INTERACTION OF HORSE LIVER ALCOHOL DEHYDROGENASE WITH ACRIDINE ORANGE

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The inhibition of horse liver alcohol dehydrogenase by acridine orange was studied as a function of the concentration of the two coenzyme and substrate forms, the inhibitor concentration, pH, and in the presence of other inhibitors of the enzyme. The changes in optical properties of the dye occurring during its binding to the enzyme (especially the absorption spectra and the fluorescence polarization) were also studied. The existence of an efficient resonance energy tranfer from the excited NADH molecule to the acridine orange molecule in the corresponding ternary complex with the enzyme has also been demonstrated.

The results obtained provide evidence showing that the binding site of alcohol dehydrogenase for acridine orange differs from the binding sites of this enzyme for both the coenzyme and the substrate. This binding site most likely is localized in a large substrate pocket of the enzyme near to the binding sites for *o*-phenanthroline and berberine and very close to the binding site for tricyclic psychochemicals.

Acridine orange and other acridine derivatives belong to compounds often used in studies on various macromolecules. The interaction has been examined of acridine dyes with nucleic acids[1], synthetic polypeptides[2], and certain enzymes (*e.g.* chymotrypsin[3], cholin esterase[4], and diamine oxidase[5]). It has been demonstrated[6,7] that acridine derivatives are capable of binding to horse liver alcohol dehydrogenase. This enzyme is inhibited by the presence of acridine dyes; the binding of these ligands is paralleled by changes in their absorption or fluorescence spectra.

This study has been aimed at the examination of the kinetics of inhibition of alcohol dehydrogenase by acridine orange and at the characterization of changes in the optical properties of this probe during its binding to the enzyme. The data obtained permitted an opinion of the character and localization of the binding site of alcohol dehydrogenase for acridine orange to be formulated. The binding site has been postulated[7] to be located at the periphery of the substrate cleft of the enzyme[8], *i.e.* in the region binding the substrate.

EXPERIMENTAL

Chemicals. Acridine orange (3,6-dimethylaminoacridinium chloride) from Loba Chemie (Austria) was purified according to Zanker[9]; the m.p. 190° (uncorrected) corresponded to re-

corded data[9]. Chloroprothixene (*trans*-9-(3-dimethylaminopropylidene)-2-chlorothiaxanthene hydrochloride) was from Farmakon (ČSSR), *o*-phenanthroline from Lachema (ČSSR). Ethylberberine (13-ethylberberinium iodide) was prepared from berberinium chloride (Merck, FRG) and purified as described elsewhere[10]. AMP was purchased from Reanal (Hungary); iodoacetic acid (Serva, FRG) was twice recrystallized from light petroleum, dissolved in water, and neutralized to pH 5·5 by 1M-NaOH. NAD and NADH were from Imuna (ČSSR) and Reanal (Hungary), respectively. Ethanol and acetaldehyde were purified by distillation.

Horse liver alcohol dehydrogenase (EE isoenzyme) was isolated by the method described earlier[11]. The enzyme concentration was determined by kinetic measurements[12] and is given in molarity (the concentration of the active sites of the enzyme is double the molarity value). The purity of the enzyme preparations determined spectrophotometrically[13] was 80–90%.

Measurement of the kinetics of the inhibition of the enzyme. Unless stated otherwise the experiments were carried out in sodium phosphate buffer at pH 7, I 0·1 and 23°C. The enzyme activity was measured in 0·1M-NaOH-glycine buffer containing 0·5 mM NAD and 10 mM ethanol. The measurement of the inhibition power as a function of pH was performed in sodium phosphate (pH 6–8), NaOH-glycine (pH 9–10), and NaOH-phosphate (pH 11) buffer. The spectrophotometric measurements were made in Cary 118 spectrophotometer, the fluorescence measurements in Aminco-Bowman standard spectrofluorimeter; for the fluororescence polarization measurements a polarization adapter was used with the fluorimeter. The kinetic inhibition measurements were carried out spectrophotometrically (the NADH increase or decrease during the initial stage of the reaction was measured at 340 nm); the inhibition data were processed graphically (double reciprocal plot).

