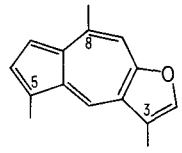
electronic spectrum was typically azulenic; λ_{max} (hexane) 280, 287, 292, 300, 315, 321, 361, 372, 380, 391, 563, 601, 612, 644sh, and 673 nm. Both UV/VIS and IR spectra are in excellent agreement with those of authentic linderazulene. The ^{1}H-NMR spectrum[4] (220 MHz, CCl_4) showed singlets for three aromatic methyl groups at δ 2.33(C-3),



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2.64(C-5) and 2.75(C-8), 3 isolated aromatic protons at 7.13(H-2), 7.28(H-9), and 8.10(H-4), and 2 coupled aromatic protons at 7.04 and 7.25 (each 1H, d, J 4Hz). Thus all the evidence agrees with structure (**I**). Furthermore the pigment formed a 1,3,5-trinitrobenzene complex, violet-black needles, m.p. 154–155 °C (154–155 °C)[3].

Naturally occurring azulenes have been found previously only in the liverwort *Calypogeia trichomanis*[5], the fungus *Lactarius indigo*[6], and possibly in the alga *Laurencia obtusa*[7].

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Studies on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by methylglyoxal[1]

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Summary. Glyceraldehyde-3-P dehydrogenase (E.C. 1.2.1.12) from rabbit muscle is rapidly inactivated by methylglyoxal following pseudo first-order kinetics. Substrate, as well as inorganic phosphate, affords protection, whereas NAD^+ is ineffective. The arginine residue implicated in the reaction is probably the anion binding site of the phosphate group of the substrate.

Several studies of the action of methylglyoxal (MG) on different cell functions have been carried out. Kun[2,3] has demonstrated that the ketoaldehyde inactivates succinate dehydrogenase, hexokinase and triose phosphate dehydrogenase; Kikuchi et al.[4] have studied the inhibition of L-glutamine: D-fructose 6-phosphate aminotransferase and recently, in our laboratory, the inactivation of fructose 1,6-P_2 aldolase from rabbit muscle was examined[5].

Materials and methods. Substrates, enzymes and coenzymes were purchased from Sigma Chemicals Co. Methylglyoxal (Fluka), distilled every time before use, was tested according to Racker's procedure[6].

Glyceraldehyde-3-P dehydrogenase (Sigma) from rabbit muscle, dialyzed overnight at 2 °C before use aganist large volumes of 40 mM triethanolamine (TEA) buffer (pH 7.6), was assayed in 50 mM pyrophosphate buffer pH 8.5, 2.5 mM EDTA, 7.5 mM sodium arsenate, 0.5 mM NAD^+ and 0.5 mM D-glyceraldehyde-3-phosphate. Protein concentration was determined according to Fox and Dandliker[7] and thiol groups according to Ellman[8]. The amino acid composition of the native and modified enzyme was determined after hydrolysis for 24 h in 6 N HCl containing 20 µl of mercaptoacetic acid at 105 °C. The hydrolyzates were taken to dryness and finally analyzed on a Spinco amino acid analyzer mod. 120 B, equipped with a high sensitivity cuvette[9].

Results and discussion. Glyceraldehyde-3-phosphate dehydrogenase is rapidly inactivated by methylglyoxal, following first-order kinetics. Saturation kinetics are observed if the first-order rate constant of inactivation is measured as a function of methylglyoxal concentration: when τ (the half-time of inactivation) is plotted vs 1/[MG], values of 2.6 mM and 0.075 min^{-1} were found for K_i and k_3 respectively: K_i is the dissociation constant for the initial reversible complex and k_3 is the rate constant for the conversion of the reversible complex to the irreversibly inhibited enzyme[10] (fig. 1). A double logarithmic plot of the reciprocal of the half-time of inactivation against inhibitor concentration[11,12] yields the reaction order of 0.94 with respect to inhibitor.

The effect of pH on the rate of methylglyoxal inactivation was examined in the pH range from 6.6 to 9.9. No maximal

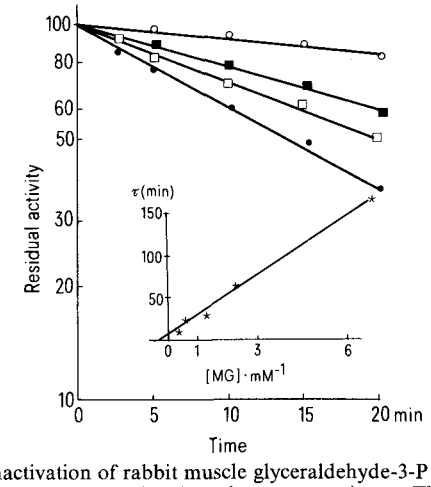

Fig. 1. Inactivation of rabbit muscle glyceraldehyde-3-P dehydrogenase at various methylglyoxal concentrations. The enzyme (0.015 mM) was incubated in 50 mM TEA buffer pH 7.5 with either 0.45 mM (○), 0.75 mM (■), 1.5 mM (□), or 2.25 mM (●) methylglyoxal. A control in absence of methylglyoxal was carried out under the same conditions. Insert: plot of the half-inactivation time (τ) vs the reciprocal of the inhibitor concentration.

