The Synthesis of Phenyl[2-³H]glyoxal

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(Received 16 October 1978)

A simple inexpensive method has been developed for the synthesis of [2-³H]acetophenone, which has been converted into phenyl[2-³H]glyoxal. The latter compound has been used to modify arginine residues in alkaline phosphatase from two sources, and also a sialidase.

Phenylglyoxal will selectively modify arginine residues in proteins (Takahashi, 1968) and the sites of modification can be studied by using phenyl-[1-¹⁴C]glyoxal (Armstrong *et al.*, 1976), which can be prepared by the oxidation of [1-¹⁴C]acetophenone by SeO₂ (Riley & Gray, 1947). However, [1-¹⁴C]acetophenone is expensive to purchase and is comparatively difficult to synthesize (Dauben *et al.*, 1950). We have synthesized [2-³H]acetophenone by a development of a method introduced for the alkylation of aliphatic carboxylic acids (Pfeffer & Silbert, 1970). In our synthesis the ³H originates from ³H₂O, considerably decreasing the cost of the isotopically labelled acetophenone. Moreover, under our labelling conditions few side reactions occur and hence the [2-³H]acetophenone can be obtained in high yield. Oxidation of the latter compound with SeO₂ leads to the formation of phenyl[2-³H]glyoxal and we have demonstrated the use of this reagent in the modification of arginine residues in some model enzymes.

Materials and Methods

n-Butyl-lithium was obtained from Ventron Alpha Products, Beverly, MA, U.S.A. and standardized by the method of Kofron & Baclawski (1976). Di-isopropylamine and hexamethylphosphoramide were obtained from Aldrich Chemical Company, Gillingham, Dorset, U.K. Phenylglyoxal was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli*, and sialidase (EC 3.2.1.18) from *Clostridium perfringens* were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The latter was purified by affinity chromatography by the method of Brossmer *et al.* (1977), followed by the method of Geisow (1975). Calf intestinal alkaline phosphatase was obtained from the Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Synthesis of [2-³H]acetophenone

To anhydrous tetrahydrofuran (2.5 ml) and diisopropylamine (0.49 g, 4.9 mmol) under an Ar

atmosphere was added 1.9 ml of 2.5 M-n-butyl-lithium in hexane and the mixture stirred for 30 min below 0°C. Acetophenone (0.27 g, 2.25 mmol) and tetrahydrofuran (1 ml) were then added and the mixture stirred for a further 15 min below 0°C after which hexamethylphosphoramide (0.9 ml, 5 mmol) was added. After 20 min, trifluoro[³H]acetic acid (0.273 ml, 2.4 mmol), prepared by treating ³H₂O (5 Ci/ml; sp. radioactivity approx. 100 mCi/mmol) with an equimolar amount of trifluoroacetic anhydride, was added and the reaction stirred for 2 h at room temperature (21°C). The reaction mixture was then acidified with 1 M-HCl at 0°C and the aqueous solution extracted with 3 × 20 ml of light petroleum (b.p. 40-60°C). The organic extracts were combined, washed with 5 × 10 ml of 1 M-HCl, dried and evaporated *in vacuo* to give [2-³H]acetophenone (0.27 g), which was not purified further, but was oxidized immediately. The position of isotope exchange was confirmed by ¹H n.m.r. spectroscopy with [2-³H]acetophenone prepared in an analogous manner from trifluoro[²H]acetic acid. The signal due to the acetyl group at 2.6 p.p.m. was greatly diminished.

Synthesis of phenyl[2-³H]glyoxal

To a solution of sublimed SeO₂ (0.27 g, 2.4 mmol) in dioxan (0.84 ml) and water (4.5 μl) at 50°C was added [2-³H]acetophenone (0.27 g, 2.25 mmol) and dioxan (0.5 ml). The mixture was heated at 80°C for 6 h and filtered through cottonwool, which was then washed with dioxan (20 ml). The filtrate and washings were evaporated *in vacuo* and dry ethanol (2 ml) was added to the resulting oily residue. After being left for 2 days at 5°C, the supernatant was carefully removed from the red precipitate that had formed and was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate (0.5 ml) and the phenyl-[³H]glyoxal purified by preparative silica t.l.c. with petrol/ethyl acetate (1:1, v/v) as solvent. The phenyl-[³H]glyoxal was eluted from the silica with ethyl acetate (50 ml), which was then concentrated *in vacuo* to 0.5 ml. The phenyl[³H]glyoxal was rechromato-

graphed as described above and the ethyl acetate removed *in vacuo* to leave an oily residue. Final traces of ethyl acetate were removed by repeated ($5 \times$) dissolution of the residue in ethanol (50ml) and evaporation *in vacuo*. Yield of phenyl[2- 3 H]glyoxal was 0.23 g (76%, based on $\epsilon_{250\text{nm}}$ of 9000 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ in ethanol) with sp. radioactivity 12.2mCi/mmol. The phenylglyoxal ran as a single u.v.-absorbing spot on silica t.l.c. with petrol/ethyl acetate (1:1, v/v) as developing solvent with the same mobility as authentic phenylglyoxal ($R_F = 0.33$). The ^1H n.m.r. phenyl[2- 3 H]glyoxal was identical with that of authentic material with a singlet at 9.55 p.p.m. due to the aldehydic proton. This singlet was absent in the n.m.r. spectrum of phenyl[2- 2 H]glyoxal, prepared as described above from [2- 2 H]-acetophenone, confirming the position of isotopic substitution. The phenyl[2- 3 H]glyoxal decomposed slowly (30% in 3 months) when stored as a 1M solution in dry ethanol at 4°C. Little decomposition occurred when the phenylglyoxal was stored as an oil or as the hydrate. The latter compound could be obtained by crystallization from a concentrated aqueous solution at 4°C.

