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Affinity Chromatography of Enzymes

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The main principles and fields of application of a new method of enzyme chromatography, the so called "affinity" chromatography, are discussed. The method is based on the employment of an insoluble ligand of the enzyme (substrate, inhibitor, cofactor, etc.), that is sorption takes place owing to specific forces associated with affinities which constitute the basis of the biological function of the enzyme. The use of sorbents based on such ligands permits a more selective and effective isolation of pure enzymes from various sources compared with the usual types of sorbents, for example ion-exchange resins. In addition the review considers certain other techniques employing the specific affinity of the enzyme for the ligand in enzyme chromatography. Affinity chromatography can also be used to fractionate the products of chemical modification of enzymes and to investigate the physicochemical properties of enzyme-ligand complexes.
The bibliography includes 144 references.

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I. INTRODUCTION

Numerous studies based on the selective binding of enzymes to ligands (substrates, inhibitors, coenzymes, etc.) for the purification of enzymes by adsorption (mainly chromatographic) methods have been published recently in scientific periodicals. Since the enzyme forms a complex with the ligand due to the so called affinity forces, the method has been given the name of "affinity" chromatography (from the Latin root "affinis"—related to, bearing a relation to), i.e. chromatographic separation of enzymes in accordance with their affinity for the substrate.

The tendency to seek selective sorbents for enzymes is itself quite understandable, since the usual types of chromatography, for example ion-exchange or gel chromatography, are not specific to the nature of enzymes; the purification of enzymes with the aid of these methods constitutes a multistage procedure and usually gives a low yield of the pure product. On the other hand, affinity chromatography normally makes it possible to extract enzymes from complex mixtures in a single stage with a high degree of purity and with a nearly quantitative yield.

Moreover, affinity chromatography is one of the most direct and at the same time simplest methods for the investigation of the formation of enzyme–ligand complexes, so that it can be used for a detailed elucidation of the physicochemical aspect of enzymatic catalysis.

The present review attempts to summarise and give a systematic account of studies in this field and to assess the potentialities of the method.

II. GENERAL PROBLEMS OF AFFINITY SORPTION

According to modern ideas, an enzymatic reaction can be represented by the following general equation

$$E + S \rightleftarrows ES \rightleftarrows EP \rightleftarrows E + P,$$

where E, S, and P are enzyme, substrate, and reaction product respectively. The equation shows that the elementary step in the simplest enzymatic reaction involves the formation of two complexes, namely the enzyme–substrate and enzyme–product complexes.

The first stage of the enzymatic reaction—the formation of the enzyme—substrate complex—take place as a result of specific interaction forces between the enzyme and substrate molecules, called the enzyme–substrate affinity. The few data obtained so far on the structures of enzymes and enzyme–substrate complexes[1-4] show that, in the binding of the enzyme to the substrate a number of non-covalent bonds of various types (hydrogen bonds, polar and non-polar interactions, etc.) are formed between them. Such multi-site contact between the enzyme and substrate molecules, leading to a high selectivity of interaction, is ensured by a specific spatial distribution of the functional groups of the enzyme, which is determined by its unique structure. In this case one usually speaks of the complementarity of the molecular structures of the enzyme and the substrate.

The enzyme–product complex is formed as a result of a chemical transformation of the substrate. Since in the course of this transformation the substrate remains bound to the surface of the enzyme molecule, evidently the enzyme–product complex is in general stabilised by the same forces (or involves the same interactions) as those involved in the stabilisation of the enzyme–substrate complex.

Apart from the complexes with the substrate and the reaction product, many enzymes form, by virtue of their functional specificity, extremely stable complexes with other types of ligands. These include coenzymes, allosteric activators, inhibitors, etc.

When the ligand is in a solid insoluble phase, it can be used as a specific sorbent for the corresponding enzyme. Like any sorbent used in chromatography, the affinity sorbent must have a fairly large surface area, a relatively high mechanical strength, and should not show a tendency to form either dense aggregates or conversely colloidal solutions. In a chromatographic column, the sorbent must allow an eluent flow rate acceptable in practice.

If the natural ligand satisfies these requirements, it can be used directly as the sorbent. However, the number of such sorbents is small and they are restricted to certain

biopolymers, mainly the substrates of digestive enzymes (starch, elastin, cellulose, etc.). In most cases ligands are soluble substances and are converted into an insoluble state by an artificial method. Here the following procedures are possible: (a) the ligand is modified chemically by the introduction of hydrophobic groups which make it insoluble; (b) the ligand is converted into an insoluble state by incorporation in an insoluble polymer which is polymerised for this purpose in a solution containing the ligand; (c) the ligand is attached (covalently or non-covalently) to an insoluble carrier. The procedure involving covalent attachment of the ligand is most frequently employed.

The main requirement which must be met by the affinity sorbent is sorption specificity. In principle, the affinity sorbent should contain only functional groups which ensure the selective binding of one specific enzyme in the absence of sorption of other substances. When the natural ligand is employed, the problem reduces to a test of its homogeneity and native properties, i.e. the absence from it of functional groups non-specific to the given ligand which may appear as a result of chemical side reactions, for example in purification or on storage.

The preparation of a synthetic sorbent requires a correct choice of the ligand, the carrier, and the method of joining the ligand to the carrier. The specificity and strength of the binding of the ligand to the enzyme may be estimated by preliminary tests using various methods, for example kinetic methods.

