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Analysis and γ-Irradiation of Polymer-Bound Catalase

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ABSTRACT

Preliminary results are given for a pyrolysis-gas chromatographic technique for estimating the enzyme content of insoluble, polymer-enzyme conjugates.
Samples of poly(diazostyrene)-bound catalase having known catalase contents were irradiated with ^{60}Co γ-rays at pH 7 in dilute, oxygenated, aqueous suspensions. Indices of radiation protection showed that the conjugation to insoluble polymer protected the enzyme from the effects of radiation more effectively than diglycylglycine. This protection is interpreted in terms of radical scavenging and the physical structure of the polymer-catalase conjugate.

INTRODUCTION

When enzymes are bound to polymeric supports by ionic or covalent bonding or by adsorption, they are usually rendered water-insoluble. Polymer-bound enzyme systems (polymer-enzyme

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conjugates, insolubilized enzymes) are of wide interest in technological and medical applications and as in vitro models of in vivo processes [1]. However, the effects of radiation on insolubilized enzymes have not been considered although such a study, as a model system, could be helpful in the investigation of irradiated tissues in which enzymes are bound to insoluble cell components [2].

In the present study, catalase (H_2O_2:H_2O_2 oxidoreductase 1.11.1.6), a widely occurring enzyme [3], was bound to poly(diazostyrene) and γ-irradiated in dilute, oxygenated, aqueous suspension.

For this investigation, an accurate, convenient, and rapid method of estimating the catalase content of the conjugate was required. The method should not be influenced by the presence of phenolic, arylamino, diazo, and related groups, and thus the Kjeldahl [4] or Folin [5] procedures could not be used. Methods based on acidic hydrolysis of the bound enzyme, followed by estimation of some or all of the resulting amino acids, although accurate and acceptable, are very time-consuming. Protein estimation from the difference in uv absorption of the solution of enzyme before and after the enzyme-polymer reaction is, in practice, often inaccurate. Consequently a method based on pyrolysis-gas chromatography was developed in which the enzyme content of the conjugate is determined from the ratio of the area of a peak arising from the enzyme to that of a peak from the polymer.

EXPERIMENTAL

Catalase was a lyophilized powder prepared from beef liver. Distilled water used in irradiations was purified by double distillation from alkaline permanganate in all-glass apparatus and irradiated for several weeks before use [6]. Styrene and p-cymene obtained from commercial sources were redistilled by standard methods before use. All other materials were AR grade.

Styrene was polymerized in solution with an equal volume of p-cymene and benzoyl peroxide (0.5% w/v) over a boiling water bath. The resulting polystyrene was isolated and purified by repeated dissolution in benzene and precipitation with methanol, before being thoroughly dried in vacuo; analysis conformed to the calculated values for C_8H_8 and the ir spectrum was identical to that of authentic polystyrene. The viscosity-average molecular weight was 76,600 [7].

Polystyrene, nitrated, reduced, and diazotized [8, 9], was thoroughly washed with phosphate buffer (pH 7). The resulting poly(diazostyrene) was reacted with catalase over 15 hr at 3-5°C in well-stirred phosphate buffer (pH 7). After reaction the supernatant liquid gave negative tests for catalatic activity (by effervescence in hydrogen peroxide) and protein (by the Biuret method [10]); control tests on solutions of catalase were positive.

The solids contents of suspensions of bound catalase were determined on aliquots of the stirred suspension by drying the washed, filtered solids to constant weight at 100°C.

Gas chromatography on pyrolysis products was performed with a Philips Series 4000 instrument incorporating a Curie Point pyrolysis unit. Dried particles of the insolubilized enzyme were clamped in a wire loop and pyrolyzed at 770°C for 10 sec. The products of pyrolysis were chromatographed as follows: column, 6 ft × 1/8 in. aluminum column packed with Porapak Q (100-120 mesh); column temperature, programed from 50 to 230°C at 10°C/min; carrier gas, nitrogen with a flow rate of 60 ml/min (measured at 50°C); detector, flame ionization. In order to calibrate the method, the ratio of the area of a peak arising from catalase to that of a peak from the polymer was determined at varying enzyme contents.

