REACTIVE POLYMERS AND THEIR USE FOR THE PREPARATION OF ANTIBODY AND ENZYME RESINS

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In recent years the synthesis of reactive high polymers has been of interest for a number of reasons. One of the aims in this field has been to produce reactive solid carriers which are capable of being bonded to physiologically active proteins by means of covalent bonds. The resulting products can be used to study many aspects of the specific reactions which take place between protein and substrate. These derivatives may yield not only information concerning the action of physiologically active proteins which are affixed to a surface, thus serving as models for processes in physiological membranes, but they may also reveal a very practical way of using some enzymes. An insoluble enzyme resin, which is formed by the reaction of a reactive carrier substance with an enzyme, can react with a substrate without contaminating the substrate solution. It can be easily separated from the solution and can be re-used. By coupling antigens or antibodies with reactive solid carriers, serologically active protein resins, which can be used as serologically specific adsorbents are obtained; they can be of great help in the isolation or separation of purified antibodies or antigens.

The proteins which will react with the carrier polymers may contain the following reactive groups[1]: free carboxyl groups (acid amino-acids or terminal carboxyl groups of amino-acids), free amino groups (basic amino-acids or terminal amino groups of amino-acids), the phenol group of tyrosine, which couples with diazonium salts, the imidazole group of histidine, the imino group in tryptophane, free thiol groups (cysteine), the disulphide link in cystine, the guanidino structure in arginine, aliphatic hydroxyl groups (serine, threonine, oxyproline, oxyglycine, oxyglutamic acid) the methylmercapto group of methionine the phenyl group in phenylalanine, also amide groups (asparagine, glutamine). In some cases the peptide links between the amino-acids are also suitable. Only those high polymer carriers with reactive groups which can react with one of the above mentioned groups of the protein, have the ability to bind with proteins chemically.

For the formation of chemical bonds between insoluble macromolecular carriers and physiologically active proteins only very mild reaction conditions can be considered: the reactions must be performed at low temperatures and in a pH range as near to neutral as possible. Therefore, only those carrier substances, which possess reactive groups capable of reacting under the above conditions can be used. The reactivity of groups is also dependent on the physical properties of the carrier substance. Hydrophilic

polymers are more likely to react with an aqueous protein solution than are hydrophobic substances.

Macromolecular carrier substances with reactive groups have been prepared by two principle methods, namely: polymerization of monomers which already contain the reactive groups, and chemical introduction of reactive groups into an originally inert polymer.

I would first like to give a short review of the different methods which have been described for binding proteins chemically to insoluble carriers. Two kinds of carriers have been used: cellulose derivatives, and totally synthetic reactive polymers. Micheel and Evers[2] affected a covalent link between carboxymethylcellulose azide and proteins. By modification of this method Mitz and Summaria[3] recently combined carboxymethyl-cellulose

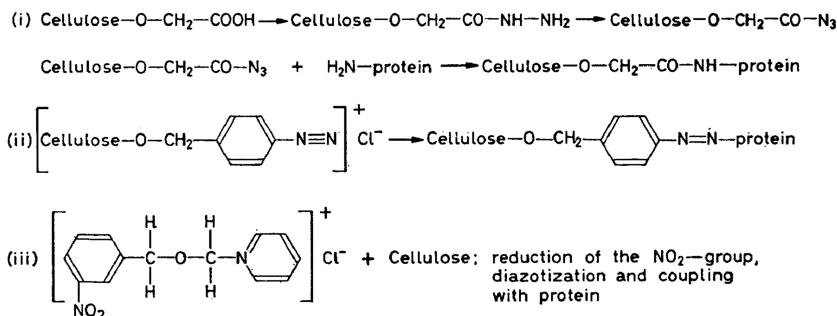

Figure 1

with enzymes (*Figure 1* (*i*)). Campbell *et al.* diazotized *p*-aminobenzyl cellulose, and coupled the product with an antigen protein[4]. Using this coupling procedure, insoluble diazobenzyl cellulose derivatives of ribonuclease and chymotrypsine were also synthesized by Mitz and Summaria (*Figure 1* (*ii*)). Using the method of Kursanow and Solodkow[5], Gurwitch *et al.*[6], and also Neslin[7], have attached antigen proteins chemically to cellulose (*Figure 1* (*iii*)).

