MgATP^{2-}$, at 25°, 0.07 M tetrabutylammonium bromide (Paetzold and Amoulong, 1966). Presumably, magnesium can combine with either free or enzyme bound ATP, but the possibility cannot be excluded that the interaction with bound ATP is less strong. This alternative could exist because the observed dissociation constant would reflect the strongest interaction between magnesium and ATP, similar as it is found for unproductive binding of substrates (Rupley, 1967).

The inhibitory effect of high concentrations of Mg^{2+} ions (>1 mM) has not been reported. Under conditions of ATP-[^{32}P]PP exchange this effect could be masked by the extent of formation of unreactive di-magnesium pyrophosphate which is reported to follow a dissociation constant 10^{-2} M (Cole and Schimmel, 1970). We found that under our conditions the variation of ionic strength, necessarily associated with variation of high concentration of magnesium, cannot account for the inhibition. Furthermore, we have observed that Mg^{2+} ions which bind at the level of the enzyme-ATP complex follow a similar concentration dependence. Our results from competition experiments with spermine indicate evidence that the site of interaction for the second magnesium ion might be close or identical with that for spermine.

Spermine interacts via two routes with the Michaelis-Menten complex of the amino acid activation reaction, both being inhibitory. One route is binding to the effector site of the enzyme and the other route is the

formation of unreactive spermine-ATP. Binding to the enzyme is associated with a decrease of the maximum reaction rate. Inhibition as a function of the effector concentration follows a value for an apparent dissociation constant that is identical with the value determined from direct titration of Ile-tRNA synthetase. Binding of spermine to the enzyme-effector site presumably induces a conformation change which results in a decreased catalytic ability of the active site of the enzyme. The enhancement of fluorescence associated with the binding is in contrast to the fluorescence quenching associated with binding of substrates and inhibitors. The enhancement together with the blue-shift of the maximum wavelength of emission indicate that the environment of the reporter group, TNS, has become more hydrophobic (McClure and Edelman, 1966; Streyer, 1965) when spermine was bound to the enzyme. This finding is in contrast to the observation that fluorescence is quenched when Ile-tRNA synthetase is titrated with L-isoleucine, ATP, or pyrophosphate, and yet the wavelength of maximum emission is shifted to the blue, but to a smaller extent. It appears likely that the ligands do not only induce different, more lipophilic environments, but also promote interaction between the fluorophor and a quencher differently. We have found that the relative degree of L-isoleucine-induced quenching is not changed by binding of spermine. This observation indicates that the binding of spermine has less, possibly a reverse, effect on the interaction between TNS and the quencher than for binding of L-isoleucine. We are, of course, aware of alternative mechanisms. Further experiments are underway for elucidation of the mechanism.

The spermine-induced conformation change apparently leaves the site for L-isoleucine intact. We have no evidence as to what extent the properties of the ATP-site are changed. We have shown (Figure 8) that magnesium and spermine compete with comparable affinities for ATP. Because of this property, binding of ATP and spermine to the enzyme are coupled reactions. As we have mentioned, there is some evidence from the titration data in Figure 7 that spermine-ATP is capable of binding to the enzyme but that the ability to induce a change of the fluorescence intensity is lost. As for the kinetics, we have shown that simple competition between magnesium and spermine may account for the variation of the Michaelis-Menten constant at various fixed concentrations of spermine, Figure 3 and Figure 4b.

There is no evidence about the location of the effector site which might accommodate both spermine and magnesium. In this connection, it is interesting that Steinmetz Kayne and Cohn (1972) have reported recently that 0.94 gram-atom of Zn^{2+} and 0.77 gram-atom of Ca^{2+} were bound per molecule enzyme. We think that these cations might interact with the site which we have called "effector" site.

In conclusion, we have found that the capability of Ile-tRNA synthetase to catalyze ATP-$[^{32}P]PP_i$ exchange is lost in the presence of spermine because the effector binds to the enzyme and to ATP (and presumably to PP_i) to leave a less reactive enzyme and an unreactive spermine-ATP. We find that enzyme bound L-isoleucyl adenylate is not accumulated when magnesium is substituted by spermine. The fact that the overall reaction, that is, the specific aminoacylation of $tRNA^{Ile}$ is still catalyzed by the enzyme, leaves the question open of whether only the accumulation of the intermediate is avoided (its generation could be rate-limiting) or whether catalysis follows a completely different pathway.

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FIGURE LEGENDS

Figure 1: Formation of Ile-tRNA synthetase-L-isoleucyl∿AMP complex at pH 8.0, 25°C. The rate constant is measured as a function of the concentration of magnesium ions. At concentrations above 1 mM, catalysis is inhibited. Data in the inset are plotted according to equation $k_o - k_{obs} = k_o (Mg^{2+})_o / [K_i + (Mg^{2+})_o]$ for concentrations higher than 1 mM. The value $k_o = 1.2\ sec^{-1}$ had been determined from the intercept for line a) in Figure 3. Initial concentrations were 0.05 µM to 0.15 µM Ile-tRNA synthetase, 43 µM TNS, 0.88 µM L-isoleucine, 11 µM ATP, 0.05 M Tris-HCl, and 0.01 M 2-mercaptoethanol.

