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# Irradiation-induced Coupling of Enzymes to Solid Phase Carriers

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Coupling of various enzymes to agarose gels, dextran gels, and polystyrene particles has been accomplished by $\gamma$-irradiation of a suspension of the carrier in the presence of enzyme. The enzymes studied were trypsin, pepsin, and ribonuclease. The main advantage with the procedure compared to the presently available coupling methods is that it may be used under a wide variety of conditions and with many different materials. The main disadvantages are the low amounts of fixed protein obtained and that some radiation damage occurs.

The effects of irradiation of ribonuclease in an agarose gel suspension and in aqueous solution were compared. In the presence of gel less aggregation of ribonuclease occurred compared to irradiation in free solution.

A great number of methods for the immobilization of biologically active substances have been developed in recent years.[1-3] Most attention has been paid to the development of methods for irreversible covalent fixation and a variety of organo-chemical reactions have been employed. However, the presently available methods have their limitations in one or several respects. The chemical reactions used generally work in a limited range of pH and are dependent on special functional groups in the carrier and the substance to be coupled. Therefore there is still need for more development work in this field.

Recently it was reported [4] that covalent coupling of dyes to macromolecules could be accomplished by $\gamma$-irradiation of water solutions containing both components. This tempted us to study the possibility of coupling proteins to solid phase carriers using radiation and further to seek more information about radiation chemistry in gel systems.

We report here a new simple method, whereby the coupling of enzymes to solid phase carriers can be performed by $\gamma$-irradiation of the carrier in the presence of enzyme. In this paper we also report some differences obtained upon irradiation of an enzyme in free solution and in a gel system.

## MATERIALS AND METHODS

The enzymes trypsin, pepsin, and ribonuclease were obtained from Sigma Chemical Company. Tosylarginine methyl ester (TAME), cytidine-2′,3′-cyclic monophosphate, RNA and hemoglobin (type II) were also from Sigma Chemical Company. Agarose gel (Sepharose 4B) and dextran gel (Sephadex G-150) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and latex particles from Serva Feinbiochemica (Heidelberg, Germany).

Irradiation was performed in a $\gamma$-cell containing $^{60}Co$. The temperature in the cell was about 20 °C and the dose rate was 30 rad/s. About 10 ml of gel was irradiated in a closed glass tube. Unless otherwise stated the gels were initially in equilibrium with air.

Washing of the gels after irradiation was performed in a small column with various buffers. The pepsin-Sepharose conjugate was extensively washed with the following solutions in the order indicated: distilled water, 0.1 M acetic acid pH 3.0 containing 1 M KCl, 0.1 M sodium acetate buffer pH 5.0 containing 1 M KCl, and 0.002 M acetic acid-sodium acetate buffer pH 4.0. The trypsin-Sephadex and the ribonuclease-Sepharose conjugates were washed with water, 0.1 M acetic acid pH 4.0 containing 1 M KCl, 0.1 M sodium bicarbonate buffer pH 8.5 containing 1 M KCl, and finally with distilled water. The polystyrene latex particles were extensively washed by repeated centrifugation using the following buffers: 0.1 M sodium formate buffer pH 3.0 containing 1 M KCl and 20 % ethanol, 0.1 M sodium bicarbonate buffer pH 9.2 containing 1 M KCl and 20 % ethanol, and finally with distilled water.

Determination of the degree of coupling was made by amino acid analysis. After careful washing with various buffers, the gels were dehydrated by washing with acetone and then dried by heating at 70 °C for 24 h. A suitable amount of dried gel was weighed and then mixed with 6 M HCl and hydrolyzed for 24 h at 110 °C in an evacuated glass tube. The amino acid analyses were performed on a Beckman Model 120 B analyzer.

Leakage and stability tests of the enzyme conjugates were done by the following procedure. The washed conjugate was stored for 1 week at 4 °C in 0.005 M acetic acid – sodium acetate buffer pH 4.7 containing 0.02 M sodium azide. The conjugate was then filtered off and washed. Both the filtrate and the conjugate were then assayed for enzymatic activity to determine the stability of the conjugate.

The activity of trypsin was measured by following the hydrolysis of 0.01 M TAME in 0.02 M $CaCl_2$ – 0.04 M NaCl in a titrator (Radiometer, Copenhagen).

The activity of pepsin was measured by adding the enzyme to a 2 % hemoglobin solution and following the hydrolysis at pH 3.0. The mixture was stirred and aliquots were removed at various times. The protein was precipitated with trichloroacetic acid followed by centrifugation. The absorption at 280 nm of the supernatant was measured.