In place of a modified cellulose, other reactive polymers have also been used. Thus, Isliker[8] described a method in which he converted carboxylic acid ion-exchange resins into a polymer acid chloride. The polymer acid chlorides were thoroughly investigated by Deuel[9] and Manecke and Heller[10] who found that these polymers also contained anhydride groups. By mixing them with antigen proteins in alkaline solution the proteins were fixed chemically onto the ion-exchange resin (*Figure 2*(*i*)). The same method was also used by Grubhofer and Schleith[11]. The latter authors have also diazotized polyaminostyrene according to the method of Bachman *et al.*[12] and coupled the product with albumin and different enzymes[13] (*Figure 2* (*ii*)). The same diazotized polyaminostyrene was also used by Manecke and Gillert[14] to synthesize antigen and antibody resins. This kind of reactive carrier has also been used by Gyenes, Rose and Sehon[15] to form antigen–polystyrene conjugates.

Brandenberger[16] synthesized a crosslinked poly-4-isocyanato-styrene, with the aid of which he succeeded in binding enzymes (*Figure 2(iii)*). He claimed this polymer to be very reactive chemically.

We investigated the reactivity and the composition of the poly-isocyanato-styrene thoroughly and developed two analytical methods for assaying the

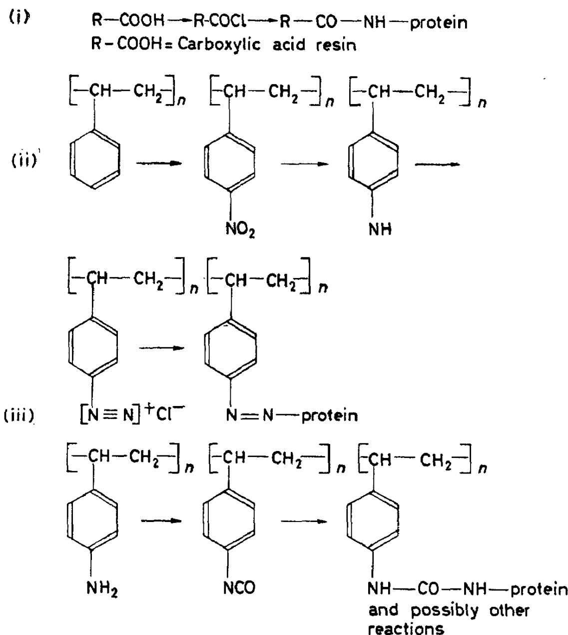

Figure 2

concentration of active isocyanate groups in the polymer[17]. The two methods gave results in good agreement with one another. By determining the amount of carbon dioxide liberated during the reaction of the isocyanate resin with water according to the reaction:

$$R\cdot NCO + H_2O \longrightarrow R\cdot NH\cdot COOH \longrightarrow R\cdot NH_2 + CO_2$$

and also by determining the amount of di-n-butylamine which was consumed by the isocyanate resin in the reaction:

$$R\cdot NCO + HN(C_4H_9)_2 \longrightarrow R\cdot NH\cdot CO\cdot N(C_4H_9)_2$$

the concentration of active isocyanate groups in the resin was assayed as *ca.* 3 meq/g resin.

Despite this high concentration of reactive groups the amount of the protein that could be bound by the polymer was rather low (see *Table 2*). By reacting the poly-isocyanatostyrene with an anti-*p*-azobenzoate antibody we obtained an antibody resin which adsorbed more *p*- than *o*- or *m*-acetaminobenzoic acid[18]. The last two isomers were adsorbed in equal quantities. A protein resin which was prepared with the same reactive carrier

and an unspecific serum adsorbed all three isomers equally. *Table 1* gives the results of these investigations.

Table 1. Adsorption of acetaminobenzoic acids by an anti-*p*-azobenzoate antibody resin (reactive carrier: poly-isocyanato-styrene)

*Antiserum**	I	I	II
Protein bound by 3 g of poly-isocyanato-styrene (mg)	51·5	49·5	45·2
Acetaminobenzoic acid adsorbed by 1 g of the protein resin (mg)			
o-acetaminobenzoic acid	2·7	2·69	3·2
m-acetaminobenzoic acid	3·05	2·85	3·48
p-acetaminobenzoic acid	4·01	3·69	3·34

* I = antiserum produced by inoculation with protein-*p*-azobenzoate antigen.
II = serum without inoculation.

