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BIOAFFINITY CHROMATOGRAPHY-METHODOLOGY AND APPLICATION

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The term "affinity chromatography" was coined in 1968 by Cuatrecasas, Wilchek, and Anfinsen [1]. All kinds of chromatography except those depending on exclusion effects are based on affinity interactions of some kind between the solutes to be separated and the solid phase. The term is therefore too indeterminate to describe a method characterized by biological or biochemical recognition phenomena. Bioaffinity chromatography is suggested [2, 3].

Bioaffinity methods were introduced at about the same time as Tsvett made his plant pigment fractionations on columns of powdered calcium carbonate, i.e., in the first decade of this century. Hedin [4, 5] seems to have been the first one to utilize specific elution with substrate (casein) to increase the separation efficiency in batchwise purification of charcoal adsorbed trypsin. A few years later Starkenstein [6] bound liver amylase to insoluble starch. In following papers by Bockestein [7], Holmberg [8], Blom et al. [9], and others in 1920-1940, rather remarkable observations were described, e.g., the adsorption dependence on temperature and the dielectric properties of the solvent medium. Even more advanced were the experiments of

Thayer [10]. He used a substrate gradient for separation of bacterial alpha- and beta-amylase. There are interesting observations and suggestions (e.g., by Schwimmer and Balls [11, 12]) that anticipated the concept of the spacer effect.

Leloir [13] in Argentina, who made such fundamental contributions to the present knowledge of the metabolic pathways in the biosynthesis of oligosaccharides and interconversion of the monosaccharides that he received the Nobel Prize, used rather advanced bioaffinity chromatography to isolate several of the enzymes involved in these interconversion reactions.

Specific immunosorption as an analytical and diagnostic tool dates back decades. One of the earliest attempts, if not the earliest, to design an immunochromatographic procedure was made by Svensson, one of the inventors of electrofocusing [14]. He adsorbed diazotized serum albumin on charcoal and tried with some success to isolate the corresponding antibodies. The most important early contributions in the immunosorption field came from Pauling's laboratory by Campbell, Lenscher, and Lerman [15].

Lerman in 1953 [16] published the first paper in which an enzyme inhibitor was attached to a solid support (cellulose) and used for selective adsorption of the enzyme (Tyrosinase). In 1960 [17, 18] Arsenis and McCormick published a series of relevant papers in which flavin compounds were covalently bound to cellulose and used to purify flavin-dependent enzymes. Among other historically important contributions I would like especially to mention those of Fritz, Werle, and their associates in Germany [19, 20], who extensively purified proteases and protease inhibitors.

In Uppsala, Rhodén, Flodin, and I started in 1959 attempts to use cross-linked dextran (Sephadex) as support for blood group substances with the hope of purifying isoagglutinins. These studies were discontinued but taken up many years later by Kristiansen, Sundberg and myself (see, e.g., Refs. 21 and 22). Miller-Anderson and I [23] synthesized avidin-Sephadex and biotin-Sephadex and studied the adsorption behavior of labeled biotin on

the former and avidin on the latter. Whereas biotin could be easily recovered from avidin-Sephadex, the avidin yield from egg-white was at best 35%. The purification factor was extremely high and could not be assessed with certainty. It was estimated to be in the range 20,000 to 100,000. On use, the adsorbent lost an intolerable part of its capacity.

The breakthrough came with the introduction of cyanogen bromide [24] as a coupling method and agarose as support [25]. Immobilized enzymes of high catalytic power were readily obtained, and the biospecific adsorbents gave very satisfactory results both in batch and column procedures.

We were very surprised indeed when the paper of Cuatrecasas, Wilchek, and Anfinsen [1] appeared only one year after we had published the BrCN method-a lagtime so short in comparison with the years it took until Sephadex was generally recognized! No doubt this fortunate situation reflects the adoptability of prepared minds.

