Acetoacetate Decarboxylase. Photochemical Oxidation Induced by Iodoacetate*

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ABSTRACT: Acetoacetate decarboxylase reacts only slowly and incompletely with iodoacetate in the dark, or when irradiated in the presence of iodoacetate under vacuum. However, when the enzyme and iodoacetate are irradiated in air, enzymic activity is destroyed. Experiments with iodoacetate labeled with ^{14}C and with ^{125}I show that little of these isotopes is incorporated during the oxygen-promoted photochemical inactivation, but the process is accompanied by oxidation of cysteine to cysteic acid.

Acetoacetate decarboxylase contains several free sulfhydryl groups in each subunit (Lederer *et al.*, 1966). In order to determine whether these sulfhydryl groups are essential to enzymic activity, an attempt was made to cause them to react with iodoacetate. Although reactions of sulfhydryl groups are best conducted in alkaline solution, the instability of the enzyme at high pH led to an attempt to conduct some experiments near neutrality. Early results were irreproducible, but it was eventually discovered that the inactivation of the enzyme in the presence of iodoacetate depends on light. Further investigation has shown that the inactivation of acetoacetate decarboxylase in the presence of iodoacetate is not caused by a carboxymethylation of the enzyme, but by a photochemical oxidation by air.

Experimental Section

Materials. Crystalline acetoacetate decarboxylase was prepared from *Clostridium acetobutylicum* by the method of Zerner *et al.* (1966). Iodoacetic acid labeled with ^{14}C was purchased from New England Nuclear Corporation, and recrystallized from hexane. Iodoacetic acid labeled with ^{125}I was prepared as follows: Carrier iodoacetic acid was added to a solution of 500 μc of $Na^{125}I$ (IsoServe, isotopic purity 98%) dissolved in 0.1 ml of 0.1 N sodium hydroxide. After 2.5 hr in the dark at 25°, 0.05 ml of 0.2 N HCl was added to neutralize the base, the solution lyophylized, and the residue extracted with, and twice recrystallized from, warm hexane. The yield of iodoacetic acid was about 50%, and the exchange was essentially complete between the inorganic iodide and iodoacetic acid. Acetopyruvic acid was prepared from the sodium salt of methyl acetopyruvate (Marvel and Dreger, 1932) by the method of Lehninger and Witzemann (1942). The compound was obtained in two modifications, plates and needles, both of which melted around 97–98° (lit. 97.7–98.5°). Other chemicals were reagent grade.

Methods. The enzyme was assayed spectrophotometrically at 270 mμ with a Zeiss spectrophotometer by a slight modification of the procedure of Fridovich and Westheimer (1962). All radioactive materials were counted with a Nuclear Chicago liquid scintillation counter, series 720. The scintillation media were the same as those previously described (Warren *et al.*, 1966). Counting efficiency for ^{14}C was determined with standard toluene from New England Nuclear Corp., and ^{125}I with material from the National Bureau of Standards. Irradiation was conducted with a G.E. sunlamp, filtered through Corning C. S. 0–51, 0–52 and 0–54 filters, 2-mm thick, to permit only wavelengths greater than 350, 320, or 300 mμ, respectively, to pass through. The lamp and the reaction tubes (20 × 150 mm Pyrex test tubes) were cooled with an air blower, so that the latter were at room temperature (23–27°). Dark reactions were conducted in tubes wrapped in aluminum foil.

Experiments in the absence of air were conducted in tubes about 20 cm long, with a side arm 5 cm long. The iodoacetate was introduced into the side arm, and the enzyme and buffer into the larger tube. The solutions were then degassed four or five times on a vacuum line, freezing the enzyme solution each time, and finally sealed under vacuum.

