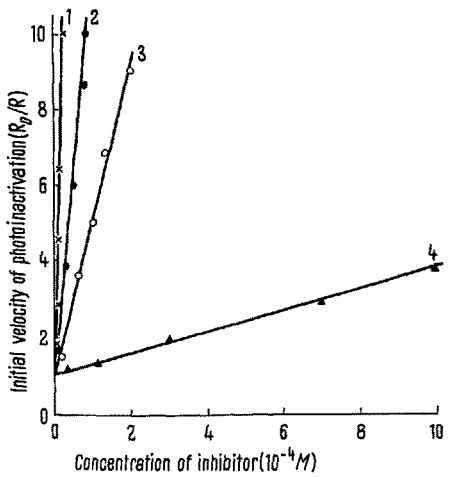
Inhibition of Riboflavin-Sensitized Photoinactivation of Taka-Amylase A by Some Inhibitors

Taka-amylase A (TAA) is easily inactivated[1,2] by the visible light in the presence of a sensitizer, riboflavin[3] (RF). The inactivation is mainly derived from the oxidation of amino acids in TAA by the photosensitizing action of RF. As described in a previous article[2], the amino acids in TAA are considered to be oxidized by the reactive oxygen in the first excited singlet state which may be produced upon dissociation of the complex of oxygen in the ground triplet state with RF in the metastable triplet state.

The addition of some substances to TAA solution containing RF strongly inhibits the photoinactivation. Paramagnetic ion, high Z atom ion[2], *p*-phenylenediamine hydrochloride (PPD) and tryptophan (T_r) were found to be strongly effective as inhibitors. The presence of these inhibitors does not affect the activity of TAA in the dark.

In the present article, the inhibition of RF-sensitized photoinactivation of TAA by these inhibitors is dealt with as regards their inhibition mechanisms. In our reaction system, the main possible mechanisms of inhibition are considered to be the quenching of the first excited singlet state of riboflavin (RF^a) or the metastable triplet state (RF^b) to the ground state (RF) by the inhibitor and the competition of the inhibitor with TAA in the oxidation process by the reactive oxygen.

The ratio of the initial velocity of photoinactivation (R_0) in the absence of inhibitor to that of (R) in the presence of inhibitor was measured as a function of the inhibitor concentration (C). The results are shown in the Figure. The relation between R_0/R and C is linear over the concentration range studied and can be expressed by the following Stern-Volmer-type equation: $R_0/R = 1 + kC$, where k is a 'quenching constant' for the photoinactivation and is equal to the reciprocal of the half-value concentration ($C_h(I)$) for the inhibition of photoinactivation. The $C_h(I)$ values determined from the curves in the Figure for each inhibitor are listed in the Table. The order of effectiveness of inhibitors is PPD > T_r > I^- > Co^{++}.



Dependence of initial velocity of photoinactivation on concentration of inhibitor. Concentration of TAA, 0.005%; concentration of RF, $3.3 \cdot 10^{-5}M$; pH 5.6 (acetate buffer); illuminated at 20 °C; exciting light, light absorbed by 445 nm-band of RF; R_0 and R, initial velocities of photoinactivation in the absence and presence of inhibitor, respectively. Activity was measured at 40 °C by the blue value method. Curve 1, PPD as inhibitor; curve 2, T_r as inhibitor; curve 3, KI as inhibitor; curve 4, $CoCl_2$ as inhibitor.

The quenching of RF fluorescence by these inhibitors also obeys the Stern-Volmer law. The obtained half-value concentration ($C_h(F)$) for the fluorescence quenching by inhibitors is given in the Table. The large ratio of $C_h(F)$ to $C_h(I)$ shows that the quenching of RF^a (lifetime[4] = 10^{-8} sec) is not the primary step in the inhibition mechanism and the quenching of RF^b may be responsible for the inhibition. If the quenching of RF^b is assumed to be the primary step in the inhibition mechanism, the lifetime of RF^b is roughly estimated to be of the reasonable order of $10^{-6} \sim 10^{-4}$ sec, since the encounter frequency for water at room temperature is of the order of $10^{10}M^{-1}$ sec^{-1} and $C_h(I)$ is of the order of $10^{-4} \sim 10^{-6}M$. The triplet species has a relatively long lifetime, and hence the possibility of colliding with the inhibitor in a very low concentration.

However, the quenching mechanism of RF^b is not necessarily the same for different inhibitors. Paramagnetic ions are known as efficient triplet quenchers. The presence of Co^{++} gives rise to the enhancement[5] of the radiationless transition probability from RF^b to RF (the breakdown of the spin-conservation rule) as the result of the spin-orbital coupling[6] due to the paramagnetic perturbation[7,8] of Co^{++}, leading to the shortening of the lifetime of RF^b. This kind of inhibition can also be observed by the presence of the other paramagnetic ions such as Ni^{++}, Mn^{++} etc., but not by the presence of the diamagnetic ones such as K^+, Na^+ etc. A charge transfer from Co^{++} to RF^b may also be a possible mechanism of quenching of RF^b, but the degree of contribution of this

Half-value concentrations for the quenching of riboflavin fluorescence[a] and for the inhibition of photoinactivation[b] of taka-amylase A

Inhibitor	Half-value concentration		$C_h(F)/C_h(I)$
	Fluorescence quenching $C_h(F)$ (M)	Inhibition of photoinactivation $C_h(I)$ (M)	
Co^{++}	$5 \cdot 10^{-2}$	$3 \cdot 10^{-4}$	$1.7 \cdot 10^2$
I^-	$3 \cdot 10^{-2}$	$2.5 \cdot 10^{-5}$	$1.2 \cdot 10^3$
PPD	$2.5 \cdot 10^{-2}$	$4 \cdot 10^{-6}$	$6.3 \cdot 10^3$
T_r	$7 \cdot 10^{-3}$	$1 \cdot 10^{-5}$	$7 \cdot 10^2$

[a] Band maximum, 534 nm; measured at 20 °C. [b] Exciting light, light absorbed by 445 nm-band of RF; illuminated at 20 °C. Concentration of TAA, 0.005%; concentration of RF, $3.3 \cdot 10^{-5}M$; pH 5.6 (acetate buffer). Activity was measured at 40 °C by the blue value method.