Insolubilized catalase was γ-irradiated in the ^{60}Co facility at the University of New South Wales. The dose rate from Fricke dosimetry, 160 krads/hr, after correction for natural decay, was used in calculations of the dose absorbed by samples of bound catalase. An aliquot (0.60 ml) of stirred suspension of the bound catalase in phosphate buffer (pH 7) was transferred with a wide-mouthed pipet to a borosilicate ampoule (i.d. 7 mm). The sample was diluted to 1.00 ml with phosphate buffer (pH 7) and oxygenated through a fine capillary for not less than 5 min. The ampoule was quickly sealed in the flame and irradiated at 15 ± 2°C for a carefully measured period. Irradiated samples were stored at 3-5°C for 24 ± 4 hr and then analyzed for residual catalytic activity by the method of Bonnichsen, Chance, and Theorell [11]; suspensions of bound catalase were transferred with wide-mouthed pipets. The activity was expressed as a percentage of the activity of an unirradiated sample held at 15 ± 2°C for a period comparable to that which samples were irradiated.

For assessment of the effects of irradiation, the D_{37} dose, the dose required to give a residual enzymic activity of 37%, was estimated from curves of residual activity vs dose. The inactivation yield, G, was calculated from Eq. (1) [12], where E_0 is the initial

$$G = E_0/D_{37} \text{ moles } l^{-1} \text{ rad}^{-1} \quad (1)$$

molar enzyme concentration in the suspension of bound catalase, and D_{37}, as defined above, is expressed in rads. Alternatively, there is Eq. (2). The protection quotient, Q, the ratio of the dose absorbed by the

$$G = \frac{(9.65E_0 \times 10^8)}{D_{37}} \text{ molecules/100 eV} \quad (2)$$

radiation protector (the polymeric support) to that absorbed by the enzyme for equal weights of each, was calculated from Eq. (3) [13],

$$Q = \frac{E_o'}{P} \quad \frac{(D_p - De)}{(D_e)} \tag{3}$$

where D_e is the dose giving 37% residual enzymic activity in the absence of protector, D_p is the dose giving the same degree of inactivation in the presence of protector, E_o' is the weight of enzyme/ml, and P is the weight of protector/ml.

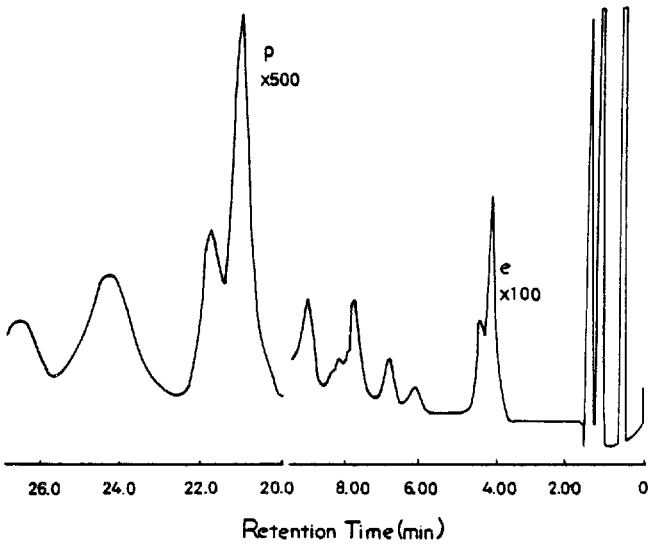

FIG. 1. Pyrogram from insolubilized catalase, showing peaks representative of the polymer and enzyme, p and e, respectively. Conditions: Mean weight ratio of enzyme to polymer, 0.106; pyrolyzed at 770°C for 10 sec, then chromatographed on 6 ft Porapak Q with 60 ml/min nitrogen. Temperature program: From 50 to 230°C at 10°C/min, then held at 230°C until completion of the chromatogram.