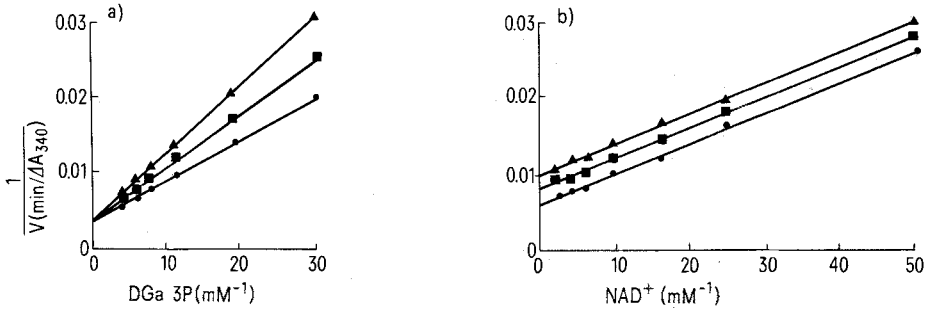

Fig. 2. The effect of substrate and coenzyme concentration on the activity of glyceraldehyde-3 phosphate dehydrogenase in the presence of methylglyoxal. *A* The enzyme (40 U/mg) was assayed directly in cuvettes containing 50 mM pyrophosphate buffer pH 8.5, 2.5 mM EDTA, 7.5 mM sodium arsenate, 0.5 mM NAD^+ in the presence of different amounts of D-glyceraldehyde-3-phosphate and 2.5 mM (■) or 5.0 (▲) methylglyoxal. An experiment without inhibitor (●) was carried out in the same conditions. *B* The enzyme activity was assayed in 50 mM pyrophosphate buffer pH 8.5, 2.5 mM EDTA, 7.5 mM sodium arsenate, 0.5 mM D-Ga3P as a function of NAD^+ concentration, in the absence (●) and in the presence of methylglyoxal 5.0 mM (■) or 8.0 mM (▲) respectively.

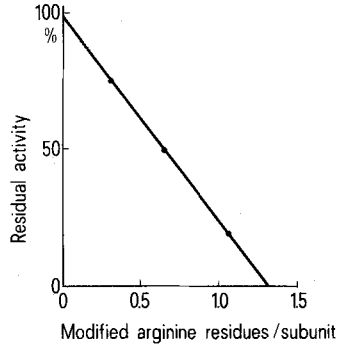

Fig. 3. Correlation between the inactivation of glyceraldehyde-3-phosphate dehydrogenase and the number of arginine residues modified by methylglyoxal treatment. The enzyme (0.025 mM) was incubated in 50 mM TEA buffer pH 7.5 (assay volume 1.0 ml) with 0.3 mM methylglyoxal. Reaction was stopped by adding suitable quantity of 6 N HCl. Reported values are the mean of the results of 3 samples.

rate was observed but inactivation rate increases with increasing pH. Glyceraldehyde-3-phosphate, stoichiometric to the inhibitor, affords a complete protection against inactivation, whereas the coenzyme has only a partial effect, independent of its concentration in the range 0.5–5.0 mM. Inorganic phosphate (36 mM) almost completely protects the enzymic activity. Kinetic studies showed that methylglyoxal is a competitive inhibitor with respect to glyceraldehyde-3-P and uncompetitive with respect to NAD^+ (fig. 2): such competition between triose phosphate and inhibitor suggests that the residue affected by modification is involved in the binding of the substrate. Methylglyoxal, therefore, can be considered an active-site directed reagent.

In order to show which amino acid is involved in the binding of the inhibitor, first of all titration of sulphydryl groups has been carried out, since it is known that glyceraldehyde-3-P dehydrogenase is a SH dependent enzyme. No difference was found between native and inactivated enzyme, and 4.0 SH groups/monomer were always titrated within the mixing time of Ellman's reagent. Addition of excess of mercaptoethanol and dithioerythritol did not restore the activity of the enzyme inactivated by methylglyoxal. These results, as well as the pH profile, which is not characteristic of the SH-dissociation, allow us to exclude the involvement of a SH moiety in the inactivation mechanism.

On the other hand, amino acid analysis showed that arginine is the only residue modified throughout the course of inactivation. Other residues did not appear to be affected by methylglyoxal treatment and in particular, no loss of lysine was detected in our determinations carried out on 5 different samples. In figure 3 a linear relationship between residual activity and loss of arginine residues is reported. In good agreement with kinetic data at complete inactivation, a loss of ~ 1.3 arginine residues/monomer has been demonstrated. It is possible to explain this result by assuming that a single most reactive arginine is responsible for the activity. The remainder of the label is more or less distributed among the other arginine residues present in the glyceraldehyde-3-phosphate dehydrogenase: more arginine residues than the essential one may be modified.

Similar results have been obtained by Nagradova's et al.[13,14] studies on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by 2,3 butanedione. In good agreement with our data, saturation kinetics and substrate protection of the holoenzyme against inactivation have been demonstrated. It also seems probable that in 2 cases the modified essential arginine residue is Arg-231. Moreover, data concerning the effect of NAD^+ can be explained by the fact that the conformation of the active site of the holoenzyme is different from that of the apoenzyme: NAD^+ enhances the rate constant of the inactivation of the apoenzyme, increasing the reactivity of the essential arginine residue, but partially protects the holoenzyme.

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