So, by comparing the amounts of the isomers of acetaminobenzoic acid which were adsorbed we found that, although the *p*-isomer was bound preferentially, an unspecific binding of the other isomers also took place. We further found that poly-isocyanato-styrene, which had been treated with protein-free water, produced a polymer which adsorbed all the three isomers of the acetaminobenzoic acid, and even showed a small selectivity for *o*-acetaminobenzoic acid. This ability to adsorb aromatic compounds can be explained by the presence of many aromatic nuclei in the polymer.

Our findings described above led us to look for reactive polymers which would be more suitable for our purpose. After many attempts[17] we synthesized a polymer which satisfied our needs. By copolymerizing methacrylic acid with methacrylic acid *m*-fluoroanilide in the molar ratio 3 : 1, and in the presence of a 1 per cent divinylbenzene, we obtained a copolymer which, after nitration, contained the 3-fluoro-4,6-dinitroanilide group as the reactive group[19] (*Figure 3*). This copolymer exhibited the great advantage of being able to bind large quantities of proteins (*Table 2*).

Table 2. Affinity for proteins of different reactive macromolecular carriers

Polymer carrier	*Protein bound* (mg/g resin)	*Ref.*
Diazotized *p*-aminobenzyl ether of cellulose	15	4
Chlorinated amberlite XE-64	96	8
Chlorinated amberlite IRC-50	15	19
Diazotized polyaminostyrene	13	13
Diazotized polyaminostyrene	12	19
Poly-isocyanato-styrene	12–15	17
Nitrated crosslinked copolymer of methacrylic acid-3-fluoranilide and methacrylic acid (molar ratio 1 : 3)	150–300	19

This property can be attributed to the good wettability of the polymer and its high reactivity. By treating this reactive copolymer with an anti-*p*-azobenzoate antibody we obtained an antibody resin which showed selective adsorption for *p*-acetaminobenzoic acid[20] (*Table 3*).

