awaits definitive biochemical and biological studies.

The control diet at Darrah Springs, diet 8, contained 19 percent cottonseed meal. In contrast, diet 9 (liver and cottonseed meal) contained 37 percent cottonseed meal when calculated on a dry-weight basis. Yet at the end of 10 months, 40 out of 50 trout in the group fed on diet 8 had gross hepatomas, but neither gross nor microscopic tumors were found in the 50 trout sampled from the group fed on diet 9. At the end of 23 months, among 65 fish fed on diet 9 only 3 gross tumors were found. These observations suggest that liver markedly inhibits the hepatoma-inducing effect of the cottonseed meal, but does not completely suppress it. Nakahara *et al.* (*10*) have demonstrated the inhibitive effect of a diet containing liver on hepatoma induction by butter yellow (dimethyl-amino-azobenzene) in rats, and a similar effect may be operating in this instance.

The results obtained with diets 2 and 4 obscure the significance of the four microscopic tumors found in diet 6. With the omission of wheat middlings in diet 4, the incidence of hepatomas was no lower than in the control group; with diet 2, no hepatomas were found, although the diet included 25 percent wheat middlings. For these reasons, we believe that this component played no significant role in the outbreak of hepatomas in California during 1960, when the incidence of tumors in very young fish was extremely high (*11*).

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Antigens and Enzymes Made Insoluble by Entrapping Them into Lattices of Synthetic Polymers

Abstract. *Biologically active, insoluble products have been prepared from soluble antigens or enzymes by entrapping them into the lattice of an insoluble, highly cross-linked synthetic polymer as it forms. Antigens, made insoluble, removed quantitatively certain or all antibiotics from complex mixtures. Seven enzymes so treated are described.*

Soluble antigens have previously been rendered insoluble either by coupling the proteins with diazotized cellulose derivatives (*1*) or by linking them to the acid chloride of a carboxylated ion-exchange resin (*2*). Also, insoluble forms of amylase, pepsin, carboxypeptidase, and ribonuclease have been obtained by coupling with diazotized polyaminostyrene (*3*), and insoluble ribonuclease was obtained by absorption on a Dowex-50 cation-exchange resin (*4*). Tryspin (*5*) and papain (*6*) were transformed into insoluble derivatives by the introduction of polytyrosyl peptide side chains. This reaction is not applicable without considerable modifications to other proteins, or protein mixtures. Insoluble proteins obtained by coupling with diazotized polymers or by attachment to ion-exchange resins either suffered partial denaturation during these reactions or exhibited partial reversibility of the absorption, under certain conditions (*5*).

We have obtained biologically active, insoluble forms of antigens, enzymes, and other macromolecular material (for example, amylopectin), by mechanically entrapping the soluble macromolecular product into the lattice of a highly cross-linked synthetic polymer by polymerizing certain synthetic monomers in aqueous solution in the presence of the biologically active macromolecular substance to be embedded. Cross-linked polyacrylamide, which polymerizes in an aqueous medium and which has been used as a supporting medium for zone electrophoresis (*7*), is eminently suited for this purpose. We have used two methods for rendering proteins or other macromolecular biologically active material insoluble, depending on the purpose for which the resulting products are to be used. For rendering proteins or other antigens insoluble (procedure I) 50 mg of antigen are added to 200 mg of acrylamide, 120 mg of *N,N'* methylenebisacrylamide, 5.6 mg of tetramethylethylenediamine, 0.5 mg of Al $NH_4(SO_4)_2 \cdot 12\ H_2O$, and 1 ml of (1*M*) tris buffer, *p*H 8.6, all diluted to 11 ml with water. At the last, 2.5 mg of potassium persulfate are added. For rendering enzymes insoluble (procedure II), 2 mg of the enzyme are added to a mixture similar to the one described for procedure I except that the acrylamide is omitted and the amount of the potassium persulfate added is 5 mg.

In both procedures, the mixture is kept for 1 hour at 35°C without agitation after the polymerization catalyst (potassium persulfate) has been added. The insoluble synthetic polymer thus formed is dispersed mechanically, centrifuged for 30 minutes in a Sorvall centrifuge, model RC-2, rotor SS-34, at 17,000 rev/min (34,000*g*), and is washed by mixing it with 20 ml water and subsequent centrifugation. A total of three washings for procedure I and 16 washings for procedure II are necessary.

Table 1. Activity of crystalline enzyme (*9*) entrapped in the synthetic polymer preparation and the residual activity remaining in solution after the removal of the synthetic polymer. The "entrapped" activity is the percentage of total amount of enzyme present during the polymerization reaction. The "residual" activity is measured by first incubating the substrate at 25° to 35°C with insoluble enzyme for periods ranging from 25 to 180 minutes, cooling the mixture to 0°C, and removing the insoluble enzyme by centrifugation and subsequent filtration, and incubating the remaining solution at 25° to 35°C for 18 to 22 hours. Residual enzyme activity is expressed in percent activity per unit of time before centrifugation.

Enzyme	Activity	
	Entrapped (%)	Residual in supernatant solution (%)
Trypsin *	4–5.5 †	0.65
α-Chymotrypsin *	4.5	0.77
Papain *	3.4–6 †	0.57
α-Amylase ‡ §	1.9	0.7
β-Amylase || §	6.55	0.3
Ribonuclease ¶	4.6	0.89
Aldolase * *	4.2	0.52

* Determined by the casein digestion method (*10*). † Two separate experiments. ‡ From hog pancreas. § Determined by the reductometric method with dinitrosalicylic acid reagent (*11*). || From sweet potatoes. ¶ From beef pancreas; activity determined by the spectrophotometric method (*12, 13*). ** From rabbit muscle; activity determined by the method of Taylor *et al.* (*14*).