Amino Acid Analyses. Amino acid analyses were performed on a Beckman 120 B amino acid analyzer. Elution patterns were obtained for the standard amino acids (Calbiochem, A grade standard mixture), for *S*-carboxymethylcysteine (Mann), *N*-carboxymethylglycine (Aldrich), *N*-carboxymethyltyrosine, leucine, and alanine (prepared in these laboratories by Dr. Jules Shafer), and impure samples of *N*-carboxymethylserine and threonine, prepared by heating the amino acid with bromoacetic acid and sodium hydroxide solution. Cysteic acid and methionine sulfone were obtained from Calbiochem, A grade. Since cysteic acid is eluted near the solvent front on the Dowex 50 column

* From the James Bryant Conant Laboratory of the Chemistry Department, Harvard University, Cambridge, Massachusetts. *Received October 18, 1965.* This work was supported by Grant GM-04712 from the Institute of General Medical Studies of the National Institutes of Health.

of the amino acid analyzer, it was identified by paper chromatography with butanol–acetic acid–water solvent (4:1:5), followed by electrophoresis at pH 3.54 with pyridine–acetic acid–water solvent at 2000 v and in a direction at right angles to that for chromatography. The cysteic acid was visualized on the dried chromatograms with ninhydrin.

Results

The results of experiments with iodoacetate are reported in Tables I–VI below. Table I shows that the enzyme is not appreciably inactivated by 0.02 M iodoacetate at pH 5.9 except in the presence of light. Table II shows that the extent of photochemical inactivation increases with increasing intensity of illumination. Table III shows that dilute solutions of the enzyme are more readily inactivated by iodoacetate and light than more concentrated solutions. Table IV shows that relatively high concentrations of iodoacetate are required to effect the inactivation, and finally Table V shows that in the presence of iodoacetate and under illumination, the extent of inactivation is much less when the enzyme solutions have been carefully degassed.

TABLE I: Effect of Wavelength on the Photochemical Inactivation of Acetoacetate Decarboxylase by Iodoacetate pH 5.93.[a]

Enzyme (10^{-7} M)[b]	Iodoacetate (M)	% Activity In the Dark	After Irradiation at 300 mμ	320 mμ	350 mμ
4.5			100		
4.5	0.0198		45	57	78
4.5	0.0198	92.4			

[a] 0.2 M phosphate buffer for 10 min, 79 cm from light source. [b] Molarity of subunits, with assumed molecular weight of 33,000.

TABLE II: Effect of Light Intensity on the Photochemical Inactivation of Acetoacetate Decarboxylase by Iodoacetate pH 5.93.[a]

Enzyme (10^{-7} M)[b]	Iodoacetate (M)	% Activity after Irradiation at 30 cm	60 cm	90 cm	120 cm
3.4	...	100	100	100	100
3.4	0.0209	8	25	52	62

[a] 0.2 M phosphate buffer for 10 min, λ >300 mμ. [b] Molarity of subunits, with assumed molecular weight of 33,000.

TABLE III: Effect of Enzyme Concentration on the Photochemical Inactivation of Acetoacetate Decarboxylase by Iodoacetate pH 5.9.[a]

Enzyme (10^{-7} M)[b]	% Activity after Irradiation: No Iodoacetate	Iodoacetate (0.0205 M)
140	100	45
70	100	27
14	100	0

[a] 0.2 M phosphate buffer for 2 hr, 70 cm from source, λ >300 mμ. [b] Molarity of subunits, with assumed molecular weight of 33,000.

TABLE IV: Effect of Iodoacetate Concentration on the Photochemical Inactivation of Acetoacetate Decarboxylase by Iodoacetate 0.2 M Citrate–Phosphate Buffers.[a]

Enzyme (10^{-7} M)[b]	Iodoacetate (M)	pH	% Activity In the Dark	After Irradiation
3.2	0.0207	5.9	95	66
3.2	0.00207	5.9	98	94
3.2	0.000207	5.9	109	103
3.2	0.0197	7.21	100	43
3.2	0.00197	7.21	102	90
3.2	0.000197	7.21	100	99
3.2	0.0189	3.8	77	3.6
3.2	0.00189	3.8	92	8.4
3.2	0.000189	3.8	89	92

[a] For 30 min, 79 cm from light source, λ >320 mμ. [b] Molarity of subunits with assumed molecular weight of 33,000.

