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Matrix-Bound Enzymes

Part I. The Use of Different Acrylic Copolymers as Matrices

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The preparation of matrix-bound enzymes is described. The matrices used are copolymers of different hydrophilic acrylic monomers, the preparation procedures of which are given. Subsequently a.) The entrapment of citrate synthase in a cross-linked acrylamide polymer is described. These preparations showed 15 % of enzymic activity in relation to free enzyme. b.) The covalent binding of trypsin to a cross-linked acrylamide-acrylic acid copolymer or the commercially available "Bio-Gel CM-100" using a water soluble carbodiimide as coupling agent has been carried out. Such matrix-bound trypsin derivatives showed 30 % of proteolytic activity towards α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide in relation to free enzyme with a coupling yield of the added enzyme amounting to 90 %. Ultracentrifugation of free trypsin treated with carbodiimide gave no indication of cross-linking of the protein under the coupling conditions used. c.) Covalent binding of trypsin to cross-linked acrylamide-hydroxyethyl methacrylate copolymers by the use of cyanogen bromide resulted in trypsin derivatives showing 35 % of proteolytic activity in relation to free enzyme with a coupling yield of 25 %. d.) A combination of the entrapment technique with the covalent binding by means of carbodiimide has been applied for trypsin using a cross-linked acrylamide-acrylic acid copolymer. Such preparations, in which trypsin is kept both entrapped and covalently bound to a matrix, gave a proteolytic activity averaging 20 % relative to free enzyme and a binding yield of added enzyme averaging 100 %. The corresponding average values from control preparations lacking carbodiimide were 20 % and 50 %, respectively. Heat stability tests (100°, 10 min, pH 7.5) of matrix-bound trypsin showed an increased stability of such preparations retaining more than double the enzymic activity after heat-treatment as compared to soluble enzyme.

The study of enzymes bound to or within synthetic matrices has in the last decade found increasing interest. Such preparations have significant practical value and offer model-systems for the study of enzymes *in vivo* occurring bound to insoluble cell material such as membranes. Two major

techniques in binding enzymes to a matrix can be distinguished: a. The entrapping of the protein within the lattice of a polymer such as cross-linked polyacrylamide [1,2] or Silastic resin.[3] b. The establishing of covalent [4-6] or ionic linkages [7] between protein and support. In the majority of studies described, stable, proteolytic enzymes have been used.[8,9] In the present study the binding of another type of enzyme, citrate synthase, has therefore been undertaken using the entrapment technique. Studies on the preparation of a matrix-bound Δ^{1-2} steroid dehydrogenase applicable in steroid transformation processes [10] as well as of polymer particles supporting a two-enzyme system, hexokinase and glucose-6-phosphate dehydrogenase,[11] have also recently been concluded in this laboratory.

Of the insoluble enzyme preparations described in which enzymes are attached by covalent or ionic linkages to polymers, in the majority of cases celluloses [12-14] or cross-linked dextran, "Sephadex",[4] as well as "Agarose" [5] have been used as matrices. Acrylic polymers appear because of their hydrophilic nature to offer a valuable alternative. In the present study the following cross-linked copolymers of this type have been used as matrices: a. Copoly-(acrylamide-acrylic acid); b. The corresponding commercially available "Bio-Gel CM-100" and c. Copoly-(acrylamide-hydroxyethyl methacrylate). To polymers a. and b. trypsin has been attached by covalent linkages using a water-soluble carbodiimide. To polymer c. trypsin has been fixed by activation of the carrier with cyanogen bromide, the general method of which has been described elsewhere.[4] Finally a combination of the entrapment technique with the covalent linking of trypsin by means of carbodiimide has been carried out with polymer a. leading to matrices in which the enzyme is kept both entrapped and covalently bound.

MATERIALS AND METHODS

Materials. Acrylamide, *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman, *N,N'*-methylenebis(acrylamide) and *β*-dimethylaminopropionitrile were purchased from Fluka, Buchs SG, acrylic acid from Ugilor, hydroxyethyl methacrylate from Röhm und Haas GMBH, Bio-Gel CM-100 (high capacity 100–200 mesh) from Bio-Rad lab, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene-sulfonate from Aldrich Chemical Co. Trypsin (2× crystallized and lyophilized, 11 000 BAEE U/mg), citrate synthase (crystalline suspens. in $(NH_4)_2SO_4$, 120 U/mg), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl, *cis*-oxaloacetate (grade 1) were purchased from Sigma Chem. Comp.