Reaction of phenylglyoxal with enzymes

(a) *Inactivation.* The alkaline phosphatases and sialidase were treated at 25°C with various amounts of a solution of phenylglyoxal hydrate (16mg) in ethanol (100 μ l) as described in Table 1. Care was taken to use the same buffers for the inactivation as were used for the assay. Samples were removed after fixed times and assayed. After inactivation, the modified enzymes were dialysed at 4°C for 36h against the buffers indicated (3×5 litres), after which portions were removed and assayed.

(b) *Protection by substrate.* The three enzymes in the presence of their substrates were treated with unlabelled phenylglyoxal hydrate and then dialysed against assay buffer as described in Table 2. The modified enzymes were then treated with a 100-fold molar excess of phenyl[2- 3 H]glyoxal for 2h, the protein was precipitated on glass-fibre discs with trichloroacetic acid (Yang & Schwert, 1972) and their ^3H content determined.

Discussion

The oxidation of acetophenone by SeO_2 proceeds via the formation of an enol selenite ester that

Table 1. *Inactivation of enzymes with phenylglyoxal*

Enzyme	Buffer	Slow inactivation		Rapid inactivation	
		Molar ratio of phenylglyoxal to protein	Time for 75% inactivation (min)	Molar ratio of phenylglyoxal to protein	Time for 100% inactivation (min)
<i>E. coli</i> alkaline phosphatase* (mol.wt. 89000)	0.125M-NaHCO ₃ (pH7.5)	10^3	256	3.3×10^3	60
Calf intestine alkaline phosphatase* (mol.wt. 140000)	0.125M-NaHCO ₃ (pH7.5)	10^3	240	3.3×10^3	60
<i>C. perfringens</i> sialidase† (mol.wt. 56000)	0.1M-Sodium acetate (pH5.3)	6×10^4	180	1.6×10^5	96

* Assayed by the method of Ghosh & Fishman (1968).

† Assayed by determining sialic acid released from sialyl-lactose by the method of Warren (1959).

Table 2. *Protection of active sites of enzymes during rapid labelling by [^3H]phenylglyoxal*
The buffers used were the same as in Table 1.

Enzyme	Substrate	Molar ratio of phenylglyoxal to enzyme	Molar ratio of substrate to enzyme	Time for reaction (min)	Molar ratio of phenyl[2- 3 H]glyoxal to enzyme	Number of arginine residues/active site
Alkaline phosphatase* (<i>E. coli</i>)	Phenyl phosphate	10^5	10^5	5	4.23	2.12
Alkaline phosphatase* (calf intestine)	Phenyl phosphate	10^5	10^5	5	3.56	1.8
Sialidase	Sialyl-lactose	10^4	1.6×10^4	5	3.74	1.85

* In the presence of MgCl_2 (5mol/mmol of enzyme).

rearranges and then decomposes to phenylglyoxal, two protons being lost during the reaction sequence (Corey & Schaefer, 1960):

In the synthesis of phenyl[2-³H]glyoxal, the retention of ³H may be assisted by favourable isotope effects operating in the two proton-abstraction steps.

The phenyl[2-³H]glyoxal was used to determine the number of arginine residues in the active centres of two alkaline phosphatases and a sialidase. The interaction of the enzymes with unlabelled phenylglyoxal was investigated over a molar range of phenylglyoxal/enzyme of 10³–10⁵ (Table 1), assaying at fixed time intervals until the inactivation was constant. The enzymes were then dialysed and reassayed and in all cases the inactivation was irreversible with molar ratios of 3 × 10³ or greater. The rate of inactivation was less at pH 5.3 than at 7.5. Once the conditions for rapid inactivation had been determined, the inactivation was carried out on the enzymes in the presence of substrates. The concentrations of the latter enzymes were derived from the turnover number for each enzyme and were chosen so as to provide maximum protection of the active site. After 5 min the substrates and excess phenylglyoxal were removed by dialysis and the enzymes were treated with phenyl[2-³H]glyoxal (Table 2). The number of molecules of phenyl[2-³H]glyoxal that bound to each enzyme molecule were then determined. This value is twice the number of arginine residues modified (Takahashi, 1968) and hence the number of arginine residues at or near the

active centres of the enzymes can be calculated. Our values for calf intestinal (1.8) and *E. coli* (2.1) alkaline phosphatase are close to that (2) obtained by Daemen & Riordan (1974) for *E. coli* alkaline phosphatase. We found that approx. 2 arginine residues are involved in the active site of the sialidase from *C. perfringens*. No data on the involvement of arginine residues in the active site of this enzyme have been reported, although we have observed (Kabayo & Hutchinson, 1977) that arginine residues are essential for the activity of sialidase from *Streptomyces griseus*.

We thank the Ernest Oppenheimer Memorial Trust for financial support (B.W.A.) and Dr. V. W. Armstrong and Dr. B. T. Golding for helpful discussions.

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