The analysis of the simultaneous effect of two inhibitors (I_1 and I_2, where I_2 stands for acridine orange) was carried out by the procedure developed by Keleti and Fajszi[14,15]. The initial rates of the enzyme reaction were measured at constant enzyme, coenzyme and substrate concentration, and at variable concentrations of the two inhibitors. Parameters D and α were determined from the data obtained. Parameter D is defined by the equation $D = 1/(v \,.\, v_{12}) - 1/(v_1 \,.\, v_2$, where v is the reaction rate in the absence of inhibitors, v_1, v_2, and v_{12} the reaction rates in the presence of I_1, I_2, or the two inhibitors, respectively. α represents the ratio of the dissociation constant of the enzyme–I_1–I_2 complex for the dissociation to the complex enzyme–I_1 and free I_1 and of the dissociation constant characterizing the enzyme–I_1 complex. If α equals 1 and D zero then the inhibitors do not affect each other during the binding; if $D < 0$ they act antagonistically, if $D > 0$ they act synergistically. If $\alpha > 1$ the ligands are mutually labilized, if $\alpha \to \infty$ they compete, and if $\alpha < 1$ they stabilize each other. The $1/v_{12}$ *versus* $[I_1]$ plots for various I_2 concentrations and secondary plots of the intercepts of the lines obtained on the $[I_1]$ (m) *versus* $[I_2]$ axis were carried out. If m is independent of $[I_2]$ then $\alpha = 1$; if m linearly increases with the increasing $[I_2]$ then $\alpha \to \infty$. The increasing hyperbola indicates that $1 < \alpha < \infty$ whereas the decreasing hyperbola indicates that $0 < \alpha < 1$.

RESULTS AND DISCUSSION

The results of the kinetic inhibition measurements of variable concentrations of all the partners participating on the reaction catalyzed by alcohol dehydrogenase are given in Table I. Together with the inhibition type the value of the inhibition constant of the intercept (K_{ii}) is also recorded; this constant can be well interpreted also in the case of two-substrate enzymes on condition that the decomposition of the binary

enzyme–coenzyme complexes is the slowest step in the reaction sequence. This constant reflects the interaction of the enzyme with the inhibitor in such situations where the enzyme is predominantly present in the binary enzyme–coenzyme complex. The value of this inhibition constant roughly corresponds to the constant characterizing the dissociation of the inhibitor from the enzyme–NADH–inhibitor complex (for ethanol oxidation) or from the enzyme–NAD–inhibitor complex (for acetaldehyde reduction).

As shown in Table I the intercepts and slopes of all Lineweaver–Burk plots are markedly affected by the presence of acridine orange (the inhibitory behavior of acridine orange toward all four reaction partners approaches the noncompetitive type). It is therefore probable that this inhibitor forms complexes with all the kinetically important forms of the enzyme. If the Theorell–Chance mechanism which alcohol dehydrogenase complies with at least to a first approximation[8] is valid, then these partners are the free enzyme and the binary complexes enzyme–NAD and enzyme–NADH.

The partial inhibition test of the inhibitor examined is presented in Fig. 1. Since for inhibitor concentrations exceeding all limits the inhibition is not complete (the intercept of the line on the $v/(v - v_i)$ axis is larger than one), acridine orange can be classified as a partial inhibitor. The partial type of inhibition does not change even

TABLE I

Inhibitory behavior of acridine orange toward alcohol dehydrogenase at pH 7·0. The concentrations of acridine orange used in the measurements were 0, 16, and 32 μM (ethanol oxidation) and 0, 5 and 10 μM (acetaldehyde reduction). K_{ii} are mean values of inhibition constants calculated from the intercepts of double reciprocal plots (reciprocal initial rate *versus* reciprocal substrate or coenzyme concentration). NC and UC stand for noncompetitive and uncompetitive type of inhibition, respectively