Figure 2: Fluorimetric titration of Ile-tRNA synthetase-ATP complex with Mg^{2+} ions at pH 8, 25°C. a) Fluorescence quenching upon titration with ATP (o) and ATP plus $MgCl_2$ (1:1) (o). Initial concentrations were 0.35 µM enzyme and 8 µM TNS. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, plus emission cut-off filter 430 nm. b) Fluorescence enhancement upon titration with $MgCl_2$ at a fixed (2.2 mM) concentration of ATP. The broken line indicates fluorescence intensity before addition of ATP. Initial concentrations were 1 µM enzyme and 5 µM TNS. c) Data from b) are replotted according to the method by Eadie (1942), assuming that fluorescence enhancement is associated with the binding of a second Mg^{2+} ion. The dimension of the abscissa is mM^{-1}. The term $(ATP)_o$ is added in order to correct for the amount of magnesium bound to ATP.

Figure 3: Inhibition of the L-isoleucine activation reaction by spermine at 25°C, pH 8.0. Linearized plots (Eadie, 1942) for k_{obs} as determined from stopped-flow experiments as function of concentration of Mg^{2+} ions at various fixed concentrations of spermine. Initial concentrations were 0.075 μM Ile-tRNA synthetase, 43 μM TNS, 0.88 μM L-isoleucine, 11 μM ATP, a) zero, b) 0.1 mM, c) 0.316 mM, d) 0.645 mM, and e) 1.45 mM spermine. Solutions contained 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol. Concentration of $MgCl_2$ was varied between 0.01 mM and 1 mM. Excitation wavelength 290 nm, slit 2 mm, emission cut-off filter, Corning #373.

Figure 4: Evaluation of the data in Figure 3. a) Linear plot for inhibition at saturating concentration of Mg^{2+} ions. The lines in Figure 3 intersect with the k_{obs}-axis at various values k_o^I and k_o in presence and absence of spermine, respectively. The difference $k_o - k_o^I$ is plotted as function of the concentration of spermine. The linear arrangement is consistent with a saturation function for spermine binding to the Michaelis-Menten complex. The inhibition constant K_I is determined from the slope. Note that saturation is not associated with complete loss of activity. b) The Michaelis-Menten constant, $K_{Mg}(app)$ for magnesium is plotted as function of the concentration of spermine. We have assumed that Mg^{2+} ions and spermine compete for ATP. The theoretical relation is given by the expression $K_{Mg}(app) = K_{Mg}\,[1 + (spermine)_o/K_{I\cdot ATP}]$. The value for the inhibition constant $K_{I\cdot ATP}$ is determined from the slope using a value K_{Mg} = 25 μM (Paetzold and Amonlong, 1966).

Figure 5: Binding of spermine to Ile-tRNA synthetase at pH 8.0, 25°C. Titration is associated with an enhancement of fluorescence intensity. The dissociation constant for the formation of enzyme-spermine was determined from the slope of the line in the inset. The maximum enhancement of fluorescence intensity corresponds to 37% of the initial fluorescence intensity (corrected for background). Initial concentrations were 0.18 μM enzyme, 10 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, emission cut-off filter 430 nm.

Figure 6: Binding of L-isoleucine to Ile-tRNA synthetase-spermine complex at pH 8.0, 25°C. Titration is associated with quenching of fluorescence intensity as described (Holler, Bennett, and Calvin, 1971). Initial concentrations were 0.14 μM enzyme, 10 μM TNS, 1.7 mM spermine, 10.2 μM to 2.6 mM L-isoleucine, 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 8 nm, emission wavelength 470 nm, slit 10 nm, plus cut-off filter 430 nm.

Figure 7: Titration of a mixture of Ile-tRNA synthetase plus spermine with ATP. Concentration of spermine was a) 0.415 mM, b) 0.248 mM, and c) 0.155 mM. Initial concentrations were 0.19 μM enzyme, 12 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Excitation wavelength 366 nm, slit 10 nm, emission 470 nm, slit 10 nm. Broken lines indicate the original levels of fluorescence for Ile-tRNA synthetase-TNS complex.

Figure 8; Determination of the dissociation constant of spermine-ATP, $K_{I \cdot ATP}$, at pH 8.0, 25°C.* An average value for the dissociation constant $K_{I \cdot ATP}$ was determined from the slopes for the two sets of data in b) according to the relation $K_{I \cdot ATP} = [\text{-slope}]\ [1 + (ATP)_o/K_{Mg \cdot ATP}(app)]^{-1}$. The value for $K_{Mg \cdot ATP}(app)$ has been determined according to $K_{Mg \cdot ATP}(app) =$ -slope from the linear plot a) as function of ATP concentration. The procedure is valid for conditions when $(spermine)_o >> (ATP)_o > (Mg^{2+})_o << K_{Mg \cdot ATP}$ and $(8\text{-hydroxyquinoline-5-sulfonate})_o >> (Mg^{2+})_o$. a) A mixture of dye plus $MgCl_2$ was titrated with ATP. Initial concentrations were 1.53 mM 8-hydroxyquinoline-5-sulfonate, 2.5 μM $MgCl_2$, and 0.019 mM to 0.19 mM ATP. b) A mixture of dye, $MgCl_2$ and ATP was titrated with spermine. Initial concentrations were 1.53 mM 8-hydroxyquinoline-5-sulfonate, 2.5 μM $MgCl_2$, 57.2 μM ATP, and 41 μM to 0.8 mM spermine. Excitation wavelength was 390 nm (slit 8 nm), emission wavelength 520 nm (slit 10 nm) plus 430 nm cut-off filter in the emission path.