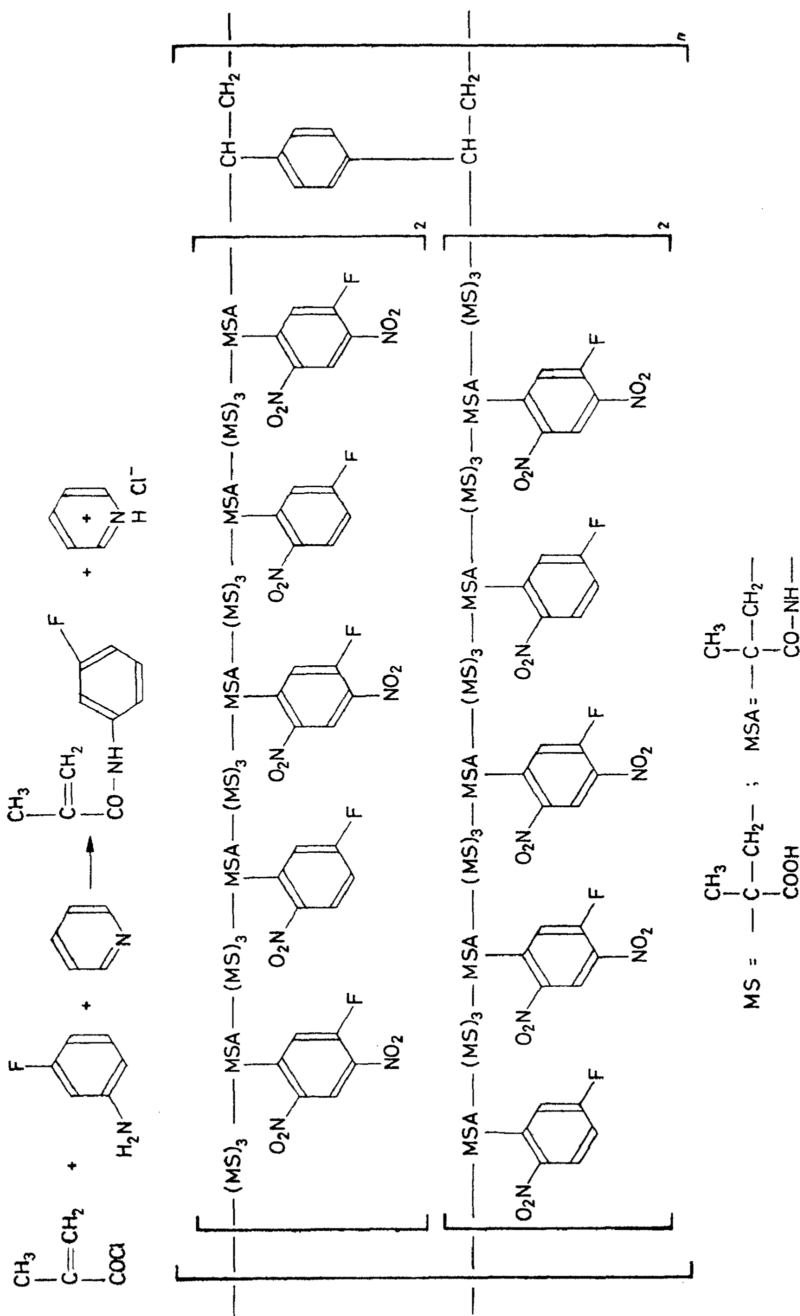

Figure 3

This hapten specific antibody resin adsorbed no *m*-acetaminobenzoic acid, and only a little *o*-acetaminobenzoic acid. It was shown in control tests, that neither the un-nitrated copolymer, nor a nitrated copolymer which had been treated with an unspecific serum, adsorbed any of the acetaminobenzoic acid isomers (*Table 3*).

Table 3. Adsorption of acetaminobenzoic acids by an anti-*p*-azobenzoate antibody resin (reactive carrier: nitrated copolymer of methacrylic acid and methacrylic acid *m*-fluoroanilide)

*Antiserum**	I	I	II
Protein bound by 100 mg nitrated copolymer (mg)	17·0	14·75	22·0
Acetaminobenzoic acid adsorbed by 100 mg protein resin (mg)			
o-acetaminobenzoic acid	0·10	0·14	0
m-acetaminobenzoic acid	0	0	0
p-acetaminobenzoic acid	0·35	0·65	0

* I = antiserum produced by inoculation with protein-*p*-azobenzoate antigen.
II = serum without inoculation.

We investigated the composition of the nitrated copolymers and found that it is possible to introduce about 1·6 nitro groups per aromatic nucleus. Our assumption that all aromatic nuclei of the copolymer (molar ratio of the components 3 : 1) were nitrated, and that 60 per cent of them were doubly nitrated, was verified by reacting this polymer with *p*-aminobenzoic acid. The amount (16·1 mg) of the acid which was bound by 100 mg of the nitrated copolymer agreed with that predicted theoretically. The copolymer reacts in N/10 aqueous sodium hydrogen carbonate with organic amino compounds, and also with proteins, in competition with water. In N/10 sodium hydroxide hydrolysis is very quick, and no reaction with amines or proteins occurs. As can be seen in *Table 4* the amount of chemically bound aminobenzoic acid to the reactive copolymer depends on the initial concentration of the aqueous amino-acid solution. The higher the concentration, the larger is the amount of bound acid.

Table 4. Reaction of a nitrated crosslinked copolymer (methacrylic acid, methacrylic acid-*m*-fluoroanilide) with *p*-aminobenzoic acid

Initial concentration (mg *p*-aminobenzoic acid per 40 ml N/10-$NaHCO_3$ solution)	*p-aminobenzoic acid bound per* 100 mg *resin* (mg)	(% of max. amount)
20	8·5	53
40·5	10·5	65
78·8	14·8	90
152	16·0	99·5

To make sure that effects other than coupling due to the reactive fluorine atoms of the polymers were not responsible for the binding of the proteins to the nitrated copolymer, we investigated the binding activity of the poly(methacrylic acid) and also of the un-nitrated copolymer. Under the

same reaction conditions as those mentioned above, no binding of proteins was observed.