Varied concentration μmol l^{-1}	Saturating concentration	Inhibition type	K_{ii} μmol l^{-1}
Ethanol (100–1 500)	NAD (500)	NC	20
NAD (10–150)	ethanol (3 000)	NC	24
Acetaldehyde (30–500)	NADH (200)	UC–NC	9
NADH (2–30)	acetaldehyde (1 000)	UC–NC	12

after the correction of acridine orange concentration for the content of dimer which is present to a significant degree at higher concentrations of the dye[16] and which is probably lacking inhibitory activity (Fig. 1). A dimerization constant equal 70 μM (ref.[16]) was considered in the corrections. Likewise, the markedly hyperbolic character of the Dixon plot of uncorrected and corrected data given in Fig. 1 (not presented) confirm the fact that acridine orange is a partial inhibitor. Acridine orange cannot therefore displace the substrate (ethanol) from binding to the enzyme yet merely leads to a decrease of the rate of its oxidation (at saturating concentrations of NAD, ethanol, and acridine orange) at pH 7 to approximately one third of its original value.

In other experiments the dependence of the inhibitory power of acridine orange on pH was examined (Fig. 2). The inhibition is maximal around pH 10. The effect of the inhibitor on the enzymatic activity abruptly decreases at higher pH-values. It appears that the uncharged form of the inhibitor (pK 10·45, ref.[17]) binds more weakly to the enzyme. The inhibitory power decreases also at low pH-values. This may be the result of the fact that alcohol dehydrogenase contains a water molecule

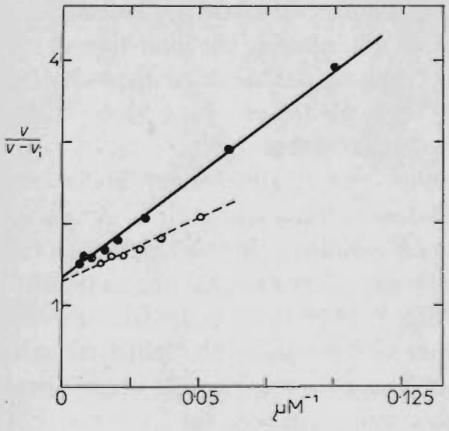


FIG. 1

Dependence of alcohol dehydrogenase inhibition by acridine orange (AO) on inhibitor concentration (μM). v is the initial rate of the enzymatic reaction in the absence of the inhibitor (10 mM ethanol, 0·5 mM NAD, pH 7·0, 10 nM enzyme), v_i is the reaction rate under identical conditions yet in the presence of the inhibitor. (●) Uncorrected value of acridine orange concentration, (○) of inhibitor in monomer form

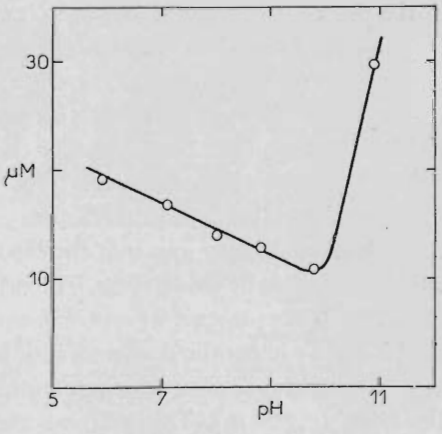


FIG. 2

pH-Dependence of alcohol dehydrogenase inhibition by acridine orange. The reaction mixture contained 10 mM ethanol, 0·5 μM NAD and 5 nM enzyme. μM is the concentration of inhibitor which brings about 50% of the inhibition caused by saturating inhibitor concentration

in its active center which is bound to the catalytic zinc atom firmly incorporated into the protein structure. The p*K*-value of this water molecule is roughly 9·6 (and drops in the presence of bound NAD to 7·6, ref.[8]). It is likely that the binding of acridine orange to alcohol dehydrogenase achieves the highest strength as a result of electrostatic attraction if a hydroxyl anion is present on the catalytic zinc atom and if acridine orange is simultaneously present as a cation; these conditions exist at pH around 10.