In order to enrich to a maximum extent compounds present in trace amounts, it is necessary to suppress nonspecific interactions. The latter may otherwise predominate and decrease the separation power, thus making the procedure less attractive. Instead of an approximately 10,000-fold purification, the result may be two or three orders of magnitude lower. This very fact explains why it took so long a time until bioaffinity chromatography became a routine procedure for protein isolation.

We have studied in detail the requirements for increasing the specificity up to a maximum. Particular emphasis has been made on the solid support or matrix and the method of anchoring the ligand to become an efficient adsorption center. The development of effective adsorbents has been described in original papers [25, 48-50, 56] and in review articles [2, 3, 26-42].

Agarose has been selected as the starting material that most closely approaches the ideal one for preparing adsorbents for biopolymers. Agar and agarose chemistry is well-known, especially

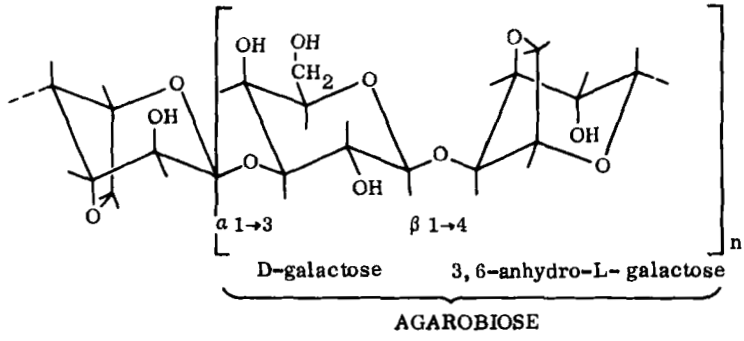
from the works of Araki in Japan [45] and Rees, Duckworth, and collaborators in England [46, 47]. The chemical structure is unique, as is also the physicochemical structure of agar(ose) gels. The former renders agar(ose) resistant to bacterial attacks and enzyme breakdown, which is unusual among polysaccharides. It also determines the macroporous gel structure. Figure 1(a) shows how the agarose polysaccharide chain is built up of agarobiose units (from Laas [48]). Figure 1(b) indicates schematically the structure of agarose gel (right) with junction zones of polymer aggregates. The gel structure composed of cross-linked separate polymer chains is shown for comparison (left).

The polysaccharide chains form parallel double helices in the gel state. The helix is internally stabilized by hydrogen bonds and, due to imperfections in the structure, each agarose chain will join 8 to 10 other molecules to form bundles. The consequence of this lining up of the polysaccharide chains will be a gel containing macroporous cavities supported by multiple strands of polymers. Such a structure appears to be unique and distinctly different from the macroporous gels of cross-linked synthetic polymers. It also explains some interesting features of importance for production of agar(ose)-based adsorbents with desirable properties.

If agarose is cross-linked in solution, e.g., at 65 to 70°C, a gel is formed which has much lower permeability for proteins than that common spontaneously formed (unpublished observation). The former kind of gel has a structure consistent with the one indicated in Figure 1(b), and similar to Sephadex and common cross-linked polymers.

If aged, spontaneously formed agar(ose) gels are cross-linked whereby the gel structure is largely preserved and fixed, so that it can survive heating to 100°C and above.

There is, however, apparently a latitude in free movement within the polysaccharide chain bundles which explains the elasticity and compressibility of the gel. By cross-linking with



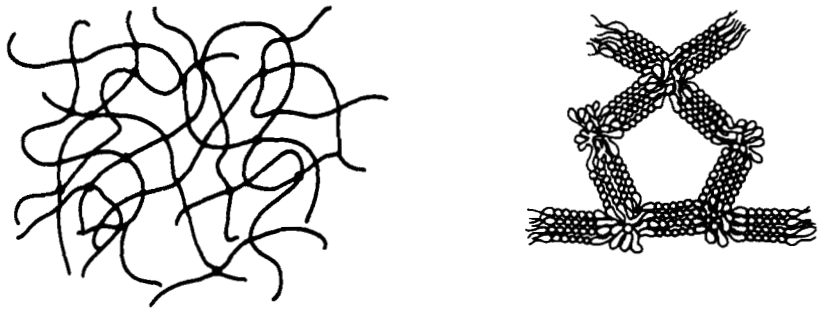


FIG. 1

(a) Main features of the structure of agarose. (b) Schematic structures of a synthetic gel (e.g., Sephadex) (left) and agaro(ose) (right) with polymer aggregates forming "junction zones" (after Låås).