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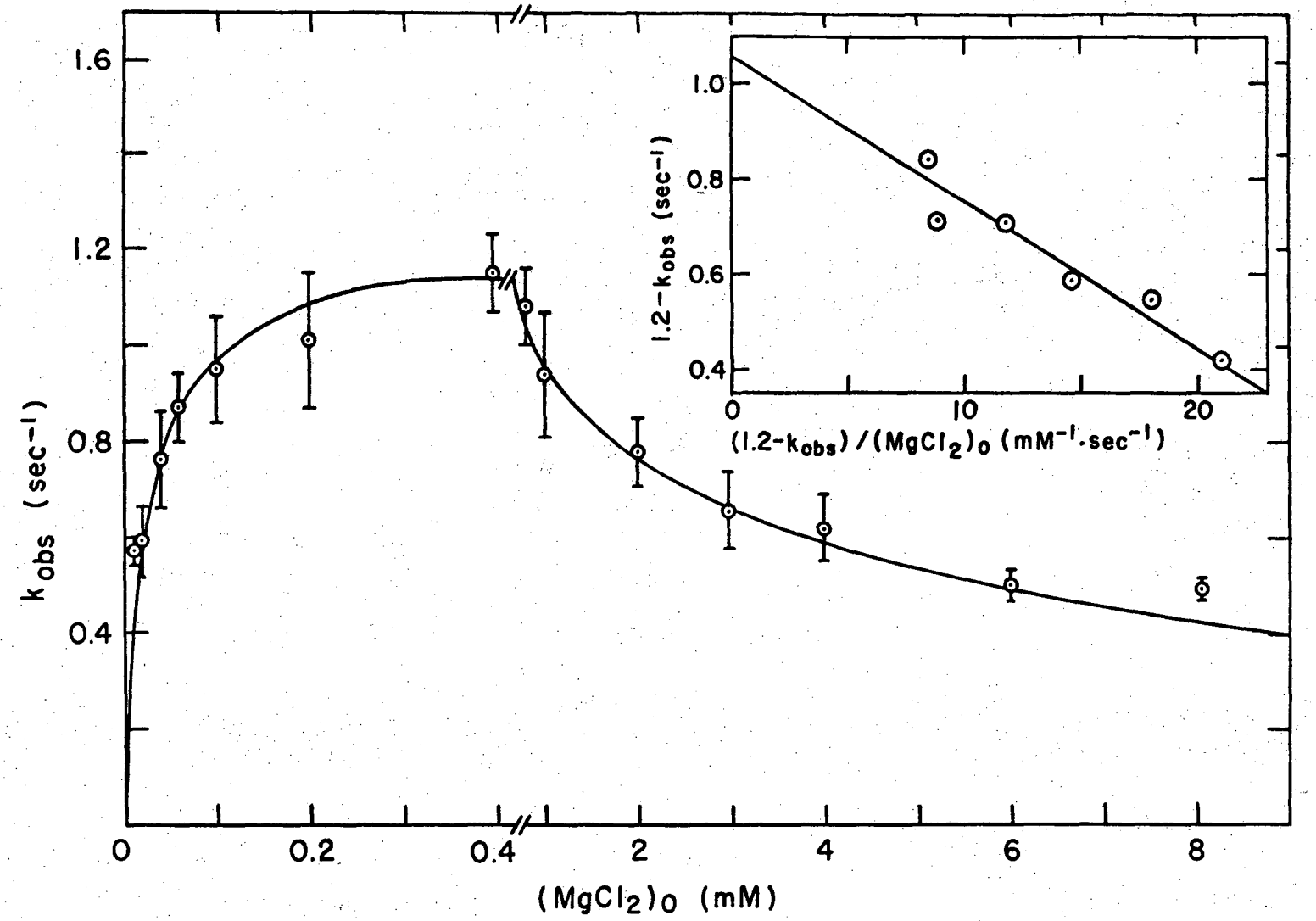
*The fluorescence intensity of 8-hydroxyquinoline-5-sulfonate was measured as a function of various concentrations of ATP (a) and of spermine (b).