All reagents were used without further purification except for acrylic acid which was distilled and hydroxyethyl methacrylate which was passed through an Al_2O_3 column (Woelm basic grade) prior to polymerization to remove traces of methacrylic acid present. $CH_3{}^{14}COSCoA$ (0.68 μC/μmole) was prepared from $(CH_3{}^{14}CO)_2O$ according to the literature.[15]

Abbreviations used: Acrylamide=AAm, acrylic acid=AA, *N,N'*-methylenebis-(acrylamide)=Bis, hydroxyethyl methacrylate=HEMA, N,N,N',N'-tetramethylethylen-diamine=TEMED, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate=CMC, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl = BAPNA, cross-linked copolymer of acrylamide-acrylic acid=copoly-(AAm-AA), cross-linked copolymer of acrylamide-hydroxyethyl methacrylate=copoly-(AAm-HEMA).

Preparation of entrapped citrate-synthase in 10 % (w/w) gel granules. 95 mg of acrylamide (AAm) and 2 mg of ammonium persulfate were dissolved in 0.75 ml of 0.1 M phosphate buffer (pH 8.0) in a small test-tube. 5 mg of *N,N'*-methylene-bis(acrylamide),

(Bis), dissolved in 0.25 ml of the above buffer, 25 μl of citrate synthase dissolved in the same buffer (=30 mU) and 5 μl of β-dimethylaminopropionitrile were added in the order given. The solution was kept at 4° and gassed with nitrogen. Polymerization proceeded within 30 min. The stiff gel obtained was subsequently passed through a 30-mesh sieve. The granules formed were then washed under stirring at 4° for two days in dist. water to remove any non or poorly entrapped enzyme.

Assay of entrapped citrate-synthase. The obtained gel granules were suspended in 2 ml of the above buffer. After addition of 4.48 μmoles of oxaloacetate and 2.24 μmoles of acetyl-SCoA dissolved in 200 μl of water the incubation proceeded in a 5 ml E-flask under stirring for 15 min at 37°. After filtering off the gel granules the filtrate was concentrated and applied to a paper chromatogram. The paper was run in the system ethylmethylketone:water:acetone:formic acid = 80:12:4:2 (v/v/v/v).[16] After scanning the chromatogram in a stripcounter the radioactivity measured of the citric acid formed (R_F-value=0.48) was integrated. Assay of soluble citrate synthase using 25 μl of enzyme solution was carried out under identical conditions except for the omission of gel granules.

Preparation of cross-linked copolymer of acrylamide-acrylic acid, 20 % (w/w). 0.95 g of acrylic acid (AA) was titrated with 3 M NaOH to pH 6.5. After addition of 0.95 g of AAm and 0.1 g of Bis the final volume was brought to 10 ml with 0.1 M phosphate buffer (pH 6.5). After addition of the catalyst system consisting of 25 mg of ammonium persulfate and 220 μl of TEMED the solution in a small test tube was carefully deaerated at 4° by applying vacuum with a water pump. Polymerization proceeded within 30 min. The gel obtained was passed through a 30-mesh sieve and the gel granules formed were then washed thoroughly under stirring with dist. water. The granules were dried with acetone on a glass filter and stored at room temperature. Alternatively the fresh gel granules prepared were used directly for coupling processes.

Preparation of cross-linked copolymer of acrylamide-hydroxyethyl methacrylate, 20 % (w/w). In this preparation the above procedure was followed. Instead of AA, the equivalent amount of hydroxyethyl methacrylate (HEMA) was added. The gel granules prepared were washed thoroughly under stirring with dist. water and were then ready for use.