The first procedure (I) yields a gel-like, insoluble synthetic polymer which can be easily and completely removed from the suspension by centrifugation; because of its particular mechanical properties, this material is not suitable, however, for accurately measuring out or pipetting samples of its suspension. The second procedure (II) results in a flocculent product which lends itself well to quantitative measurement, but separation of the solid from the liquid phase is more difficult.

To use insoluble antigens for the absorption of antibodies, the solid material obtained after the last washing (procedure I) is mixed with 1 ml of an antiserum (for example, rabbit antiserum against whole human serum), and the suspension is kept under constant agitation (by means of a Boerner oscillating platform) for 2 hours at room temperature. The insoluble antigen is then removed by centrifugation at 34,000*g*, and the protein concentration of the remaining solution is adjusted to that of the antiserum before treatment by lyophilizing and dissolving the residue in water.

The photographs of the antibody-antigen reactions in Fig. 1 show that antibodies against a mixture of a large number of antigens (whole human serum) can be quantitatively removed by this method (Fig 1*A*, upper and lower right), while the antibody against an additional antigen, which was absent from the mixture of insoluble antigens used (bovine serum albumin), is totally unaffected (Fig. 1*A*, upper and lower left, lower right). Inversely, a single antibody can be selectively removed from a complex mixture of antibodies (against whole human serum and bovine serum albumin) when its pure antigen (crystalline bovine serum albumin) is available (Fig. 1*C*).

For reactions with insoluble enzymes, a portion of the suspension of insoluble enzyme (procedure II) is mixed with the appropriate substrate and buffer solutions, and the mixture is incubated under continuous agitation by means of a Boerner oscillating platform. The enzyme reaction is arrested by centrifugation of the mixture at 34,000*g* and subsequent filtration of the supernatant solution through a Whatman No. 1 filter.

Between 2 and 6 percent of the enzyme activity, present during the polymerization reaction, appeared in the exhaustively washed synthetic polymer

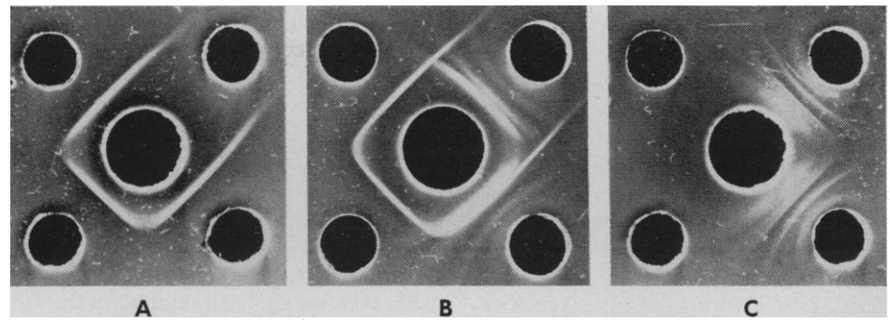

Fig. 1. Antigen-antibody interactions by the agar plate diffusion technique of Ouchterlony. All center wells (in *A*, *B*, and *C*) contain rabbit antiserum specific for whole human serum and for crystalline bovine serum albumin. *Center well in A*, antiserum treated with insoluble human serum protein (prepared from pooled whole human serum). *Center well in B*, antiserum untreated (control). *Center well in C*, antiserum treated with insoluble bovine albumin (prepared from crystallized bovine serum albumin). *The outside wells* have the same arrangement in *A*, *B*, and *C*, that is, upper and lower left, bovine serum albumin (157 μg per ml); upper right: whole human serum (1:128 dilution); lower right, mixture of whole human serum (1:128) with bovine albumin (157 μg per ml).

(see Table 1). After centrifugation and filtration, more than 99 percent of the enzyme activity had been removed from the mixture in all cases (residual activity was less than one percent of the activity before centrifugation).

The enzymes rendered insoluble maintained their activity when stored at 0° to 4°C. A ribonuclease preparation, for instance, exhibited 90 percent of the original activity after one month. There was no indication that the enzymatic properties of the insoluble products were in any way different from those of the corresponding soluble materials and, in particular, the *p*H optima of activity were unchanged. In the tests made, contact with the substrate did not release even traces of any of the entrapped enzymes.

Freshly prepared insoluble papain was active in the absence of added sulfhydryl agents or of cyanide, and was not activated by any of these materials; this is comparable to the papain derivative described by Cebra *et al.* (*6*). Our insoluble papain preparation completely lost its enzyme activity when stored for 3 days at 0° to 4°C (air was not excluded); however, it could be reactivated by cysteine.

Biological activity of antigens or enzymes requires contact with antibodies or substrates, respectively, but most antibodies, and many enzyme substrates, are macromolecular substances and hence are unable to penetrate into the particles of the cross-linked polymer for the same reason that the trapped antigens or enzymes cannot get out. It appears likely therefore that, while the entrapped antigen or enzyme may be located both inside and near the surface of the insoluble particles, only the latter portion exhibits biological activity. Actually, considerably more than 2 to 6 percent of the original enzyme (or antigen) may be entrapped (Table 1) but may not exhibit biological activity for reasons of steric hindrance (*8*).

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