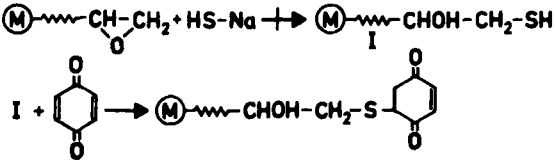
bifunctional reagents of a certain chain length, the bundles will be locked and the strands will lose most of their motility. Consequently, the gel will be mechanically reinforced by such cross-links. Divinylsulfone is a cross-linking agent that will increase the gel strength to an extent that makes possible flow rates in beds of beaded gel 10 to 30 times higher than can be obtained with common agarose gels.

It is possible to cross-link an agar(ose) gel, consisting of 2% matrix, with divinylsulfone to increase the matrix density so it approaches twice the original value without significantly affecting the permeability. This can only be explained on the assumption that the large internal cavities are empty and cannot be bridged over either by single polysaccharide strands or by the cross-linker.

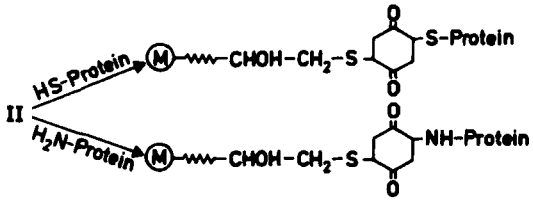
The unique structure is also reflected in the substitution conditions for small and large molecular size solutes. When reactive groups are introduced in the agar(ose) matrix, only a small part of them will be available for attachment of macromolecular substances. A major part will be available for substitution with small-sized solutes such as glycine, but a sizable portion of the groups may not be readily accessible even to glycine [50]. Presumably only reactive groups at the cavity linings will take part in protein coupling. The spacer effect, so much discussed, may be of importance for projecting the electrophilic groups of the activated matrix out into the bulk solution of the cavities and preventing coupling to groups located in regions where the ligands would be sterically hindered to interact with the counter ligands.

Every extra manipulation with the matrix should be regarded with suspicion. Occasionally the adsorbent may be improved, but frequently the opposite is true. The spacer may or may not relieve the obstruction imposed by the matrix. The concept of the spacer action was immediately accepted in most quarters. However, precautions are recommended in selection of spacer substance and interpretation of results in terms of biospecificity. It was an important discovery and a milestone when O'Carra and Barry [37] showed that the thiogalactoside-agarose lacks the presumed specificity toward β-galactosidase. The spacer itself was found to be responsible for the affinity. It is now clear that the spacer, if the introduction of such a structure is deemed necessary, should be hydrophilic, flexible, and neutral. Wilchek

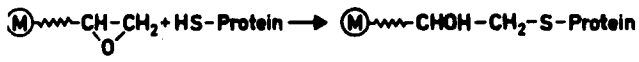
and Cuatrecasas have designed different kinds of spacer substances [51, 52]. A ligand spacer unit containing a spacer terminal nucleophilic group, NH_2 or SH, can be synthesized and subsequently be attached to the matrix. This technique is preferable to those where the spacer is built up from the matrix by solid phase synthesis. The latter will frequently result in the introduction of adsorption centers that cannot easily be removed. For example, the intermediates often contain amino or carboxyl groups. Condensation reactions such as those based on carbodiimide for water extraction will not go to completion and in addition they may introduce new substituents which in turn may cause nonspecific adsorption to occur. We have preferred to use a spacer forming reagent in the activation procedure. This is clearly demonstrated by the bisoxirane attachment [44, 50]. The weak point in the bisoxirane attachment is a sluggish reaction with many proteins in an acceptable pH-range. We are presently attempting to improve the conditions for coupling by inserting an intermediate stage with elevated reactivity compared to that of oxirane, e.g., over benzoquinone:



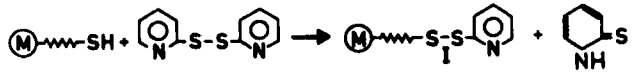
Coupling with proteins will then proceed over SH or NH_2 at lower pH:

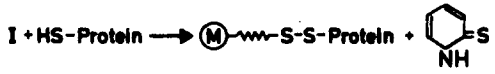


The method of attachment is frequently of crucial importance for the successful use of an immobilized ligand. Since the immobilization problem has been covered in detail in many reviews [3, 26-42], it may suffice in this context to point out that we are guided by the principle of avoiding introduction of substituents which will increase nonspecific adsorption. This implies that reagents which introduce ionic or ionogenic groups should be avoided. Also, highly hydrophobic or aromatic reagents are not permitted. A further restriction is mandatory: the linkages formed should be stable. When we consider all these requirements and the fact that an introduced spacer must have balanced hydrophilic (amphipatic) properties, we realize that very few reagents are possible. Divinylsulfone, dibromodiacetyl, n-butane-1, 4-diol-bisglycidyl ether, polymerized glutaraldehyde, and benzoquinone may serve as examples of such reagents. Cyanogen bromide, the most commonly used reagent for protein coupling, reacts in a complicated fashion to yield mixtures of imido carbonate-, isourea-, and carbamate linkages, where the two first mentioned are charge preserving. Bisoxiranes may be used for the coupling of proteins via thiol groups:



Another interesting coupling procedure is based on thiol-disulfide interchange. This method is introduced by Brocklehurst and collaborators in Professor Crook's laboratory in London [54]. A thiol containing gel is activated by 2,2'-dipyridyl disulfide:





The method was further studied by Carlsson and Axén in my laboratory [55].

The leakage problem is of much concern, especially in connection with adsorbents for immunoassays. It arises as a consequence of unstable attachment, solubilization of matrix substance, or enzymic cleavage of ligand or parts of the ligand. We have cross-linked the agar to suppress leakage due to solubility. Cross-linked agarose is now commercially available (Pharmacia Fine Chemicals). Cyanogen-bromide-activated agarose yields a variety of connector groups, one or two of which appear not to be completely stable. Multipoint attachment can bring down the leakage of ligand substance, but this is likely to create new problems: steric hindrance and extinction of ability to form adsorption complex. Another way is to use an agent for activation that will secure formation of an extremely stable bond. Bisoxirane is such an agent.

Isolation of proteins and naturally occurring substances is just as much an art as a science. Bioaffinity chromatography evokes the hope to short-cut isolation procedures. Arguments in favor of such hopes are not lacking, but there are indeed still many problems to be solved.

Prefractionation may be necessary even if adsorbents are available for application of biospecific methods. Among possible alternatives the best fractionation route is seldom obvious. Also, employment of highly selective adsorption procedures may require additional fractionation methods, since the desorbed material may consist of a mixture of many substances, all with affinity for the ligand, but differing in molecular size, distribution of surface exposed ionic or hydrophobic groups, etc. Only experience makes possible the design of optimal fractionation conditions for each particular separation problem.

A problem of great importance for the fractionation strategy concerns the selection of ligand. Should the ligand be highly specific or selective, or should it exhibit group specificity? If

the former is desirable, one has to synthesize or isolate the ligand substance from natural sources. This in itself may be just as difficult as to use nonspecific methods for isolation of the desired substance. If a reusable adsorbent can be obtained, it may be worthwhile to engage in lengthy procedures for the preparation of the efficient bioadsorbent. In other cases it may be more time and labor saving to prepare group specific adsorbent and instead increase the specificity in chromatography by employing specific elution techniques.