Coupling of trypsin to cross-linked copolymer of acrylamide-acrylic acid, 20 % (w/w). 70 mg of dry gel granules were stirred in 20 ml of 0.5 M HCl for 30 min at room-temperature. Subsequently the granules were washed on a glass filter with dist. water followed by 0.1 M phosphate buffer (pH 6.5). When using Bio-Gel CM-100 the beads were left overnight in the above buffer prior to coupling, no treatment with HCl was made. The swollen gel granules were then transferred to a small beaker. After addition of 8.75 mg of trypsin dissolved in 3.5 ml of the above buffer the suspension was stirred for 5 min. Subsequently 40 mg of the carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC), was added and stirring continued for 18 h. The formed enzyme-gel granules were then filtered off, tansferred to a beaker and washed for 30 min using 20 ml each of dist. water, 0.1 M $NaHCO_3$, 0.001 M HCl and 0.5 M NaCl. After a final washing on filter with dist. water, the enzyme-gel granules were kept in 0.15 M borate-HCl, 0.02 M $CaCl_2$ buffer (pH 7.5). Both coupling and washing procedures were conducted at 4°.

Coupling of trypsin to cross-linked copolymer of acrylamide-hydroxyethyl methacrylate, 20 % (w/w). Wet gel granules (corresponding to 70 mg of dry polymer) were transferred to a 10 ml beaker and suspended in 2 ml of dist. water. Subsequently 2 ml of a BrCN solution (100 mg/ml water) were added under slight stirring during 8 min keeping the pH of the suspension at 11 with 4 M NaOH. The gel granules were then quickly washed on a glass filter with cold 0.1 M $NaHCO_3$. The now activated gel granules were then transferred to a 10-ml beaker and 1 ml of 0.1 M $NaHCO_3$ containing 8.75 mg of trypsin was added and the coupling process permitted to proceed under slight stirring for 18 h at 4°. The subsequent washing procedure followed the description given above.

Combined entrapping and covalently binding of trypsin to cross-linked copolymer of acrylamide-acrylic acid, 20 % (w/w). In 0.5 ml of 0.1 M phosphate buffer (pH 6.5) were dissolved 0.05 g of Bis and 50 mg of trypsin. After addition of 0.48 g of AAm and a solution of 0.48 g of AA the pH of which had been adjusted with 3 M NaOH to 6.5, 200 mg of CMC were added. The final volume was brought to 5 ml with the above buffer. After addition of the catalyst system consisting of 12 mg of ammonium persulfate and 100 μl

of β-dimethylaminopropionitrile, the solution was carefully deaerated by applying vacuum with a water pump until polymerization started. The gel obtained was left for 18 h at 4°. The polymerization process was carried out in a 20 ml beaker at 4°.

Two cylindric portions were then cut out of the middle of the gel block formed. Both upper and lower sections of the gel cylinders were cut off to assure that no surface-attached trypsin was taken. The enzyme gel was then quickly passed through a 30-mesh sieve and washed following the procedure given for cross-linked copoly-(AAm-AA). A control gel in which CMC had been omitted was prepared under identical conditions.

Determination of the amount of bound enzyme. From the protein contents found in filtrate and washings of the above preparations as determined by the method of Lowry,[17] the amount of bound enzyme was calculated. No interference in the protein determinations from either gel or washing solutions was observed except for the combined entrapped-covalently bound enzyme preparations. Since here the gels cannot be washed prior to coupling as is the case with the other gel preparations, contaminations present result in too large protein values. However, by subtracting the "protein" content in blanks obtained with gels to which no enzyme had been added, the true protein value could be calculated.

Determination of enzymic activity of matrix-bound trypsin preparations. A portion of trypsin-gel particles corresponding to 7 mg of dry polymer was transferred to a 25-ml E-flask containing 4 ml of 0.15 M borate-HCl, 0.02 M $CaCl_2$ buffer (pH 7.5). The suspension was equilibrated at 25° for 15 min under stirring with a magnetic stirrer at a speed setting found optimal for the enzymic reaction (130 rpm, dimensions of Teflon bar used: 0.4 × 2.7 cm). Subsequently 8 ml of a solution containing 3.2 mg of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) in the above borate buffer was added. The incubation solution was immediately pumped with the aid of a peristaltic pump to a flow-cuvette placed in a spectrophotometer and back again to the reaction vessel. From the increase in absorption at 410 mμ read at 30-sec intervals during the first minutes of enzymic reaction, the enzyme activity could be determined using a standard curve of *p*-nitroaniline as reference. The details of this procedure, found ideally suited for continuously measuring enzymic activity of matrix-bound enzymes, are given in part II.[11]

Analytical ultracentrifugation of carbodiimide-treated trypsin. To a solution of 35 mg of trypsin in 3.5 ml of 0.1 M phosphate buffer (pH 6.5) were added 200 mg of CMC and kept for 12 h at 4°. 1 ml of this solution was subsequently analyzed in a Spinco centrifuge (model E) adapted with an An-D rotor. The temperature was kept at 5° and the rotor run at 59 780 rpm. Photographs were taken at 4 min intervals after reaching full speed. The control was run with the above solution in which CMC had been omitted.