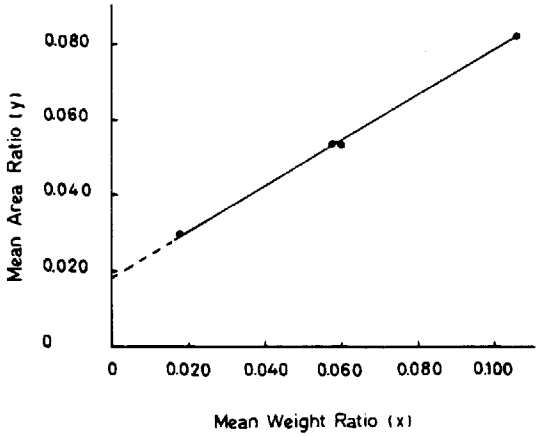

FIG. 2. Calibration curve for pyrolysis-gas chromatography. y is the mean area ratio of a peak representative of the enzyme to one representative of the polymer; x is the mean weight ratio of enzyme to polymer from ion-exchange chromatography of the hydrolysate of the bound catalase [7].

RESULTS

Pyrolysis-Gas Chromatography

A typical pyrogram of insolubilized catalase is shown in Fig. 1. The results of calibration of the mean area ratios of the chromatograms against protein content, determined by ion-exchange chromatography [7] are shown in Fig. 2; each value for the mean area ratio shown is the mean of 5-7 replicate pyrolyses.

Irradiation

The residual activities of samples of irradiated, insolubilized catalase are plotted as a function of absorbed dose in Fig. 3.

The parameters used to assess the radiation sensitivity of insolubilized catalase are summarized in Table 1.

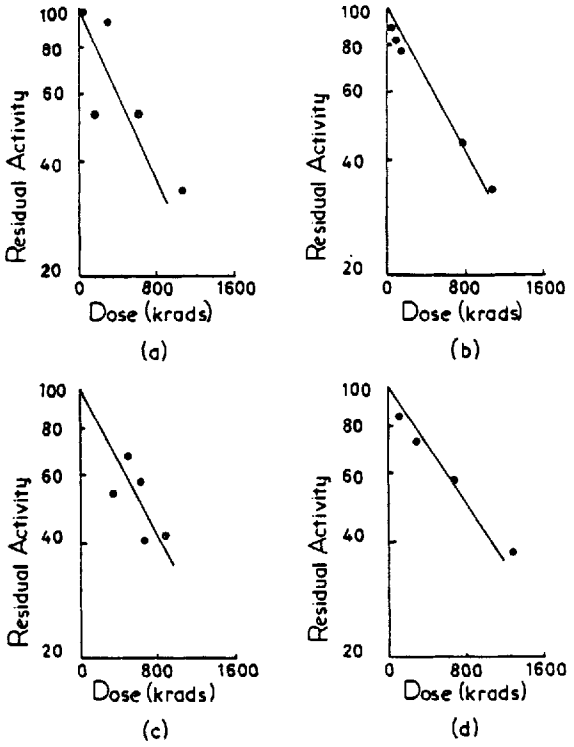

FIG. 3. Residual activity-dose curves for insolubilized catalase. The curves apply to samples with the following catalase concentrations: (a) 0.90×10^{-6} M, (b) 0.82×10^{-6} M, (c) 0.82×10^{-6} M, and (d) 3.9×10^{-6} M.

DISCUSSION

Pyrolysis-Gas Chromatography

Neither the design of the pyrolysis unit nor the physical nature of the conjugates allowed the pyrolysis of known weights of sample. As it is inherently more accurate, it was decided to use an internal standard for the estimation of the catalase content. Consequently, the results of the pyrolysis experiments were expressed as the ratio of the area of a peak representative of catalase to that of a peak

TABLE 1. Radiation Sensitivity of Catalase

	Catalase concentration ($M \times 10^6$)			
	0.90	0.82	0.82	3.9
Suspensions of Insolubilized Catalase				
Mean weight ratio, enzyme to polymer[a]	0.058	0.018	0.018	0.106
Weight of irradiated conjugate (mg)	4.0	12	12	10
D_{37} dose (krads)	780	900	910	1150
Protection quotient, Q[b]	0.73	0.29	0.29	0.45
Inactivation yield, G[b] (molecules/100 eV) $\times 10^3$	1.4	1.0	1.0	3.8
Solutions of Free Catalase[c]				
D_{37} dose (krads)	192	187	187	337
Solutions of Free Catalase and $(Gly)_3$[c,d]				
D_{37} dose (krads)	305	295	295	750
Protection quotient, Q[b]	0.028	0.026	0.026	0.11
Inactivation yield, G[b] (molecules/100 eV) $\times 10^3$		19.8		

[a]Taken from the results of Hourigan, Melrose, and Shaw [7].