Mosbach [57, 58], Dean and Lowe [59], O'Carra [37], Tesser [60], and others have prepared nucleotide containing adsorbents for purification and isolation of enzymes with nucleotides as cofactors or modifiers. Such group-specific adsorbents are extremely versatile. From a practical point of view the gain in convenience far overshadows the negative consequences of a lower affinity. Cofactor adsorbents can be further developed in other areas, e.g., pyridoxial derivatives for transaminases [41] and specific elution techniques can be further advanced. The methods of attachments can also be much simplified.

We find numerous examples in the literature of group-specific adsorbents directed toward proteolytic enzymes with substrate analogs or inhibitors as ligands. Conversely, enzyme inhibitors may be isolated by chromatography on beaded immobilized enzymes (for references see the reviews 2, 3, 26-42).

Attachment of monosaccharides to the matrix will result in adsorbents which are group specific for the carbohydrate ligand. For example, by coupling galactose or lactose to agarose, a highly selective adsorbent is obtained for lectins which can react with terminal galactose. The saccharide coupling can be easily accomplished by divinylsulfone:

$$\text{Ⓜ-OH} + H_2C{=}CH{-}SO_2{-}CH{=}CH_2 \rightarrow \underset{\text{I}}{\text{Ⓜ-O-}CH_2{-}CH_2{-}SO_2{-}CH{=}CH_2}$$

$$\text{I} + ROH \longrightarrow \text{Ⓜ-O-}CH_2{-}CH_2{-}SO_2{-}CH_2{-}CH_2{-}O{-}R$$

The lectins in turn may serve as group-specific adsorbents for mono-, oligo-, and polysaccharides, glycoproteins and presumably also for glycolipids. The lectin-agarose gels may turn out to be extremely useful for isolation of membrane fragments and viruses [61, 62].

The reciprocal interrelationships between hormones and hormone receptors can be studied by bioaffinity chromatography and used to advantage for isolations of the latter [63-65].

Low molecular weight biologically active compounds such as vitamins and steroid hormones are frequently bound to carriers in blood and other fluids for nutrient and waste product distribution. In immobilized form these substances can be used for isolation or analysis of their corresponding complex partners, i.e., estradiol-agarose for isolation of estradiol carriers [66]. Since steroids are highly hydrophobic, we encounter in the corresponding adsorbents an inherent weakness or limitation of the bioaffinity methods. The ligand is amenable to interact in a nonspecific way with hydrophobic compounds including many proteins. If the matrix is highly substituted with steroid ligands, it will become a strongly hydrophobic adsorbent. Presumably the nonspecific interaction can be suppressed by mixing the solvent with moderately polar substances such as ethylene glycol and dimethylsulfoxide. The pitfall has not always been realized by those who have used the bioaffinity methods.

Immunosorption expands the affinity methods to almost unlimited fields of application. Antigen and antibody isolation by immunological recognition dependent procedures have been practiced since early years of this century. However, technical improvements in connection with enzyme-affinity chromatography have also greatly improved immunosorption methods. They can be used for purification of small molecular size compounds as well as for macromolecules. I may only mention the brilliant work of Wilchek, Gival, and co-workers on the development of affinity procedures for isolation of peptides from protein digests [67, 68].

I have touched upon some weak points at the present stage in bioaffinity chromatography. Some of them may not be so serious in the laboratory and may soon be oversome. Other qualification requirements must also be met in industry, notably the economics. Mechanically rigid, inexpensive supports or matrices may not be easy to produce. In attempts to find such materials it is likely that complications will arise due to extensive nonspecific adsorption. For example, ceramic matrix materials may show excellent mechanical properties but intolerable adsorption characteristics. Because of their unique properties, agar and agar derivatives are likely to continue to be the supports of choice, if other polysaccharides or even synthetic polymers such as Speron find their special fields of application.