In order to study the cause of the photochemical inactivation by iodoacetate, the reaction was studied with iodoacetate labeled with ^{14}C and with ^{125}I, and the extent of the incorporation of radioactivity into the protein was measured. The reaction was also studied in the presence of acetopyruvate and in the presence of thiosulfate. Acetopyruvate was added to test an effective enzyme inhibitor, which can protect the enzyme against inactivation when an attempt is made to alkylate the enzyme by borohydride reduction in the presence of substrate (Fridovich and Westheimer, 1962; Warren *et al.*, 1966). Thiosulfate was tried on the assumption

TABLE V: Effect of Oxygen on the Photochemical Inactivation of Acetoacetate Decarboxylase by Iodoacetate.[a]

Enzyme (10^{-7} M)[b]	Iodoacetate (M)	Time (Hr)	% Activity: In the Dark, Evacuated	% Activity: In the Dark, Not Evacuated	% Activity: After Irradiation, Evacuated	% Activity: After Irradiation, Not Evacuated
4.3	...	1.0			100	100
4.3	...	2.0			100	100
4.3	0.020	1.0	66	101	61	4
4.3	0.020	2.0	69	89	51	0

[a] pH 5.9 (0.2 M phosphate buffer), 70 cm from light source, $\lambda > 300$ mμ. [b] Molarity of subunits, with assumed molecular weight of 33,000.

TABLE VI: Incorporation of ^{14}C and ^{125}I into Acetoacetate Decarboxylase on Irradiation in the Presence of Labeled Iodoacetate.[a]

Enzyme (10^{-7} M)[b]	Iodoacetate (M)	Acetopyruvate (M)	Thiosulfate[c] (10^{-4} M)	Irradiation	% Activity	Atoms Isotope per 34,000 mol wt units: ^{14}C	Atoms Isotope per 34,000 mol wt units: ^{125}I
93				$\lambda > 300$ mμ	100		
93		0.019		$\lambda > 300$ mμ	94		
93	0.0193			$\lambda > 300$ mμ	18	0.27	0.16
93	0.0193	0.019		$\lambda > 300$ mμ	93	0.077	0.031
93	0.0193			None	89	0.16	0.022
93	0.0193	0.019		None	88	0.11[d]	
93	0.0193		~8	$\lambda > 300$ mμ	86	0.12	0.034

[a] pH 5.9, 0.2 M phosphate buffer. Irradiation (when used): 2 hr, 70 cm from source. [b] Molarity of subunits, with assumed molecular weight of 33,000. [c] Average of four runs. The thiosulfate concentration was varied from 6.45×10^{-4} M to 1.16×10^{-3} M. [d] One determination only.

that iodine atoms, generated photochemically, might be responsible for inactivation. In order to make the experiment a significant one, the concentration of thiosulfate was restricted to one-twentieth that of the iodoacetate. Since thiosulfate is an active nucleophile with respect to iodoacetate, it can react stoichiometrically with it. If it were present in quantities equivalent to that of the iodoacetate, it might prevent inactivation by a trivial and irrelevant mechanism. The results are presented in Table VI. The amounts of radiocarbon and radioiodine incorporated into the enzyme are substantially below one atom per subunit, and the amount of radiocarbon introduced in the dark is an appreciable fraction of that introduced on irradiation. Therefore, the photochemical inactivation cannot depend upon carboxymethylation of the enzyme. Thiosulfate effectively prevents the inactivation of the enzyme on irradiation in the presence of iodoacetate. Since thiosulfate, present in low concentration, presumably reacts with iodine atoms, these experiments also suggest a mechanism for inactivation other than carboxymethylation.

Experiments with iodoacetamide showed that it is somewhat more effective than iodoacetate in the photochemical inactivation, whereas bromoacetate and chloroacetate are ineffective at wavelengths greater than 300 mμ. These data are consistent with a reaction initiated by absorption of light into the iodoacetate or iodoacetamide. Neither chloroacetate nor bromoacetate absorbs appreciably above 300 mμ, whereas iodoacetate (λ_{max} at 261 mμ) has an extinction coefficient of 79 at 300 mμ and of 16 at 320 mμ; the absorption of iodoacetamide is displaced relative to that for iodoacetate to longer wavelengths by about 6 mμ, with consequently higher extinction coefficients above 300 mμ.