In an effort to cast more light on the localization of the binding site of alcohol dehydrogenase for acridine orange, the inhibition experiments were carried out in the presence of other inhibitors whose binding to the enzyme had been characterized in more detail[7,8]. These experiments were carried out with AMP, which binds to the same site as the coenzyme, with iodoacetate, binding to the so-called "anion-binding site" in the neighborhood of the catalytic zinc atom, with *o*-phenanthroline, binding directly to the catalytic zinc atom and competing both with the coenzyme and the substrate, and with ethylberberine and chloroprothixene, which bind to two sites in the substrate pocket of the enzyme close to the catalytic zinc atom.

Figs 3–5 show three typical examples of the behavior of the compounds tested in the presence of acridine orange (I_2). All the examples examined are summarized in Table II. As obvious, AMP and iodoacetate do not influence the inhibition of the enzyme by acridine orange, therefore the binding sites for these compounds (*i.e.* the coenzyme-binding site and the anion-binding site) are not most likely in the nearest vicinity of the binding site of the enzyme for acridines.

The other two ligands tested (*o*-phenanthroline and ethylberberine) labilize the binding of acridine orange to the enzyme; this demonstrates that their binding sites lie close to each other and that the binding site for acridine orange is localized in the substrate pocket of the enzyme. The primary and secondary plots assume an unusual character in the presence of *o*-phenanthroline (Fig. 4) and ethylberberine (not shown). At higher *o*-phenanthroline and acridine orange concentrations the initial reaction rate in the presence of both compounds is higher than in the presence of *o*-phenanthroline itself ($v_{12} > v_1$). This extreme case of antagonism between the inhibitors can occur[15] if at least one of the inhibitors is partial (I_2) and if the concentration of inhibitor I_1 is higher than some critical concentration. Both conditions are fulfilled in our case since acridine orange (I_2) is a partial inhibitor and the effect mentioned takes place at higher *o*-phenanthroline concentrations (I_1) only. Acridine orange thus acts to a certain degree, as an agent "liberating" alcohol dehydrogenase from the inhibitory effect of *o*-phenanthroline (and likewise of ethylberberine).

Acridine orange kinetically competes with chloroprothixene (Fig. 5). Since, however, acridine orange and chloroprothixene markedly differ in their effect on the rate of the enzyme carboxymethylation and on the "steroid activity" of alcohol dehydrogenase isoenzymes, the binding sites of the enzyme for these compounds are not entirely

TABLE II

Analysis of kinetic effect of certain compounds on interaction of acridine orange with alcohol dehydrogenase. The conditions of the measurement were analogous to those described in Figs 3—5. Parameters α and D are defined under Experimental and their evaluation was carried our according to ref.[15]

Inhibitor I_1	α	D	Effect of inhibitor I_1 on binding of acridine orange to enzyme
AMP	1	0	none
Iodoacetate	1	0	none
o-Phenanthroline	>1	<0	labilization[a]
Ethylberberine	>1	<0	labilization[a]
Chloroprothixene	$\rightarrow\infty$	<0	competition[a]

[a] Antagonism.

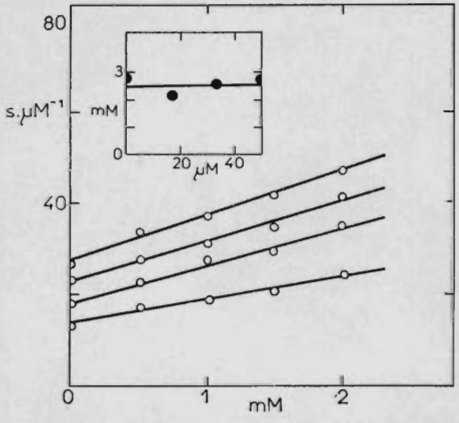


FIG. 3

Effect of AMP on alcohol dehydrogenase inhibition by acridine orange (AO). The reaction mixture contained 10 mM ethanol, 0·2 mM NAD and 10 nM enzyme. The concentration of acridine orange corresponding to the lines in the primary graph is (from the bottom), 0, 16, 32, and 50 μM. The secondary plot represents the values of intercepts on the [AMP] axis for the individual lines of the primary graph as a function of acridine orange concentration (AO)