REFERENCES

[1] P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, Proc. Natl. Acad. Sci. U. S. A., 61, 636 (1968).
[2] J. Porath, Biochimie, 55, 943 (1973).
[3] J. Porath, and T. Kristiansen, in The Proteins, Vol. 1, 3rd ed. (H. Neurath and R. L. Hill, eds.), Academic, London, 1975, pp. 95-178.
[4] S. G. Hedin, Biochem. J., 2, (1907).
[5] S. G. Hedin, Z. Physiol. Chem., 50, 497 (1907).
[6] E. Starkenstein, Biochem. Z., 24, 210 (1910).
[7] P. T. Bockestein, Acta Brevia Neerl., Physiol. Pharmacol. Microbiol., 2, 132 (1932).
[8] O. Holmberg, Biochem. Z., 258, 134 (1933).
[9] J. Blom, A. Bak, and B. Brae, Z. Physiol. Chem., 250, 104 (1937).
[10] P. S. Thayer, J. Bacteriol., 66, 656 (1953).
[11] S. Schwimmer and A. K. Balls, J. Biol. Chem., 179, 1063 (1949).
[12] S. Schwimmer and A. K. Balls, Ibid., 180, 883 (1949).
[13] L. F. Leloir and S. H. Goldenberg, Ibid., 235, 919 (1960).
[14] H. Svensson, Kem. Min. Geol., 17A (5), (1943).
[15] D. H. Campbell, E. Leuscher, and L. S. Lerman, Proc. Natl. Acad. Sci. U. S. A., 37, 575 (1951).
[16] L. S. Lerman, Ibid., 39, 232 (1953).
[17] C. Arsenis and D. B. McCormick, J. Biol. Chem., 239, 3093 (1964).
[18] C. Arsenis and D. B. McCormick, Ibid., 241, 330 (1966).
[19] H. Fritz, H. Schult, M. Hutzel, M. Wiedermann, and E. Werle, Hoppe-Seyler's Z. Physiol. Chem., 348, 308 (1966).