In order to investigate how the affinity of the nitrated copolymers for proteins depends on their composition, copolymers of different molar ratios of the two main components were prepared. The copolymers thus obtained were nitrated. It was striking that the copolymers containing a large amount of methacrylic acid showed the lowest degree of nitration. This is due to the fact that these copolymers do not swell in acid solution, because of the many acrylic acid groups in the copolymer. Since they react with proteins it is evident that nitration of these copolymers takes place mostly on the surface layers, so that the reactive dinitro-*m*-fluoroanilide groups are found on the surface, while the inner regions are not nitrated.

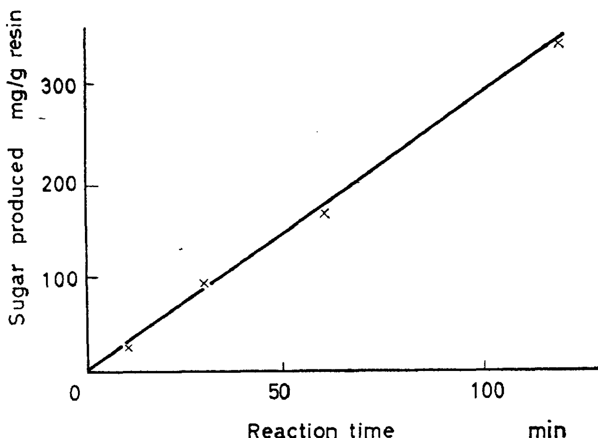

Figure 4. Rate of formation of invert sugar by β-h-fructosidase resin

The results of these investigations are summarized in *Table 5.* It will be seen that the ratio of 1 : 3 of methacrylic acid-*m*-fluoroanilide to methacrylic acid during the copolymerization gives the most effective carriers. The " dilution boundary " for the fluorine compound lies at the molar ratio 1 : 5. Higher concentrations of methacrylic acid *m*-fluoroanilide do not give a higher amount of bound protein. In all copolymerizations 1 per cent divinylbenzene was present as a crosslinking agent. The degree of nitration also depended very much on the grain dimensions of the copolymer. Higher

Table 5. Nitrated copolymers of methacrylic acid and methacrylic acid-*m*-fluoroanilide; influence of composition on affinity for proteins

No.	*Molar ratio of methacrylic acid-m-fluoroanilide to methacrylic acid in the copolymer*	*Degree of nitration* (number of NO_2-groups per benzene nucleus)	*Protein bound* (mg/100 mg resin)	
			Ovalbumin (mg)	*Rabbit serum proteins* (mg)
1	1 : 2	1·45	24·0	31·5
2	1 : 3	1·6	23·0	38·0
3	1 : 5	1·3	22·0	22·9
4	1 : 10	1·0	15·0	14·0

degrees of nitration were usually obtained with smaller grains. The nitrated copolymers were of lemon-yellow colour; they swelled in alkaline solutions and were insoluble.

The nitrated crosslinked methacrylic acid-*m*-fluoroanilide methacrylic acid copolymers were used to synthesize enzyme resins. By reacting the nitrated copolymer with an aqueous solution of β-h-fructosidase (Invertin, Merck) in N/10 sodium hydrogen carbonate an enzyme resin was obtained which showed enzymatic activity[19]. The rate of the formation of invert sugar is shown in *Figure 4*. The enzyme resin reacted with a 6·5 per cent sucrose solution in an N/10 acetate buffer at a pH of 4·5.

The stability of this enzyme resin was also investigated. Three different fructosidase resins were prepared and stored in an N/10 acetate buffer at pH 4·5. At different intervals of time they were reacted with a sucrose solution for one hour and their enzymatic activities determined (*Table 6*).