XBL724-4610

Fig. 1 - Holler

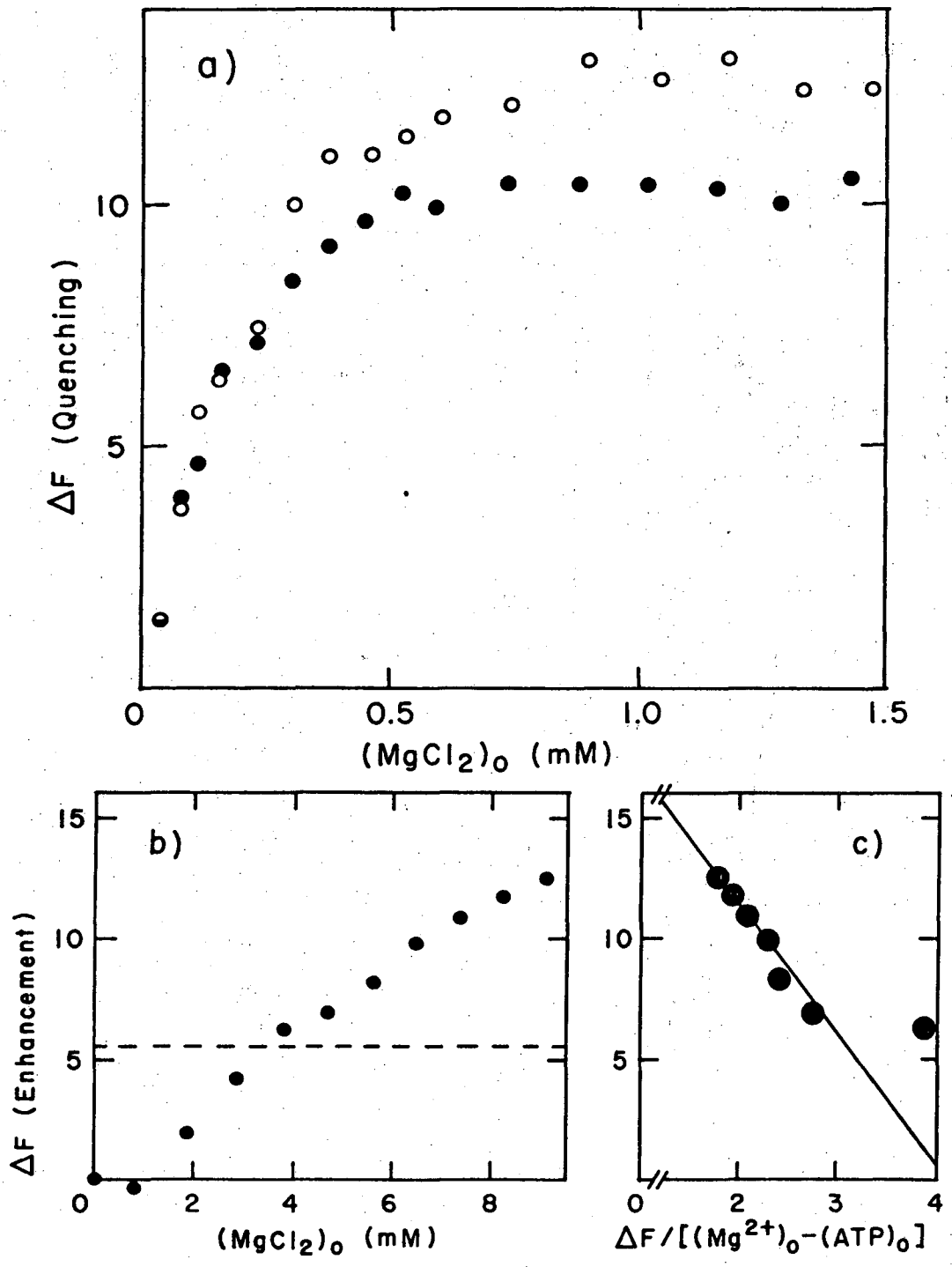