The activity of ribonuclease was determined by following the hydrolysis of cytidine-2',3'-cyclic monophosphate [5] in 0.1 M KCl at pH 6.9 in a titrator or by the spectrophotometric method of Kunitz [6] using RNA as substrate.

## RESULTS

*Coupling of trypsin to dextran gel.* Swollen Sephadex G-150 gel samples in 0.1 M acetic acid-sodium acetate buffer pH 4.7 containing 0.04 M $CaCl_2$ and various concentrations of trypsin were irradiated with 1 Mrad of $\gamma$-radiation. After careful washing, the amount of fixed protein and the esterolytic activity against TAME at different pH-values were determined for each conjugate. It was found that pH for optimum activity was 7.7 for the free enzyme and 9.3 – 9.5 for the enzyme conjugates. This type of pH-shift has earlier been shown to occur with enzymes chemically coupled to gel matrices.[7–9] It probably means that pH-equilibrium is not obtained in the gel particle. The release of $H^+$ during the reaction is rapid and causes a lower pH in the interior of the gel particle than in the external solution.[8] The ratio of the activity of bound to free enzyme was calculated for each conjugate on the basis of the activity at pH-optimum. The results obtained are shown in Table 1. It is evident that an increased protein concentration during irradiation results in lower amounts of fixed protein, but gives an adduct with a higher specific activity.

*Table 1.* Coupling of trypsin to Sephadex G-150 gel and the enzymatic activity of the conjugates.

| Enzyme concentration (mg/ml) | Amount of fixed protein (mg/g dry conjugate) | Activity ratio bound to free enzyme (%) |
|---|---|---|
| 2 | 10 | 12 |
| 10 | 8 | 21 |
| 30 | 7 | 27 |

In a separate experiment Sephadex G-150 gel was irradiated as described above but in the absence of trypsin. Immediately after irradiation trypsin was added to give a final concentration of 10 mg trypsin/ml gel and the mixture was incubated for 24 h at room temperature. Amino acid analysis of a part of the gel after washing and drying showed that no protein had coupled to the gel.

An experiment was also performed, where Sephadex G-150 gel in the presence of 10 mg trypsin/ml was irradiated as described above but in the absence of oxygen. Deoxygenation was accomplished by allowing a stream of oxygen-free argon to pass through the solution for 2 h. The same degree of coupling and also the same activity of the coupled enzyme were obtained as compared to aerobic irradiation.

*Coupling of pepsin to agarose gel.* Sepharose 4B gel containing 1.0 mg pepsin/ml gel in 0.1 M acetic acid-sodium acetate buffer pH 4.0 was irradiated with 0.5 Mrad. After careful washing part of the gel was dried and subjected to amino acid analysis. The amount of fixed protein was 7.1 mg pepsin/g of dry gel corresponding to about 16 % of the pepsin present in the gel during irradiation. The enzymatic activity of a part of the gel was determined at pH 3.0 using hemoglobin as substrate. On the basis of the activity for the free and the coupled enzyme at pH 3.0, it could be calculated that the relative activity was 14 %. Stability test on a part of the gel was done after storage and repeated washing. No leakage

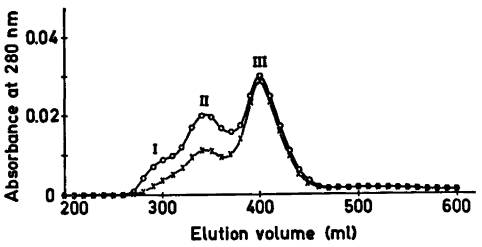


*Fig. 1.* Elution diagrams of Sephadex G-50 gel filtrations (column 3.2 × 90 cm) of ribonuclease irradiated in 0.1 M Tris-HCl buffer pH 7.4 in the presence of Sepharose 4B gel and in free solution.
Elution patterns: ( × ) irradiation in the presence of gel: (O) irradiation in free solution.

of enzyme or loss of enzymatic activity of the conjugate could be detected.