Table 6. Stability of the enzymic activity of β-h-fructosidase resins on keeping

Preparation of the β-h-fructosidase resins 100 mg copolymer + 20 ml invertin solution (10 ml invertin (Merck) + 80 ml N/10 $NaHCO_3$), 5 h stirring at room temperature *Reaction of the washed enzyme resins* Enzyme resin (from 100 mg copolymer) + 15 ml of a 10% sucrose solution in N/10 acetate buffer at pH 4·5 (1 h)	*Un-nitrated copolymer (control test)*	*Nitrated copolymer (large particles)* (ϕ=0·2-0·5mm)	*Nitrated copolymer (small particles)* (ϕ=0·1-0·2mm)
Invert sugar formed after 1 h reaction time (mg)			
1st day	11·4	40·2	157·4
2nd day	0	22·8	154·0
3rd day	0	28·2	100·8
7th day	0	24·0	91·0
14th day	0	22·0	98·0

On the first day the un-nitrated copolymer showed a small activity, which was caused by a small amount of adsorbed enzyme. By the second day the activity had already been lost. The nitrated copolymers gave enzyme resins which showed much higher initial activities. After the third day the activities were still of the same order. As might be expected, the smaller

Table 7. Treatment of the nitrated copolymer (methacrylic acid, methacrylic acid-*m*-fluoroanilide) with diastase in N/10$NaHCO_3$ solution

	Grain size of the resin (ϕ = 0·2–0·1 mm)	(ϕ < 0·1 mm)
Maltose, produced by 1 g diastase resin in 30 min (mg)	23	108·5
Diastase, bound per 1 g diastase resin (mg)	13·2	39·8
Maltose, which would be produced by the amount of bound diastase if it were in solution, in 30 min (mg)	1,097	3,280

grains with the larger total surface had the higher activity. The control experiment with the un-nitrated copolymer convincingly showed that the enzyme was chemically linked to the nitrated copolymer.

The nitrated crosslinked copolymer was also reacted with diastase (0·05 per cent in N/10 sodium hydrogen carbonate)[21]. This resulted in a diastase resin which also showed enzyme activity. The activity was determined according to the method of Willstätter[22] for diastase solutions: after reaction of the enzyme resin with a 1 per cent starch solution at 37° and pH 6·8 for 30 minutes the amount of maltose produced was measured (*Table 7*). The enzyme activity of the diastase bound to the macromolecular carrier was *ca.* 40 times lower than that of an equal amount of free diastase. This reduction can be attributed to the heterogeneous nature of the reaction between the solid enzyme resin and the substrate in solution. The activity of the enzyme on the resin may also be reduced.

The stability of the activity of the diastase resin is shown in *Table 8*. The same samples of the diastase resin were reacted with a starch solution at different times, and the amount of maltose produced was determined.

Table 8. Stability of the enzymic activity of the diastase resin

Grain size (ϕ) (mm)	*Maltose produced in 30 min* (mg/g diastase resin) 1st day	3rd day	7th day	14th day
0·2–0·1	23	22	19	17·7
< 0·1	108·5	108·5	71·7	60·5

After 14 days, *ca.* 40 per cent of the enzyme activity had been lost. A diastase solution which was stored under optimal conditions lost 30 per cent of its activity over the same period.

As can be seen from *Table 7*, little diastase was linked to the carrier substance. This results from the low solubility of the diastase (a 0·05 per cent solution was used). By treating the nitrated copolymer with an aqueous diastase suspension in N/10 sodium hydrogen carbonate, an enzyme resin with a higher activity was obtained (*Table 9*). It was estimated that

Table 9. Stability of the enzymic activity of a diastase resin produced by reaction of a diastase suspension with the nitrated copolymer

Time (days)	*Maltose produced in 30 min* (mg/g diastase resin)
1	191
2	189
5	180
9	170
13	169

about double the amount of diastase was now linked to the carrier. An attempt was also made to react the nitrated copolymer with the enzyme in five per cent sodium acetate, but only 33·8 mg of diastase were bound per g of the carrier (grain size $\phi < 0{\cdot}1$ mm).