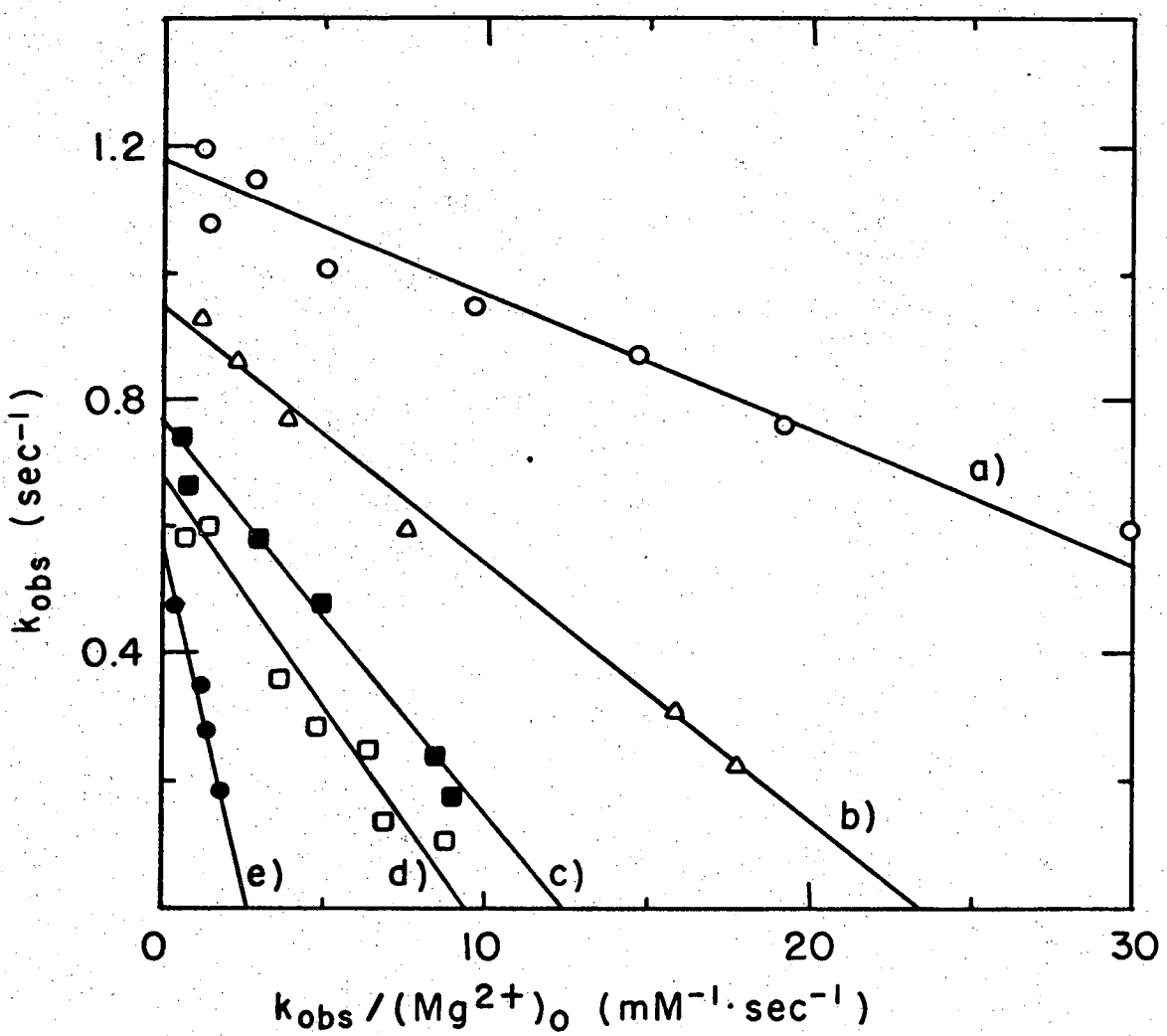
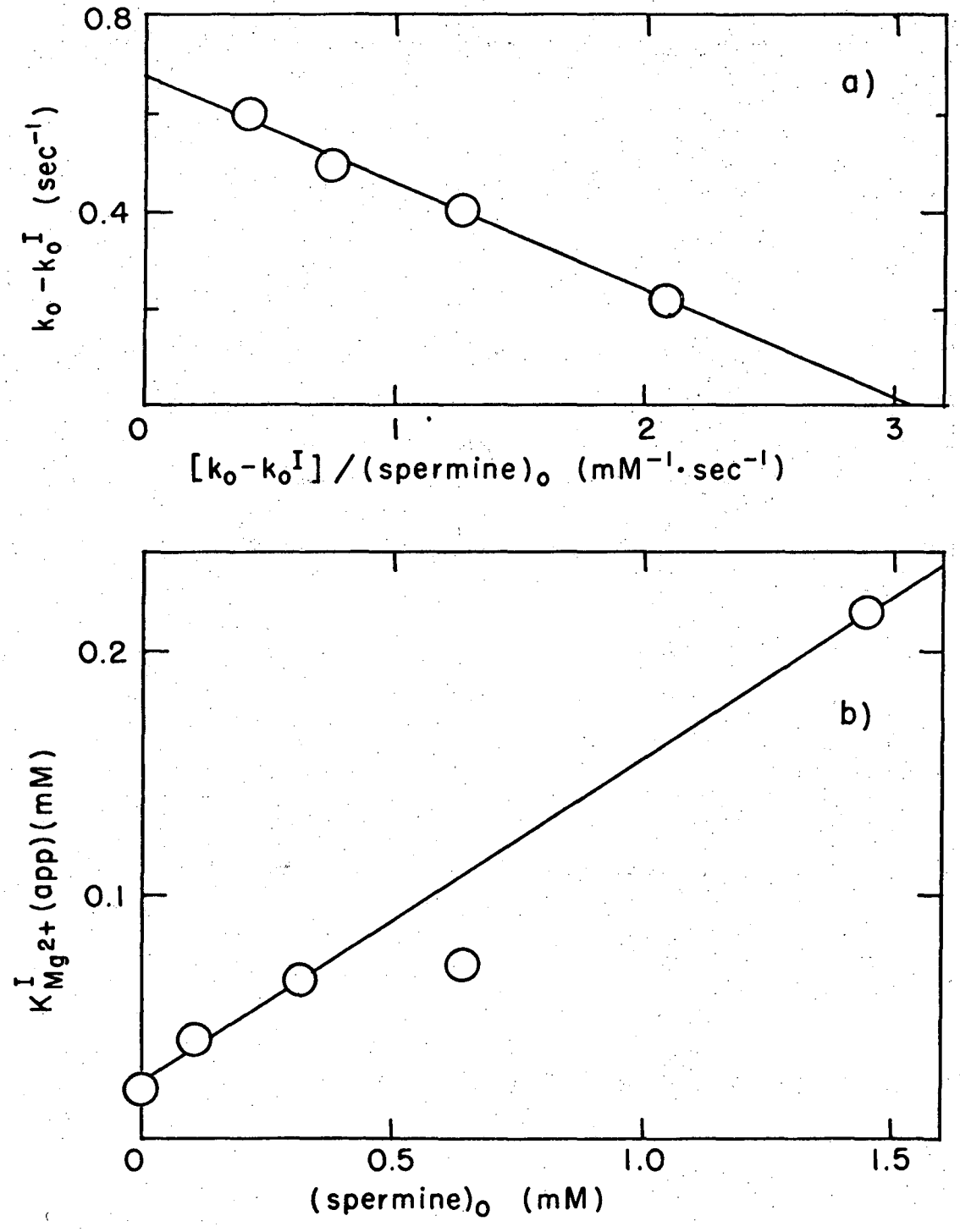