*Effects of irradiation on the system ribonuclease-agarose gel.* In order to obtain more information about the reactions taking place in protein-agarose gel systems upon irradiation, a comparative study was made in which ribonuclease was irradiated in agarose gel suspension and in dilute solution. A sample of 4 ml settled Sepharose 4 B gel in 0.1 M Tris-HCl buffer pH 7.4 containing 1 mg ribonuclease/ml gel and a sample of 4 ml of a solution containing 1 mg ribonuclease/ml of 0.1 M Tris-HCl buffer pH 7.4 were irradiated with 0.5 Mrad of $\gamma$-radiation. After irradiation the gel sample was eluted and the eluate was run on a Sephadex G-50 column. The sample irradiated in the absence of gel was also subjected to gel filtration on Sephadex G-50 using 0.1 M ammonium acetate buffer pH 4.7 for elution. Fig. 1 shows the elution diagrams obtained for the two samples. Fraction III in the figure eluted at the same volume as native ribonuclease. By comparison with a calibration curve, it was found that fraction II eluted at the volume expected for the dimer of ribonuclease. The composition of the two samples, with respect to the various products formed, was calculated from the UV-absorption values of the two elution diagrams. The enzymatic activities of the various fractions were determined using RNA as substrate. The specific activities of the various fractions compared to native ribonuclease were calculated and the results obtained are shown in Table 2. The values given are approximate

*Table 2.* The composition of ribonuclease with respect to the various products formed during irradiation in the presence or absence of Sepharose 4B gel, and the specific activity of the products compared to native ribonuclease.

| Product | Fraction of products (%) | Specific activity compared to native (%) |
|---|---|---|
| In the presence of gel | | |
| Polymer | 6 | 0 |
| Dimer | 27 | 6 |
| Monomer | 67 | 44 |
| In the absence of gel | | |
| Polymer | 10 | 0 |
| Dimer | 37 | 8 |
| Monomer | 53 | 46 |

since peak division is difficult and there may be differences in the extinction coefficients of the different types of material. Despite this, it is evident that both dimer and larger aggregates are formed upon irradiation both in the presence and absence of gel, but that more aggregates are formed in the absence of gel. The Sepharose 4B gel from the experiment described above was further analyzed for protein content and enzymatic activity. The gel was extensively washed and a part of it was subjected to amino acid analysis. The results obtained showed that 20 % of the ribonuclease present in the gel during irradiation had been coupled to the gel. Activity determinations on the gel using cytidine-2′,3′-cyclic monophosphate as substrate showed that the activity was 29 % compared to the activity of the same amount of enzyme in free solution. The pH for optimum activity was about the same as for the free enzyme. Stability test of a part of the gel was done after storage and repeated washing. No leakage of enzyme or loss of enzymatic activity of the conjugate could be detected.

*Coupling of ribonuclease to polystyrene particles.* A 10 % suspension of polystyrene latex particles (diameter 0.81 $\mu$m) in 0.1 M Tris-HCl buffer pH 7.4, containing 10 mg ribonuclease per ml of suspension, was irradiated with 1 Mrad of $\gamma$-radiation. The latex particles were then extensively washed and the enzymatic activity towards cytidine-2′,3′-cyclic

monophosphate was determined on a part of the latex suspension. It was found that the pH-optimum for particle-bound ribonuclease was about the same as for the free enzyme. A part of the suspension was dried and subjected to amino acid analysis. The result obtained shows that 6 mg ribonuclease was coupled per gram of latex particles. The activity of coupled enzyme compared to the same amount of free enzyme was 14 %. A part of the enzyme conjugate was subjected to stability test after storage and repeated washing. No leakage of enzyme or loss of enzymatic activity of the conjugate could be detected.

In a control experiment a 10 % suspension of latex particles containing 10 mg ribonuclease per ml of suspension was treated as described above but not subjected to irradiation. No enzymatic activity towards cytidine-2′,3′-cyclic monophosphate could be detected in this sample.

## DISCUSSION

The results show that it is possible to couple proteins to solid phase carriers using $\gamma$-radiation. It had been previously shown that $\gamma$-irradiation of solutions containing macromolecules and dyes [4] induced formation of macromolecule-dye adducts. It has also been shown that irradiation of solutions containing mixtures of proteins gives some hybrid molecules.[10] The mechanisms for these coupling reactions are probably very similar. On irradiation of a gel-protein system, radicals will be formed both in the gel matrix and in the protein. These radicals may be primary, that is, the result of direct ionization, or secondary, as the result of a reaction between the component and radicals from the solvent. The radicals formed are usually very reactive and special emphasis can be placed on the radical – radical reactions, which are very fast. It is probable that the actual coupling reaction is a radical – radical reaction as suggested below,[4] where a sequence of reactions is given in simplified form (MH = matrix; PH = protein).