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mechanism to the inhibition remains for future investigation.

In the presence of ions of large atomic number such as I^-, the collisional perturbation effect of spin-orbital coupling in the π-electron orbitals of RF by I^- favours intersystem crossing[5] in the excited RF (high Z atom effect[2,9]). The resulting shortening of the lifetime of RF^b explains the inhibition. Furthermore, I^- has a donor activity, and a charge transfer from I^- to RF produces the RF^- radical[10] which is non-fluorescent and non-phosphorescent. This kind of charge transfer may reduce the production of RF^b by diminishing the overall quantum yield of photoinactivation.

PPD is a strong quencher of RF^b and is photo-oxidized in the presence of O_2. The possible mechanism of deactivation of RF^b may be a charge transfer from PPD to RF^b.

Another type of inhibition mechanism can be seen in the inhibition reaction by T_r. T_r has a considerable ability to form a charge-transfer complex[11] ($RF^- \cdot T_r^+$) (non-fluorescent and non-phosphorescent) with RF in the ground state. The overall quantum yield of production of RF^b is reduced by the formation of this complex to some extent. T_r is easily oxidized by the photosensitization of RF. Since we have no evidence that RF^b is selectively quenched by T_r, it may be natural to consider that a preferential destruction of the reactive oxygen is the main mechanism of inhibition.

The inhibition mechanisms mentioned above are considered to be typical. At low inhibitor concentration, only one of the factors[12] is sensitive and R_0/R-C relation is linear. However, at high inhibitor concentration, several factors may be mixed up; deviations from the linear relation between R_0/R and C may then occur. Further details will be described in a future paper.

Zusammenfassung. Durch Gegenwart von Co^{++} oder J^- oder Tryptophan oder *p*-Phenylendiamin wird die durch Riboflavin sensibilisierte Photoinaktivierung der Taka-Amylase A stark gehemmt. Die möglichen Mechanismen der Hemmungswirkung dieser Inhibitoren werden diskutiert.

G. Tomita

Institute of Biophysics, Faculty of Agriculture, Kyushu University, Fukuoka (Japan), August 3, 1966.

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[12] The factors mean the quenching of RF^a and RF^b and the destruction of the reactive oxygen in the oxidation process, by the inhibitors.

Effects of Histones on Embryonic Cells

Since histones are intimately associated with the genetic material, DNA, they are suspect as regulators of gene activity[1-4]. Assuming that foreign histones might reach the nucleus of a cell exposed to a histone solution one might be able to demonstrate specific effects on gene loci or the gene activating mechanism. Such effects have been proposed for treated embryonic organs[5,6] and for whole embryos when treated with preparations containing histones at various concentrations[7-9].

We were prompted, therefore, to examine the effects of histones on organ cultures of chick embryonic skin, and its ectodermal organ derivative, the down feather. 2 histone preparations were used: whole calf thymus histone (CTH) and a diazotized sample of the same (DCTH). Unfractionated calf thymus histone was prepared from the ethanol-washed deoxyribonucleohistone by extraction with 0.2 *N* HCl[10]. A sample of this histone was diazotized and coupled with sulfanilic acid[11] in order to diminish its positive charge. If CTH is added to an extract medium a precipitate is immediately formed. Therefore, 7-day-old chick embryo back skin was dissected into bilateral halves, each 2 mm^2, rolled inward so that the mesenchyme portion was on the outside, and incubated for periods up to 1 h in Tyrode solution in a shaking water bath. To $^1/_2$ of each bilateral pair CTH was added to the incubation medium in concentrations ranging from 10 $\mu g/cm^3$ to 5 mg/cm^3. After the incubation, the skin pairs were cultured on stainless steel rafts in Falcon culture dishes on an embryo extract (20%)-Tyrode solution medium for periods of up to 6 days. Most of the tissues exposed to the highest CTH concentration of 5 mg/cm^3 became necrotic (Figure 1). Those tissues exposed to a

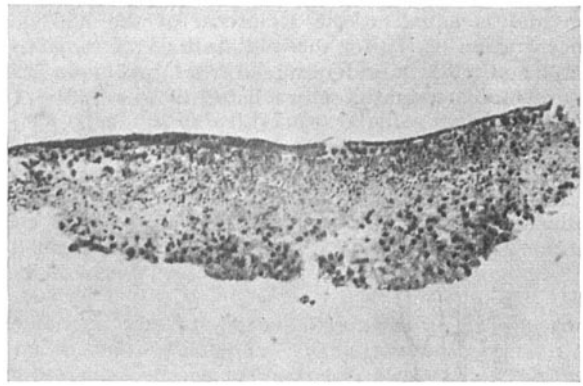

Fig. 1. Chick embryo skin grown in organ culture for 3 days after incubation in 5 mg/ml CTH for 1 h.

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