a)
ΔF (Quenching)
10
5
0
0.5
1.0
1.5
(MgCl2)o (mM)
b)
ΔF (Enhancement)
15
10
5
0
2
4
6
8
(MgCl2)o (mM)
c)
15
10
5
0
2
3
4
ΔF/[(Mg2+)o-(ATP)o]
XBL724-4611

Fig. 2 - Heller



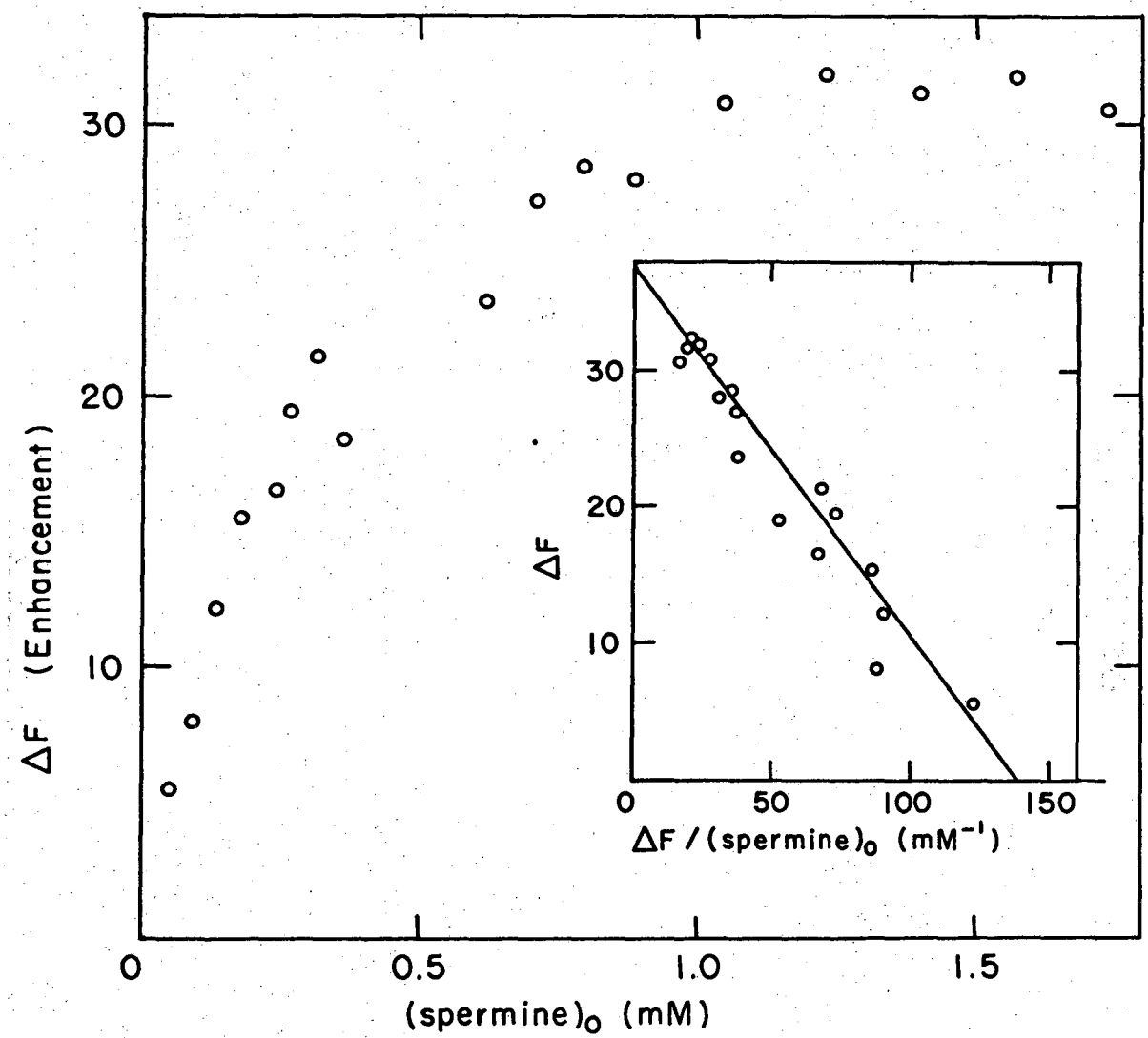
XBL724-4612