[20] H. Fritz, G. Wunderer, and B. Dittman, Ibid., 353, 893 (1972).
[21] T. Kristiansen, L. Sundberg and J. Porath, Biochim. Biophys. Acta, 184, 93 (1969).
[22] J. Porath, Nature (London), 218, 834 (1968).
[23] J. Porath, Gamma Globulins, Proceedings of the Nobel Symposium, 3rd., Halsted, New York, 1967, 1967, p. 287.
[24] R. Axen, J. Porath, and S. Ernback, Nature (London), 214, 1302 (1967).
[25] J. Porath, R. Axén, and S. Ernback, Ibid., 215, 1491, (1967).
[26] P. Cuatrecasas and C. B. Anfinsen, Methods Enzymol., 22, 345 (1971).
[27] J. Porath, and L. Sundberg in The Chemistry of Biosurfaces, Vol. 2 (M. L. Hair, ed.) Dekker, New York, (1972), p. 653.
[28] G. Feinstein, Biochim. Biophys. Acta, 236, 74 (1971).
[29] F. Friedberg, Chromatogr. Rev., 14, 121 (1971).
[30] R. H. Reiner and A. Walch, Chromatographia, 4, 578 (1971).
[31] J. Chudzik and A. Koj, Postepy Biochem., 18, 73 (1972).
[32] V. Kocemba-Sliwowska, Ibid., 18, 59 (1972).
[33] H. H. Weetall, in The Chemistry of Biosurfaces, Vol. 2 (M. L. Hair, ed.), Dekker, New York, (1972), p. 597.
[34] H. O. Orth and W. Brummer, Angew. Chem., Int. Ed. Engl., 11, 249 (1972).
[35] P. Cuatrecasas, Adv. Enzymol., 36, 29 (1972).
[36] O. Pihar, Chem. Listy, 65(7), 713 (1971).
[37] P. O'Carra, S. Barry, and T. Griffin, Biochem. Soc. Trans., 1, 289 (1973).
[38] H. Guilford, Chem. Soc. Rev., 1973, 249.
[39] H. H. Weetall, Sep. Purif. Methods, 2(2), 199 (1973).
[40] M. Wilchek, Adv. Exp. Med. Biol., 42, 15 (1974).
[41] P. O'Carra, in Industrial Aspects of Biochemistry (B. Spencer, ed.), Fed. of European Societies, North Holland, 1974, p. 174.
[42] J. Turkova, J. Chromatogr., 91, 267 (1974).
[43] M. J. Comer, D. B. Craven, A. Atkinson, and D. G. Dean, Eur. J. Biochem., 55, 201 (1975).
[44] W. B. Jakoby and M. Wilchek, eds., Methods Enzymol., 34B, (1974).
[45] C. Araki, Bull. Chem. Soc. Japan, 29, 543 (1956).
[46] D. A. Rees, Chem. Ind., 16, 630 (1972).
[47] M. Duckworth and W. Yaphe, Carbohydr. Res., 16, 189 (1971).
[48] T. Laas, "Agar Derivatives for Chromatography, Electrophoresis and Gel-bound Enzymes," Dissertation, Uppsala, 1975.
[49] J. Porath, T. Laas, and J.-C. Janson, J. Chromatogr., 103, 49 (1975).
[50] L. Sundberg and J. Porath, Ibid., 90, 87 (1974).
[51] P. Cuatrecasas, J. Biol. Chem., 245, 3059 (1970).
[52] M. Wilchek, FEBS Lett., 33, 70 (1973).
[53] J. Brandt, L.-O. Andersson, and J. Porath, Biochim. Biophys. Acta, 386, 196 (1975).
[54] K. Brocklehurst, J. Carlsson, M. P. J. Kierstan, and E. M. Crook, Biochem. J., 133, 573 (1973).

[55] J. Carlsson, R. Axen, K. Brocklehurst, and E. M. Crook, Eur. J. Biochem., 44, 189 (1974).
[56] J. Porath, J.-C. Janson, and T. Laas, J. Chromatogr., 60, 167 (1971).
[57] K. Mosbach, H. Guilford, P.-O. Larsson, R. Ohlsson and M. Scott, Biochem. J., 125, 20 (1971).
[58] K. Mosbach, H. Guilford, R. Ohlsson, and M. Scott, Ibid., 127, 625 (1972).
[59] C. R. Lowe and P. D. G. Dean, FEBS Lett., 14, 313 (1971).
[60] G. J. Tesser, H.-V. Fisch, and R. Schwyzer, Ibid., 23, 56 (1972).
[61] T. Kristiansen, in Protides of the Biological Fluids, XXIII Colloquium, Bruges 1975 (H. Peeters, ed.), Pergamon, Elmsford.
[62] M. J. Hayman, J. J. Skekel, and M. J. Crumpton, FEBS Lett., 29, 185 (1973).
[63] P. Cuatrecasas, Proc. Natl. Acad. Sci. U. S. A., 69, 1277 (1972).
[64] E. Karlsson, E. Heilbronn, and L. Widlund, FEBS Lett., 28, 107 (1972).
[65] J. Schmidt and M. A. Raftery, Biochem. Biophys. Res. Commun., 49, 572 (1972).
[66] J. C. Nicolas, M. Pons, B. Descomps, and A. Crastes de Paulet, FEBS Lett., 23, 175, (1972).
[67] D. Givol, Y. Weinstein, M. Gorecki, and M. Wilchek, Biochem. Biophys. Res. Commun., 38, 827 (1970).
[68] M. Wilchek, V. Bocchini, M. Becker, and D. Givol, Biochemistry, 10, 2828 (1971).