[b]The phosphate buffer (pH 7) absorbed a total of 145 krads during irradiation [14] and the values of Q and G have been corrected accordingly.

[c]Taken from the results of Hourigan and Melrose [14].

[d]The results of irradiation of catalase solutions containing 20 mg/ml of diglycylglycine.

representative of the polymeric carrier, the latter serving as an internal standard.

Clearly, the accuracy and precision of the analysis would be facilitated if the chromatographic peaks representative of the carrier and enzyme were resolved, symmetrical, large, and not too different in size; this was achieved using the stationary phase Porapak Q under the conditions given in the Experimental Section.

As can be seen from Fig. 2, the mean weight ratio of enzyme to polymer, x, and the mean area ratio, y, are linearly related, the equation of the line being $y = 0.018 + 0.60x$.

The pyrolysis method was calibrated against the results of ion-exchange chromatography of the hydrolyzate of the bound enzyme because the latter is an accepted method.

On the basis of the preliminary results given here, the pyrolysis method shows promise as a technique for the routine estimation of the catalase content of conjugates prepared from the same sample of poly(nitrostyrene) so long as at least 5 replicate pyrolyses are performed on each sample. Relatively, this is not inconvenient as a pyrolysis can be completed in about $\frac{1}{2}$ hr and contrasts with the established ion-exchange method which takes, in all, about 2 days (on specialized equipment).

Irradiation

The significance of the γ-irradiation of suspensions of polymer-bound catalase can be seen when the results are compared with those obtained by the γ-irradiation of oxygenated, dilute, aqueous solutions of free catalase [14] (see Table 1). The several indices of radiation sensitivity show that the conjugation to insoluble polymer has protected the enzyme from the effects of γ-irradiation more effectively than diglycylglycine which protects catalase by a combination of radical scavenging and repair processes [14].

The most significant radiation-produced radicals in aqueous solution are $OH^{\cdot}$, $H^{\cdot}$, and e_{aq}^{-} [15]. Unfortunately, the reactivity of these radicals with poly(diazostyrene) has not been investigated, previously; however, their reactivity can be inferred from studies on comparable low molecular weight compounds. The electrophilic radicals, $OH^{\cdot}$ and $H^{\cdot}$, are so reactive with benzene derivatives in aqueous solution (rate constants of the order of 10^{9} to 10^{10} M^{-1} sec^{-1}) that the nature of the substituent in the aromatic ring has little influence on the relative reactivity [16-18]. The nucleophilic hydrated electron, e_{aq}^{-}, is very reactive toward benzene derivatives containing electron-withdrawing substituents [19] and would therefore be expected to be readily scavenged by poly(diazostyrene).

Nakken [20] has suggested that the effectiveness of radical scavenging protectors could be enhanced by linking the protector to the target compound. This provides a possible explanation for the radiation protection afforded by the insolubilization of catalase.

Further, in poly(diazostyrene)-bound catalase, the surface of the catalase molecule is apparently grafted by many insoluble poly(diazostyrene) chains (up to 100) [7]. Consequently, the protection of the catalase molecule from radiation-produced radicals should be enhanced by this physical arrangement.

In addition, it is possible that the binding to polymer has induced protection for the catalase by stabilizing it against the conformational changes and denaturation which often follow the attack of radiation-produced radicals on proteins [21, 22].

The γ-irradiation of poly(diazostyrene)-bound catalase is an in vitro model of the situation existing during the irradiation of living cells in which enzymes are bound to cell components within a heterogeneous system. As such, the results of this study have significant implications in radiobiology.

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