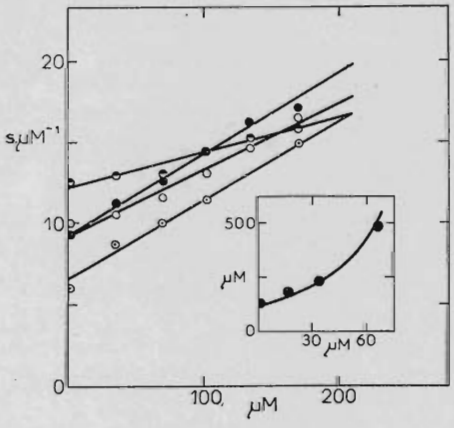


FIG. 4

Effect of *o*-phenanthroline on alcohol dehydrogenase inhibition by acridine orange. The reaction conditions are identical to those shown in Fig. 3; the acridine orange concentration is 0 μM (⊙), 16 μM (●), 32 μM (○), and 64 μM (◓)

identical[7]. These binding sites obviously lie close to each other or even partly overlap. In view of the inability of acridine orange to displace the substrate from the binding to the enzyme (Fig. 1) and in view of the relatively high hydrophility of the binding site of the enzyme for this compound[7], it is probable that the binding site of alcohol dehydrogenase for acridine orange is localized on the periphery of the substrate pocket between the catalytic zinc atom and the water phase bordering region.

The binding of acridine orange by alcohol dehydrogenase is paralleled by changes in the optical properties of this fluorescing dye whose long-wave absorption maximum lies at 490 nm. The absorption spectrum of the ligand changes (a bathochromic shift and a hyperchromic effect are observed), the fluorescence intensity of the attached dye slightly increases[7] (λ_{exc} 490 nm, λ_{em} 520 nm), and the fluorescence polarization is markedly increased[7] (at the excitation and emission wavelengths given the polarization value of the free dye is zero and assumes a value of about 0·41 if the enzyme is saturated). Likewise chirality is induced during the binding to the enzyme (a marked Cotton effect in the 470–510 nm range).

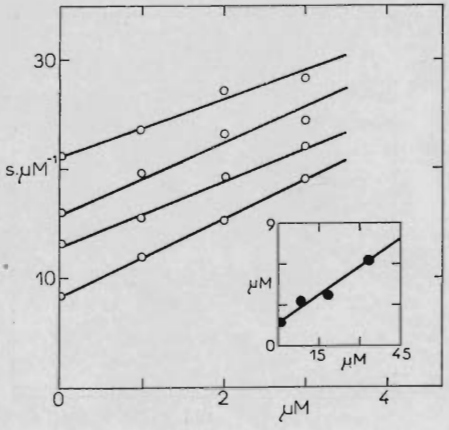


FIG. 5

Effect of chloroprothixene on alcohol dehydrogenase inhibition by acridine orange. The reaction conditions are the same as those described in Fig. 3; the acridine orange concentration corresponding to the lines in the primary graph is (from the bottom) 0, 9, 18, and 34 μM