Determination of heat stability of matrix-bound trypsin preparations. Heat stability of the trypsin matrix-bound to both copoly-(AAm-AA) and copoly-(AAm-HEMA) was examined by heating the enzyme gel granules (amount of gel corresponding to 70 mg of dry polymer) for 10 min at 100°. During heating the granules were kept suspended in 4 ml of 0.15 M borate-HCl buffer (pH 7.5) under stirring. After cooling, the enzymic activity was assayed as described in the previous section. To test the heat stability of soluble enzyme, a solution of trypsin was prepared containing the same enzymic activity as used in the above heat treatment of matrix-bound trypsin and treated under identical conditions.

RESULTS AND DISCUSSION

Of the different matrix-bound enzymes described in the literature the majority represents various hydrolytic or oxidative enzymes. In the shorter first part of this investigation the immobilization of a representative of another class of enzymes has been tried. Citrate synthase has been chosen as example of a "synthase" catalyzing citric acid formation from oxaloacetate and acetyl-coenzyme A. The enzyme was entrapped within a polymer lattice of acryl-amide cross-linked with Bis as described elsewhere.[2] An enzymic activity of about 12 – 15 % in relation to soluble enzyme was measured, a figure which is rather high considering the fact that diffusion of the large acetyl-coenzyme A

molecules is probably sterically hindered within the gel lattice. No loss of activity was observed after storing the enzyme gel granules in 0.1 M phosphate buffer (pH 8.0) for a week at 4°.

In the major part of the present study trypsin was bound by covalent linkages to matrices built up of copolymers derived from different acrylic monomers (Fig. 1). Such polymers appear ideally suited as carriers for enzymes for the following reasons: a. Their hydrophilic nature, which should help retaining the native structure and activity of the bound enzymes; b. They are easily prepared; c. Because of the variety of acrylic monomers available with varying carbon-chain length and different functional groups, preparation of "tailor-made" copolymers should be possible; d. Furthermore, copolymers such as acrylamide-acrylic acid (*e.g.* Bio-Gel CM-100) are already commercially available.

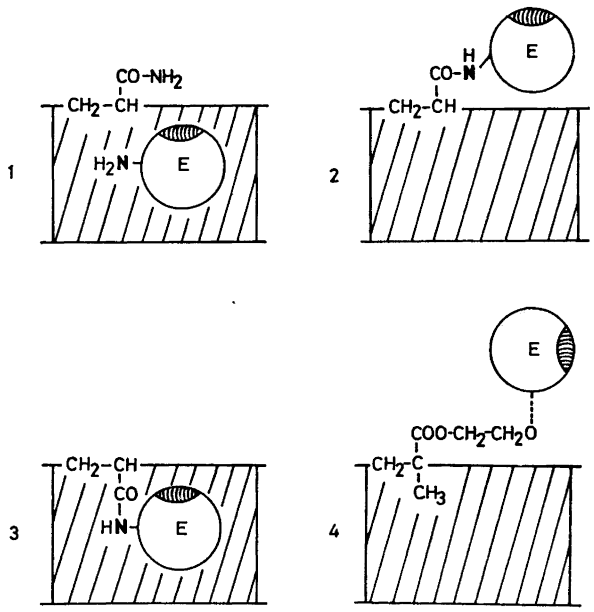

Fig. 1. Schematic representation of the four different types of enzyme-binding to a matrix as used in this study. 1. Entrapment of the enzyme within a cross-linked acrylamide lattice; 2. Coupling of the enzyme through covalent linkages to a cross-linked copoly-(acrylamide-acrylic acid) matrix using a water soluble carbodiimide; 3. Combination of the techniques used in 1. and 2. 4. Coupling of the enzyme through covalent linkages to a cross-linked copoly-(acrylamide-hydroxyethyl methacrylate) matrix using cyanogen bromide (details of the precise nature of the bonds involved are not yet known, formation of imino carbonic acid esters has been suggested [4]).