$$MH \rightarrow M\cdot + H\cdot \quad (1a)$$

$$PH \rightarrow P\cdot + H\cdot \quad (1b)$$

$$P\cdot + M\cdot \rightarrow MP \quad (2)$$

The coupling reaction (2) would result in a covalent bond between the protein and the matrix, which is in good agreement with the observed stability of the conjugates formed. The adduct formation according to reaction (2) is, of course, not the only possible fate of the M· and P· radicals. They can, for instance, react with OH radicals from water yielding chemically altered protein or matrix. Reaction (2) can probably be regarded as very rapid but it is also clear that it is strongly dependent on the concentrations of the two radicals. Thus, the intensity of the irradiation should be of importance for the coupling yield. The amino acid residues in the protein participating in the coupling process are probably mainly the radiosensitive, sulfur-containing and aromatic amino acids. The degree of exposure of the amino acid residues to solvent is probably also important.

During the past ten years considerable effort has been made to develop methods for coupling proteins and other substances to solid phase carriers. At present there are many such methods available. However, these are limited in one or several respects, for example a limited range of pH, carrier, *etc.* Irradiation coupling method has the advantage that it can be used under a wide variety of conditions. Radical reactions are usually less sensitive to the environmental conditions than other types of reactions, allowing a broader working range. Another advantage of irradiation coupling is that it probably is possible to use many different carriers. Only a few carriers have been studied in this investigation, but it is likely that irradiation coupling can be performed with most kinds of organic material since formation of radicals by $\gamma$-irradiation is a property shared by all organic materials. There are two disadvantages with the method. One is that the coupling yields are fairly low, the other is that a certain amount of radiation damage [11] occurs both to the matrix and to the protein. Those disadvantages may be important for some applications but not for others.

Preparation of immobilized enzymes by use of electron-beam irradiation [12] and also by $\gamma$-irradiation [13,14] has been reported earlier. However, in these cases enzymes were entrapped in a gel matrix formed during irradiation and

the possibility of covalent fixation of the enzyme to the gel was not thought to occur.[13] A disadvantage with enzyme conjugates prepared in that way is the low specific activity towards high molecular weight substrates because of restricted diffusion of high molecular weight substances in the gel. Another disadvantage is the difficulty of eliminating enzyme leakage using the entrapment method.[14]

The comparison between irradiation of ribonuclease in solution and in agarose gel shows that less aggregation occurs upon irradiation in a gel. The explanation is most probably that in the absence of gel the formed ribonuclease radicals can react with each other and aggregates are formed. In the presence of gel, however, many ribonuclease radicals will react instead with radicals in the gel matrix. Thus, less aggregates are formed and coupling of ribonuclease to the gel matrix occurs. Less aggregation in the presence of gel may also be due to protection by the carbohydrate against attack by solvent free radicals on the ribonuclease.

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## REFERENCES

1. Silman, I. and Katchalski, E. *Annu. Rev. Biochem. 35* (1966) 873.
2. Porath, J. and Kristiansen, T. In Neurath, H. and Hill, R. L., Eds., *The Proteins*, Academic, New York 1975, Vol. 1, p. 95.
3. Zaborsky, O. R. In Weast, R. C., Ed., *Immobilized Enzymes*, CRC Press, Cleveland, Ohio 1973.
4. Andersson, L.-O. *Nature (London) 222* (1969) 374.
5. Josefsson, L. and Lagerstedt, S. *Methods Biochem. Anal. 9* (1954) 39.
6. Kunitz, M. *J. Biol. Chem. 164* (1946) 563.
7. Porath, J., Axén, R. and Ernback, S. *Nature (London) 215* (1967) 1491.
8. Axén, R. and Ernback, S. *Eur. J. Biochem. 18* (1971) 351.
9. Gabel, D., Vretblad, P., Axén, R. and Porath, J. *Biochim. Biophys. Acta 214* (1970) 561.
10. Radola, B. J. *Biochim. Biophys. Acta 160* (1968) 469.
11. Augenstine, L. G. *Adv. Enzymol. 24* (1962) 359.
12. Maeda, H., Suzuki, H. and Yamauchi, A. *Biotechnol. Bioeng. 15* (1973) 827.
13. Maeda, H., Yamauchi, A. and Suzuki, H. *Biochim. Biophys. Acta 315* (1973) 18.
14. Maeda, H. and Suzuki, H. *Biotechnol. Bioeng. 17* (1975) 119.

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