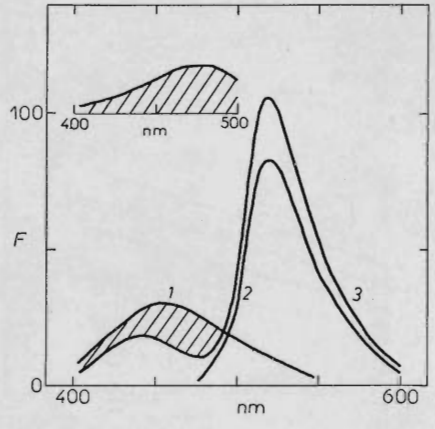


FIG. 6

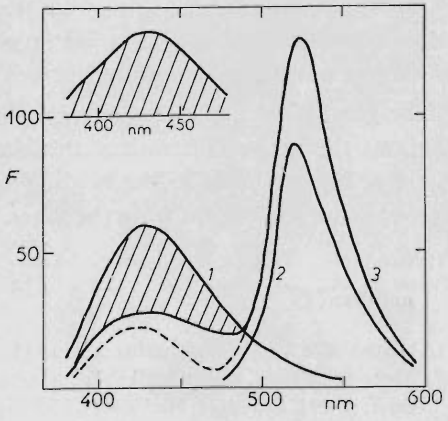
Effect of acridine orange on fluorescence of free NADH. F is the fluorescence intensity in relative units, λ_{exc} 350 nm. *1* emission spectrum of 2 μM NADH; *2* 30 μM acridine orange, and *3* a mixture of 2 μM NADH with 30 μM acridine orange. The top part of the figure shows the efficiency of NADH fluorescence quenching by acridine orange as a function of wavelength. The hatched area corresponds to the energy transferred from NADH to acridine orange

These changes in optical properties can be utilized for a more detailed characterization of the binary enzyme–acridine orange complex, *i.e.* for the determination of the interaction stoichiometry and the binding constant. The highest value of the signal corresponding to complex formation (*e.g.* a difference in absorbance at 520 nm) can be observed in mixtures of alcohol dehydrogenase and acridine orange of constant total molar concentration if the molar ratio of acridine orange to the enzyme equals 2. The molecule of the enzyme therefore contains two binding sites for this ligand; most likely each of the two identical enzyme subunits bonds one molecule of the dye. It follows from the analysis of the Scatchard graph representing polarization-fluorescence data (not given) that the dissociation constant of the enzyme subunit–probe binary complex is roughly 19 μM. This value does not change in the presence of an excess of NADH whereas in the presence of 1 mM-NAD it is approximately 10 μM.

The values of dissociation constants determined are in agreement with the inhibition constant $I_{0.5}$ shown in Fig. 1 (16 μM at pH 7) and especially with the constants given in Table I. Acridine orange thus binds to alcohol dehydrogenase by a firm bond which is not affected by the presence of NADH yet slightly strenghtened by bound NAD. A similar, apparently paradoxical stabilization of the cationic ligand in the presence of NAD has been observed with the binding of chloroprothixene to this enzyme. An important role in the stabilization of the bonds of both these cations, whose binding sites lie close to each other and not far from the catalytic zinc atom, plays the above mentioned shift of pK of the water molecule attached

FIG. 7

Effect of acridine orange on fluorescence of NADH bound to alcohol dehydrogenase. F is the fluorescence intensity in relative units, λ_{exc} 350 nm. *1* Emission spectrum of a mixture of 2 μM NADH and 1·5 μM enzyme; *2* mixture of 30 μM acridine orange and 1·5 μM enzyme; *3* mixture of 30 μM acridine orange, 2 μM NADH, and 1·5 μM enzyme (the record is corrected for the effect of the free dye on NADH fluorescence in the 450—490 nm range, the uncorrected experimental curve is drawn in a dashed line). The top part of the figure shows the efficiency of energy transfer from bound NADH to bound acridine orange as a function of wavelength. The hatched area corresponds to the energy transferred from bound NADH to bound acridine orange



to the zinc atom by the effect of bound NAD. The presence of the hydroxyl anion can increase the affinity of these ligands for the enzyme by electrostatic attraction.