In the present study the enzymes have been bound to a matrix using a water soluble carbodiimide or cyanogen bromide. The attachment of antigens to carboxymethyl-cellulose with the aid of either *N,N'*-dicyclohexylcarbo-

diimide or a water soluble carbodiimide by the activation of the carboxyl groups of the carrier has already been investigated by Weetall and Weliky.[12-14] The same authors have also coupled enzymes to these celluloses; but have not quoted data on either coupling yield or enzymic activity. In the present investigation trypsin was coupled, with the aid of a water soluble carbodiimide (=CMC), to a copolymer of acrylamide-acrylic acid by simply stirring the enzyme-carrier-CMC suspension at 4° for 18 h. The copolymer used had either been prepared in the laboratory or been purchased (Bio-Gel CM-100). As seen from Table 1, a coupling yield amounting to about 90 % of added enzyme has been obtained with a retained enzymic activity of about 30 % as compared to soluble enzyme.

Table 1. Chemical binding of trypsin to copolymers.

Polymer	Amount of bound protein (mg protein/g dried polymer)	Coupling yield based on added amount of protein (%)	Proteolytic activity (μmole/min/mg protein)	Proteolytic activity Ratio bound to free enzyme (%)
Copoly(AAm-AA)	114	92	0.12	32
"Bio-Gel CM-100"	116	93	0.10	28
Copoly-(AAm-HEMA)	30	24	0.13	35

It has been stated that the coupling agent might lead to an internal cross-linking of the protein molecules themselves.[18] To check this possibility soluble trypsin was treated with CMC in the same manner as used in the standard procedure of enzyme coupling to matrices, however, in order to facilitate the possible formation of enzyme aggregates higher concentrations of both enzyme and CMC were used. Subsequent analysis using an analytical ultracentrifuge revealed no additional peak in the Schlieren pattern obtained as compared to a reference trypsin solution to which no carbodiimide had been added. This result indicates strongly that aggregation to any extent of the trypsin molecules prior to coupling is not taking place. The possibility of cross-linking of protein molecules once bound to the carrier can of course not be excluded; however, here we would be dealing with enzyme molecules arranged in a type of monolayer fashion around the matrix. From what is said above, the method of binding enzymes to acrylic copolymers by the use of water soluble carbodiimides appears to offer several advantages including simplicity, binding efficiency, high retention of activity and coupling under mild conditions. Using this method other proteins, such as cytochrome *c*, have also been coupled in high yields to these copolymers in the course of this investigation.

A combination of covalently binding enzymes to polymers according to the above method and their simultaneous entrapping within the same polymer

has also been studied using trypsin. The enzyme gel granules obtained in which the enzyme is now kept both embedded within the gel lattice and bound by amide linkages to the gel, showed on the average 20 % of enzymic activity as compared to the corresponding amount of soluble enzyme (Table 2). Control

Table 2. Combined entrapment and chemical binding of trypsin to copolymers.

Polymer	Amount of bound protein (mg protein/g dried polymer)	Coupling yield based on added amount of protein (%)	Proteolytic activity (μmole/min/mg protein)	Ratio bound to free bezyme (%)
Copoly-(AAm-AA)+CMC	50	100	0.063	17
Copoly-(AAM-AA)−CMC	26	52	0.067	18

preparations to which no carbodiimide had been added, carried on the average the same activity. However, in the latter case only 50 % of the added amount of enzyme was present within the gel lattice after the washing procedure had been terminated as compared to 100 % for the diimide treated preparation. In addition during the assay a continuous leakage of trypsin out of the gel (−CMC) was noticed, a situation more likely to occur considering the relatively low molecular weight of trypsin (MW 23 800). Such a diffusion of trypsin out of the gel network was also observed when entrapped in granules of a cross-linked polyacrylamide gel (20 % w/w, containing 5 % of Bis of the total amount of monomer used). Any leakage of enzyme out of carbodiimide-treated preparations was not observed.