Fig. 3 - Holler



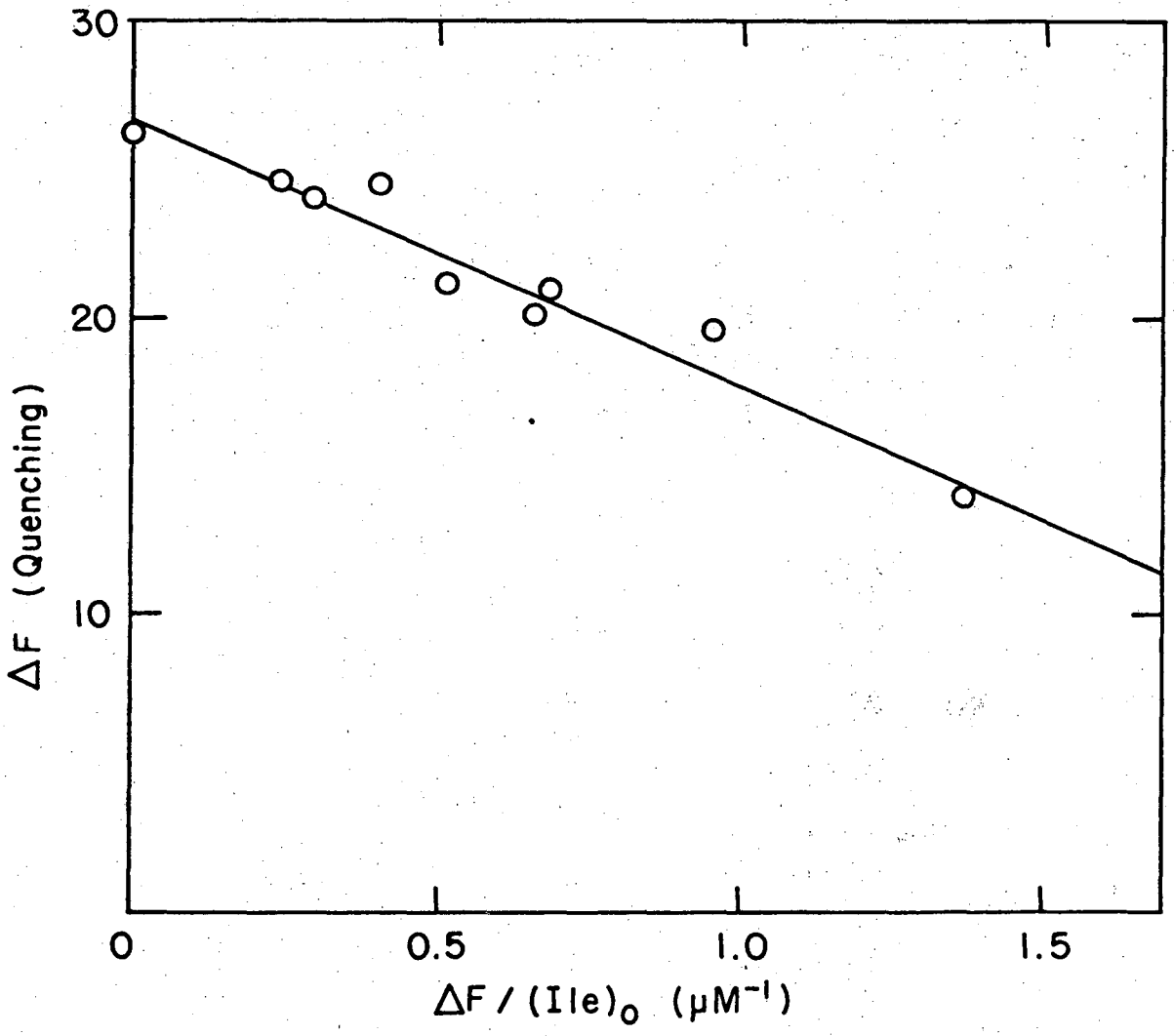
XBL724-4613

Fig. 4 - Holler



XBL724-4614

Fig. 5 - Holler



XBL724-4615

Fig. 6 - Holler

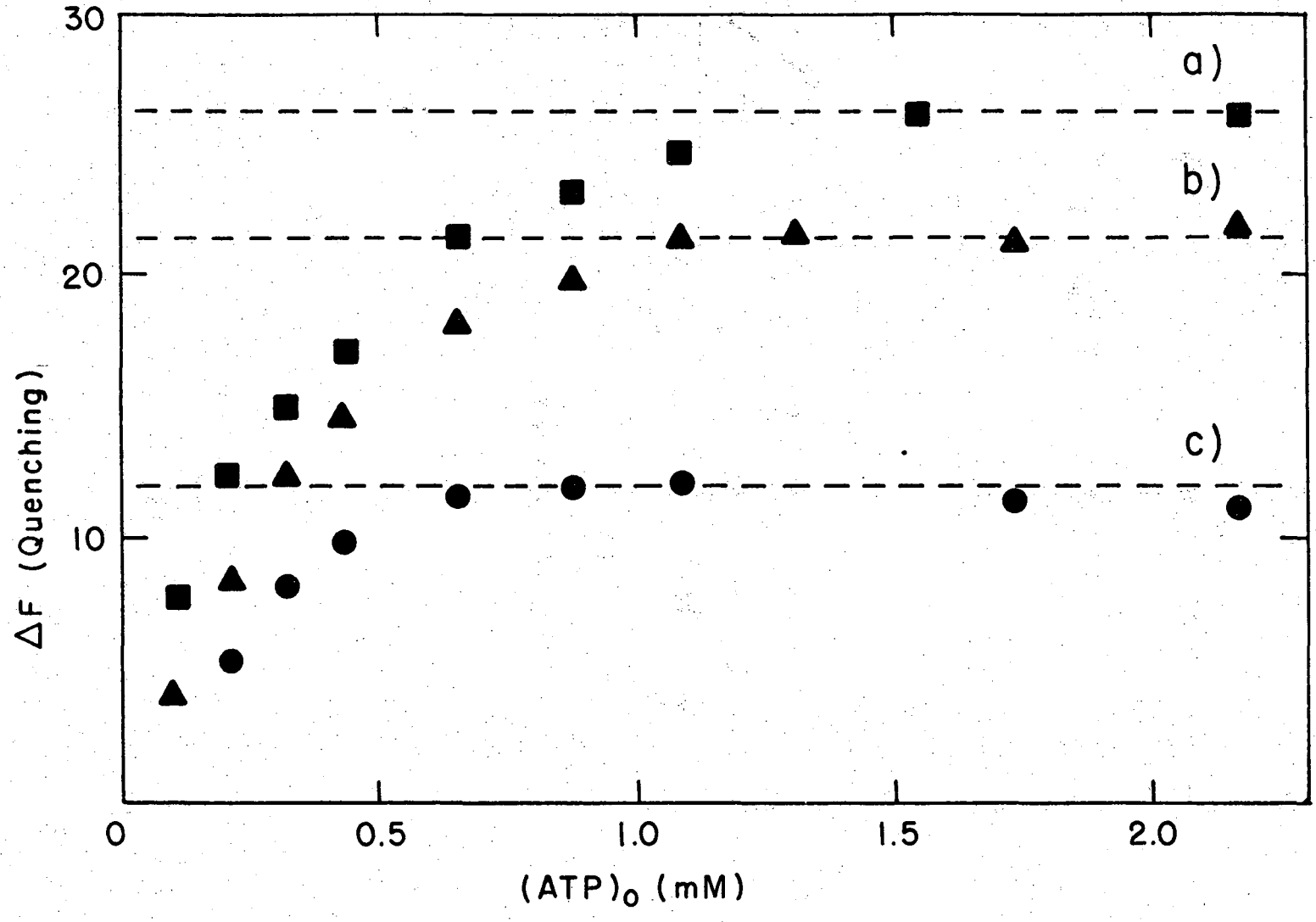


Fig. 7 - Holler

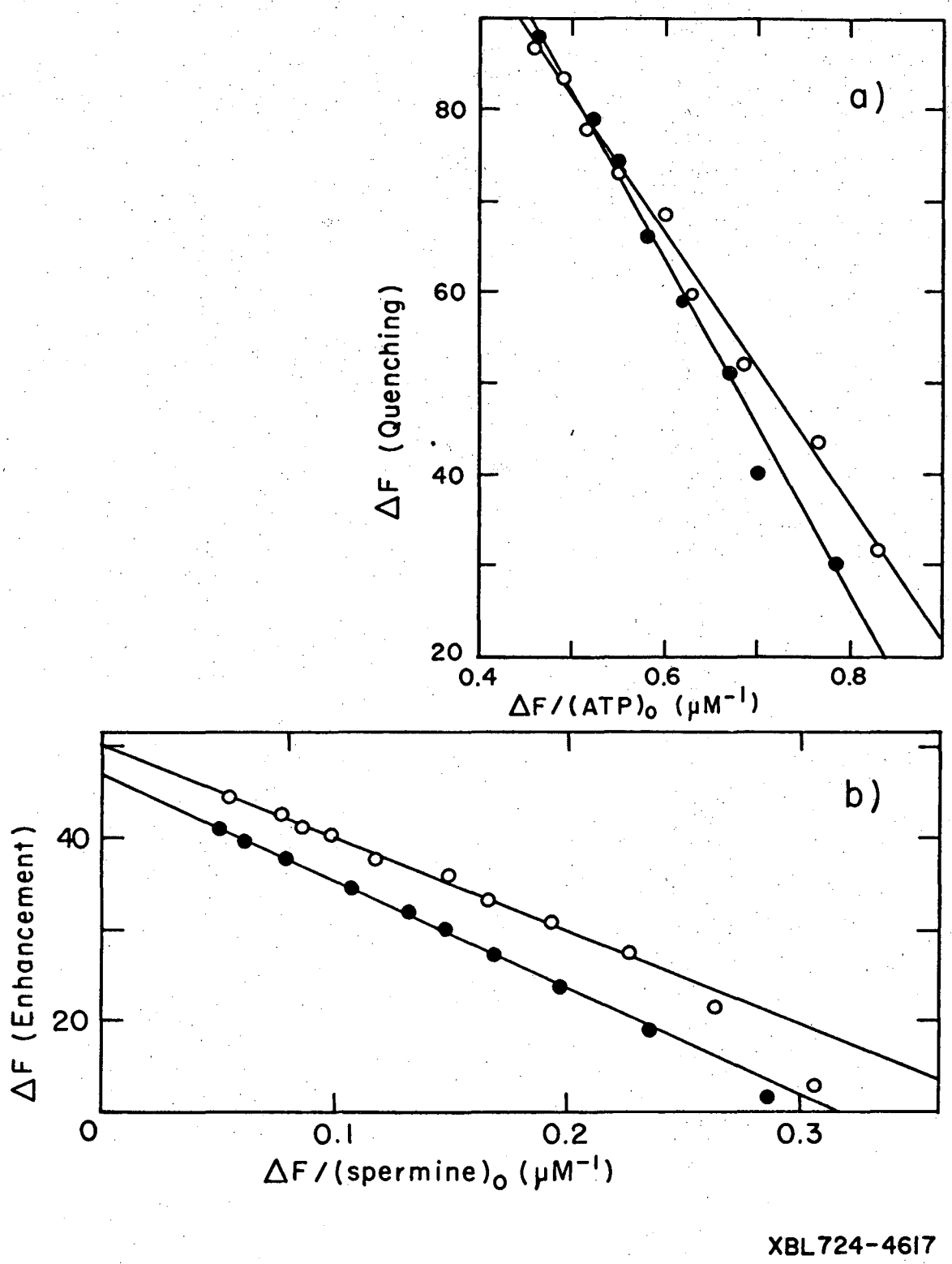

a)
80
60
40
20
ΔF (Quenching)
0.4
0.6
0.8
ΔF/(ATP)$_0$ (μM^{-1})
b)
40
20
0
ΔF (Enhancement)
0.1
0.2
0.3
ΔF/(spermine)$_0$ (μM^{-1})
XBL724-4617

Fig. 8 - Holler

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