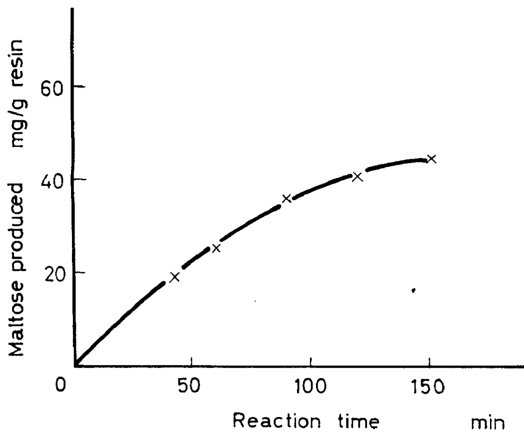

Figure 5. Variation with time of maltose formation by diastase resin

The variation with time of the production of maltose by the diastase resin–starch reaction is shown in *Figure 5.*

Reaction of the nitrated copolymer with pepsin (0·5 per cent in N/20 sodium hydrogen carbonate) similarly led to an enzymatically active pepsin resin[21]. The activity of the pepsin resin was determined by the method of Anson[23] for pepsin solutions: the amount of tyrosine produced after treatment of a 2·5 per cent haemoglobin solution with the enzyme resin for 10 minutes was determined (*Table 10*). The loss of activity resulting from

Table 10. Reaction of a nitrated copolymer with pepsin in N/20-$NaHCO_3$ solution

	Grain size of the resin (ϕ = 0·2–0·1 mm)	(ϕ < 0·1 mm)
Tyrosine produced per g pepsin resin in 10 min (mg)	10·2	14·5
Pepsin bound per g pepsin resin (mg)	272	324
Tyrosine which would be produced by the amount of bound pepsin if it were in solution (estimated)* (mg)	50·5	60·2

* 10 mg of pepsin and 100 mg of haemoglobin produce 1·86 mg of tyrosine within 10 minutes.

combination of pepsin with the reactive carrier was less than that observed with the other enzymes mentioned above. The stability of the pepsin resin can be seen from *Table 11.* It was found that, after a reaction time

Table 11. Stability of the pepsin resins

Time (days)	*Tyrosine produced per g pepsin resin in 10 min* (mg) (ϕ = 0·2–0·1 mm)	(ϕ < 0·1 mm)
1	10·2	14·5
2	9·45	13·0
4	7·6	11·95
8	9·45	14·0
12	6·53	11·4
16	7·22	12·45

of 10 minutes, the equilibrium between the enzyme resin and the substrate had already been reached.

The above mentioned enzyme resins were all produced by treating the nitrated copolymer with a holoenzyme. It was also of interest to investigate whether an apoenzyme chemically linked to a macromolecular carrier would show enzymatic activity after the addition of a coenzyme. If this were so it would be possible to separate easily the apoenzyme resin from the coenzyme after the reaction with the substrate, and to re-use the apoenzyme resin.

An apoenzyme resin was produced by treating the nitrated copolymer with a 0·15 per cent solution of alcohol dehydrogenase in N/20 sodium hydrogen carbonate[21]. The enzyme resin was stored, after rinsing, in a phosphate buffer (pH 7·5 at 0°). The activity of the alcohol dehydrogenase, and of the alcohol dehydrogenase resin, was determined by the method of Thorell and Bonnichsen[24]: the amount of DPNH produced by reaction of the enzyme, or enzyme resin, with DPN and alcohol was measured (*Table 12*).

Table 12. Alcohol dehydrogenase resin

DPNH produced per 100 mg alcohol dehydrogenase resin in 3 min (mg)	14·3
Alcohol dehydrogenase bound per 100 mg resin (mg)	16·2
DPNH which would be produced by the amount of bound alcohol dehydrogenase if it were in solution, in 3 min (mg)	698·0

The stability of this enzyme resin was also investigated (*Table 13*). After 14 days it had lost 50 per cent of its original activity. An alcohol dehydrogenase solution stored under optimum conditions lost 50 per cent of its activity after 20 days. For further characterization of this enzyme resin its rate of production of DPNH was investigated (*Figure 6*).