Acridine orange intensively absorbs around 460–500 nm, *i.e.* in a range where NADH shows fluorescence; hence the fundamental condition is fulfilled of the energy transfer from the excited NADH molecule to acridine orange in the ternary complex of enzyme–NADH–acridine orange. Since acridine orange itself shows an intensive fluorescence around 520 nm, it can irradiate the energy received from NADH in the form of sensibilized fluorescence. Fig. 6 shows that the quenching of NADH fluorescence and the sensibilization of acridine orange fluorescence take place in the absence of the enzyme. The plot of the efficiency of energy transfer on wavelength is asymmetric and maximum quenching is observed in the range of the absorption maximum of acridine orange. This indicates that the dye quenches by the simple absorption of the light irradiated by NADH molecules.

An even more marked quenching of NADH fluorescence (Fig. 7) (which is bound to approximately 85% to the enzyme under the conditions given) and a higher sensibilization of acridine orange fluorescence (less than 60% of the binding sites of the enzyme for this compound are occupied under the conditions given, *i.e.* the concentration of bound orange is 1·7 μM) occur in the presence of the enzyme. A comparison of Figs 6 and 7 shows that the NADH fluorescence quenching by bound acridine orange is almost 50 times more efficient than the quenching by the unbound dye. The symmetrical shape of the plot of the efficiency of the energy transfer on wavelength (after the correction for the effect of free orange on NADH fluorescence, Figs 6 and 7) shows that predominantly a resonance energy transfer takes place in the presence of the enzyme.

The efficiency of this transfer between these fluorophores is big and amounts to roughly 95% if the enzyme–NADH complex is saturated with acridine orange. This value obtained by extrapolation, however, is considerably inexact, especially because of the corrections for the energy transfer from NADH to free acridine orange; the latter is in the concentration range exceeding 50 μM present to a considerable degree as a dimer of different optical properties[16,17].

The great efficiency of the energy transfer from the coenzyme to acridine orange confirms the above concepts of the binding of this dye to alcohol dehydrogenase. Acridine orange binds to the binding site which differs from the coenzyme-binding site yet is not too distant from the latter.

REFERENCES

1. Hiromi K.: Studia Biophysica *57*, 129 (1976).
2. Myhr B., Foss J. in the book: *Fluorescence Technique in Cell Biology* (A. Thaer, M. Sernetz, Eds), p. 291. Springer, Berlin–Heidelberg–New York 1973.
3. Wallace R. A., Kurtz A. N., Niemann C.: Biochemistry *2*, 824 (1963).

4. Patočka J., Bajgar J., Fusek J.: This Journal *42*, 2980 (1977).
5. Mason S. F.: J. Chem. Soc. *351* (1950).
6. Einarsson R., Zeppezauer M.: Eur. J. Biochem. *59*, 295 (1975).
7. Kovář J., Dürrová E., Skurský L.: Eur. J. Biochem. *101*, 507 (1979).
8. Brändén C. I., Jörnvall H., Eklund H., Furugren B.: The Enzymes *11*, 113 (1975).
9. Zanker V.: Z. Phys. Chem. (Wiesbaden) *199*, 225 (1952).
10. Pavelka S., Kovář J.: This Journal *41*, 3654 (1976).
11. Skurský L., Kovář J., Čermák A.: 12th FEBS Meeting, Dresden, Abstr. 2953 (1978).
12. Dalziel K.: Acta Chem. Scand. *11*, 397 (1957).
13. Bonnichsen R.: Acta Chem. Scand. *4*, 715 (1950).
14. Keleti T., Fajszi C.: Mathemat. Biosci. *12*, 197 (1971).
15. Fajszi C., Keleti T.: Symp. Biol. Hung. *18*, 105 (1974).
16. Balcarová Z., Janovská E., Kleinwächter V., Koudelka J., Löber G.: Studia Biophysica *27*, 205 (1971).
17. Wittekind D., in the book: *Fluorescence Technique in Cell Biology* (A. Thaer, M. Sernetz, Eds), p. 95. Springer, Berlin—Heidelberg—New York 1973.

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