From what is said above the use of polymers carrying both embedded and covalently bound enzymes seems to offer certain advantage since here the enzyme molecules remain protected within the gel lattice against the attack of, *e.g.*, proteolytic enzymes present in incubation mixtures, at the same time because of their covalent attachment being prevented from leaking out of the gels. It can be assumed that in these entrapped enzymes a major part of the enzymic activity is due to enzyme molecules near the gel surface. This has recently been shown by Bernfeld [19] to hold true for a highly cross-linked, somewhat similar gel preparation.

The method of coupling enzymes to dextran gels using cyanogen halides, as has been worked out by Porath and Axén,[4,5] has been applied in the present study to a copolymer of acrylamide-hydroxyethyl methacrylate. The coupling yield obtained was about 25 % of the trypsin added with an enzymic activity of about 35 % relative to the corresponding amount of soluble enzyme (Table 1). By varying the amount of monomers present as well as of the degree of cross-linking such gel preparations might be a complement to the "Sephadex" and "Agarose" preparations hitherto used as matrices. The intermediate products are in the latter case believed to be primarily imino carbonic acid esters.[4]

Table 3. Heat stability of trypsin and matrix-bound trypsin preparations.

Sample	Proteolytic activity					
	μmole/min/ mg protein	%	μmole/min/ mg protein	%	μmole/min/ mg protein	%
Non-heated (pH 7.5)	0.37	100	0.12	100	0.13	100
Heated (pH 7.5)	0.056	15	0.05	42	0.04	31
	free trypsin		copoly-(AAm-AA) matrix-bound trypsin		copoly-(AAm-HEMA) matrix-bound trypsin	

As seen in Table 3, matrix-bound trypsin preparations show at a pH of 7.5 considerably higher heat stability (exceeding 100 %) as compared to free enzyme. It seems obvious that the covalent fixation of the trypsin molecules at different sites of the matrix will prevent or impede autodigestion or thermal denaturation. Furthermore, as may be the case with copolymers of the type acrylamide-acrylic acid, the higher hydrogen-ion concentration present in the micro-environment of the bound enzyme as compared to the external solution, may also be responsible for the higher stability obtained. Similar effects have already been found with other negatively charged polyelectrolyte gels to which enzymes have been attached.[20]

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REFERENCES

1. Hicks, G. P. and Updike, S. J. *Anal. Chem.* **38** (1966) 726.
2. Mosbach, K. and Mosbach, R. *Acta Chem. Scand.* **20** (1966) 2807.
3. Pennington, S. N., Brown, H. D., Patel, A. B. and Knowles, C. O. *Biochim. Biophys. Acta* **167** (1968) 479.
4. Axén, R., Porath, J. and Ernback, S. *Nature* **214** (1967) 1302.
5. Porath, J., Axén, R. and Ernback, S. *Nature* **215** (1967) 1491.
6. Kay, G. and Crook, E. M. *Nature* **216** (1967) 514.
7. Tosa, T., Mori, T., Fuse, N. and Chibata, I. *Biotech. Bioeng.* **9** (1967) 603.
8. Silman, I. H. and Katchalski, E. *Ann. Rev. Biochem.* **35** (1966) 873.
9. Goldstein, L. and Katchalski, E. *Z. Anal. Chem.* **243** (1968) 375.
10. Mosbach, K. and Larsson, P. O. *Biotech. Bioeng.* **12** (1970) 19.
11. Mosbach, K. and Mattiasson, B. *Acta Chem. Scand.* **24** (1970) 2093.
12. Weliky, N., Weetall, H. H., Gilden, R. V. and Campbell, D. H. *Immunochem.* **1** (1964) 219.
13. Weetall, H. H. and Weliky, N. *Nature* **204** (1964) 896.
14. Weliky, N. and Weetall, H. H. *Immunochem.* **2** (1965) 293.
15. Simon, E. J. and Shemin, D. *J. Am. Chem. Soc.* **75** (1953) 2520.
16. Reio, L. *J. Chromatog.* **1** (1958) 338.

17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
18. Kay, G. *Process Biochemistry*, August (1968) 36.
19. Bernfeld, P., Bieber, R. E. and MacDonnel, P. C. *Arch. Biochem. Biophys.* **127** (1968) 779.
20. Levin, Y., Pecht, M., Goldstein, L. and Katchalski, E. *Biochemistry* **3** (1964) 1905.

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