Table 13. Stability of the alcohol dehydrogenase resin

Time (days)	*DPNH produced in 3 min* (mg/100 mg resin)
1	14·35
2	11·95
4	10·40
7	9·30
11	8·08
14	7·53
15	6·90

Several authors have tried to resolve racemates with macromolecular adsorbtives with, for example, the help of optically active macromolecules[25–27]. It is known that (±)-mandelic acid can be resolved *via* its diastereoisomeric compound with (−)-ephedrine into its optical antipodes. By reaction of our nitrated copolymer with (−)-ephedrine we produced an

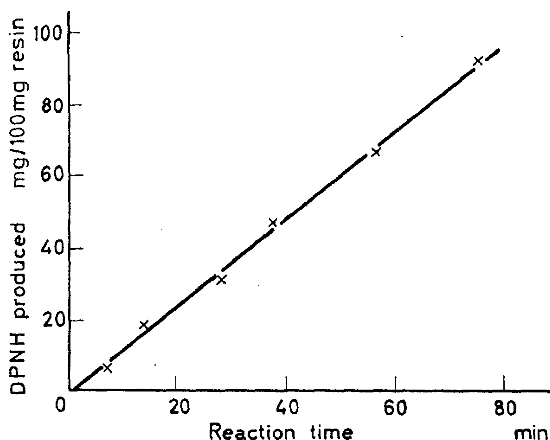

Figure 6. Rate of DPNH formation by alcohol dehydrogenase resin

optically active resin[21]. When 70 ml of a 4 per cent solution of (±)-mandelic acid was passed through a column (20 cm × 1 cm) filled with 10 g of dialysed (—)-ephedrine resin it was found that the first fractions of the effluent were

Table 14. Chromatography of (±)-mandelic acid on an (—)-ephedrine resin column

Fraction	*Mandelic acid* (mg/ml)	*Degree of rotation*	*Active mandelic acid* (mg/ml)
4	0·08	0·0	0·0
5	0·95	0·075	0·51
6	4·5	0·20	1·35
7	14·2	0·30	2·03
8	17·1	0·30	2·03
9	17·1	0·25	1·69
10	17·1	0·225	1·52
11	17·6	0·20	1·35
12	18·2	0·175	1·18
13	16·8	0·125	0·85
14	19·4	0·15	1·01
15	20·2	0·15	1·03
16	21·2	0·15	1·01
18	20·8	0·15	1·01
20	20·8	0·15	1·01
22	20·8	0·15	1·01
24	20·8	0·15	1·01
26	21·2	0·15	1·01
28	22·0	0·15	1·01
30	23·9	0·15	1·01
32	23·9	0·15	1·01
34	25·6	0·15	1·01
36	26·4	0·15	1·01
38	26·8	0·15	1·01
40	28·4	0·125	0·85
42	29·8	0·125	0·85
44	29·6	0·125	0·85
46	29·6	0·125	0·85
48	29·6	0·125	0·85
50	34·6	0·10	0·68
52	35·0	0·0	0·0
54	36·8	0·0	0·0

enriched with (—)-D-mandelic acid. The maximum degree of rotation was — 0·3° (*Table 14*). Very soon, however, the racemate solution was eluted.

It was also established, that 1 g of the (—)-ephedrine resin adsorbed $6{\cdot}20 \times 10^{-4}$ moles of acid when in contact with a (+)-L-mandelic acid solution, but only $5{\cdot}45 \times 10^{-4}$ moles when in contact with a (—)-D-mandelic acid solution. This difference in adsorption amounts to 12·1 per cent.

The enzyme resins and the specific adsorbtives are expected to serve as models for the study of the physiological processes in living cells. Permeability, and problems of transport, arise not only at the cell boundaries, but also in the interior of the cell. Electron microscopic investigations have shown that big membrane surfaces are built up in cells. The principle of spatial order of co-operating enzymes is, for instance, very clearly found in the mitochondrium, the " chemical factory " of the cell. We still know very little about all these inter-relations. Hopkins described life as " a dynamic equilibrium in a multiphase system ". Enzymology has studied the first part of this definition–the reactions and flow equilibria. Electronic microscopy has shown us the multiphase nature of these systems. The task now is to fill the space in between.

Summary

Data are given on the composition and reactivity of some high polymers which can act as chemically reactive carriers. Their chemical reaction with aqueous solutions of proteins is described.

Enzyme and antibody resins were synthesized by reacting enzymes and antibodies with a nitrated copolymer of methacrylic acid and methacrylic acid *m*-fluoroanilide. The activity and stability of these resins were investigated. An optically active polymer was prepared with (—)-ephedrine. By means of this polymer a partial resolution of (±)-mandelic acid was effected.

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