The enzyme is also inactivated by low concentrations of iodine in the dark. In the presence of 6×10^{-5} M iodine, the enzyme (6.84×10^{-7} M in 0.2 M phosphate buffer at pH 5.9) retains only 19% activity after an hour. Furthermore, photolysis of iodoacetate does yield iodine; a solution of 0.0388 M iodoacetate, after 2-hr photolysis with light of wavelength greater than 300 mμ, had decomposed into iodine to an extent of about 0.1% (*i.e.*, enough decomposition to produce 5×10^{-5} M iodine). However, the amount of iodine

produced on photolysis with light of wavelength greater than 320 mμ was negligible. When a solution of iodoacetate was irradiated for 2 hr with light of wavelength greater than 320 mμ, and then added in the dark to acetoacetate decarboxylase in phosphate buffer at pH 5.9, the enzyme was inactivated only to the extent of 15%.

Enzyme which had been photochemically inactivated in the presence of iodoacetate was hydrolyzed and subjected to amino acid analysis. Only small amounts of carboxymethyl amino acids were found, but cysteic acid was produced in amounts from 20 to 30 times those produced (by accidental oxidation during analysis) in control experiments. As explained in the Experimental Section, the identity of cysteic acid was confirmed with paper chromatography and electrophoresis. Semiquantitative experiments suggested that the amount of cysteic acid formed in an inhibited enzyme approximated 0.7 mole per subunit.

Discussion

The data presented above show that the inactivation of acetoacetate decarboxylase by iodoacetate only occurs under illumination, and is ineffective except in the presence of air. Furthermore, even in air, thiosulfate prevents inactivation. The experiments with iodoacetate labeled with ^{14}C and ^{125}I show that the inactivation does not result either from carboxymethylation or iodination of the enzyme.

The action of light on alkyl iodides (Bates and Spence, 1931; Christie, 1958; Lampe and Noyes, 1954; West and Paul, 1932) and in particular the effect of air on the photochemistry (Hacobian and Iredale, 1950) suggest that the first reaction is the photochemical dissociation of the halide to a free radical and an iodine atom

$$ICH_2\text{–}CO_2^- \xrightarrow{h\nu} I\cdot + \cdot CH_2\text{–}CO_2^-$$

Both the iodine atom and the carboxymethyl radical are reactive species, which might attack the enzyme. Presumably one or both of these is swept from solution by thiosulfate, so that inactivation is prevented when that reagent is present during irradiation of the reaction mixture. A reasonable reaction which may be postulated for the iodine atom is attack at one of the sulfhydryl groups of the enzyme

$$E\text{–}SH + I\cdot \longrightarrow E\text{–}S\cdot + H^+ + I^-$$

In the absence of air, this reaction is insufficient to inactivate the enzyme, or at any rate quantitatively insufficient to inactivate it efficiently. But in the presence of air, the sulfhydryl radical may combine with oxygen, and may be oxidized, probably in a chain reaction, to the sulfonic acid group of cysteic acid.

The data do not lend themselves to the interpretation that the photochemical reaction leads to the production of iodine, which in turn inactivates the enzyme. Illumination of iodoacetate prior to introducing it into the enzyme solution does produce iodine which cause inactivation, but such inactivation is slight relative to that produced by photolysis of a solution of iodoacetate and enzyme. The importance of air in the photochemical decomposition of alkyl iodides was shown earlier by Hacobian and Iredale (*loc. cit.*) who found that the quantum yield of iodine on photolysis of iodoacetate is negligible in the strict absence of air, but approaches 0.5 in air. Similarly in our experiments, the oxidation of the enzyme could be initiated by iodine atoms but strongly promoted by air.

The photochemical oxidation of the enzyme in the presence of air inactivates the enzyme and converts a cysteine molecule to cysteic acid. The details of the role of the sulfhydryl group in the activity of this enzyme (or, alternatively, of the sulfonic acid group in preventing enzymic activity) are unknown, but protection of the enzyme by acetopyruvate suggests that a sulfhydryl group may be near the active site. At present, the generality of the discovery of a photochemical reaction of iodoacetate is uncertain. In previous examples of inactivation of enzymes by iodoacetate, some specific group in the enzyme (*e.g.*, a sulfhydryl group) has been carboxymethylated. Further, these reactions have proved reproducible, so that no photochemical reaction need be invoked or should be suspected. Nevertheless, the photochemical oxidation found here may prove applicable to other systems.

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