In order that the ligand which is bound effectively in solution should retain this capacity when attached to the carrier, it is necessary that the attachment should involve groups not participating in the interaction with the enzyme. Exact information about this may be obtained by the X-ray diffraction analysis of the corresponding enzyme – ligand complexes. These data are important for practical affinity sorption, since they constitute the basis for the most complete utilisation of the affinity forces and help to avoid an empirical search in the preparation of the sorbent. However one should note that information of this kind is still extremely limited [1-4].

When the ligand is attached to the carrier, one should also make sure that the functional groups responsible for complex formation are not only free but also accessible for binding to the enzyme in solution. Here we are concerned with possible steric hindrance in the approach of the enzyme molecule to the active surface of the sorbent. In particular, such hindrance may arise if the site of attachment of the ligand to the carrier is in the immediate vicinity of its functional groups involved in binding to the enzyme. In such cases it is desirable to attach the ligand to the carrier via intermediate reagents with a suitable length of molecular chains which function as a kind of "arm" which separates the active groups of the ligand from the surface of the sorbent.

In choosing the carrier one should bear in mind that it must not have a significant influence on sorbent specificity, i.e. that it must not contain functional groups which might give rise to the sorption of extraneous substances. The fact that the ligand is bound to the enzyme as a rule via several non-covalent linkages itself guarantees to some extent a minimal binding of impurities by such a sorbent, in which the ligand is attached to a fairly neutral carrier. Carriers of this type may include, for example, molecular sieves (Sephadex, Biogel, etc.) the very principle of whose action is designed to ensure the absence of non-specific sorption of proteins. Moreover, the choice of the neutal carrier makes it possible not only to eliminate the sorption of impurities but also to avoid a possible non-specific binding of the enzyme itself. In practice the specificity and efficiency of synthetic and natural sorbents must be tested experimentally in each case.

The next problem concerns the conditions governing the sorption and desorption of the enzyme. As stated above, the formation of an enzyme–ligand complex is due to the specific steric structure (conformation) of the enzyme protein molecule, which may change when the conditions in the medium are altered. Therefore it is extremely difficult to assess beforehand the optimal sorption and desorption conditions. On the basis of general considerations, one may expect that the optimal sorption of the enzyme on an affinity sorbent will occur close to the conditions governing its optimal catalytic activity and any departure from these conditions will lead to a weakening of the stability of the enzyme–ligand complex and to desorption of the enzyme.

If electrostatic interactions between the ionogenic groups of the enzyme and the ligand are important for their mutual binding, the stability of such a complex will vary with the pH of the medium and will decrease with increasing ionic strength. The stability of hydrogen-bonded complexes is probably an inverse function of temperature. Moreover, the stability of such complexes decreases in the presence of agents capable of forming hydrogen bonds (for example urea, guanidine, etc.). A similar approach is evidently applicable also to complexes stabilised by "hydrophobic" contact. Thus one of the desorption procedures consists of the weakening of individual types of bonds between the enzyme and the ligand in the complex. In the presence of bonds of different types, the decomposition of the complex requires a combined action of a variety of factors; one must bear in mind that the desorption conditions can then be very severe and can lead to denaturation of the enzyme.

Another desorption procedure, which is more appropriate to enzyme–ligand complexes, involves a weakening of the overall affinity of the enzyme for the ligand by altering the conditions in the medium (pH, ionic strength, temperature, etc.); for example, the conformation of the enzyme can be altered reversibly from complementary to non-complementary. Since the conformation of the protein molecule is as a rule senstive to the medium and the changes in it are of a cooperative type, these reversible conformational changes can occur under extremely mild conditions where the native structure of the enzyme is preserved. In addition, the sorbed enzyme may be desorbed competitively under the influence of the free ligands present in solution.

It is noteworthy that the study of the effect of each individual factor in the medium on the binding of the enzyme to the ligand can result in certain cases in definite deviations from the above general sorption and desorption rules. In principle, the attainment of optimal results in experiments on the isolation and purification of enzymes requires such preliminary study for each individual enzyme. In this case the optimisation of the conditions in the medium can not only ensure an efficient absorption and desorption of the enzyme itself but can also promote the elimination of possible impurities.

Yet another problem concerning the stability of the sorbent must be examined. This includes, first, the stability in relation to the conditions in the medium and, secondly, the resistance to the action of the enzyme. The first factor can be as a rule neglected since the choice of the conditions in the medium is determined in the first place by the stability of the enzymes themselves; it can be

taken into account only in the stage where the sorbent is prepared for the experiment or during its regeneration, which, when necessary, can be carried out under more severe conditions than sorption and desorption†.

As regards the stability of the sorbent to the enzyme, one should consider that it can be chemically transformed under the influence of the enzyme in those cases where a sorbent based on the substrate is employed. Evidently increased contact time between the sorbent and the enzyme can significantly decrease the capacity and specificity of the former although this factor is not common to all enzyme–substrate systems. In particular, one cannot rule out the possibility that under certain conditions in the medium the enzyme retains fully its affinity for the substrate but does not exhibit catalytic activity. In general preference should be given to sorbents where the ligand employed is not the substrate but a competitive inhibitor, which forms with the enzyme a so called "non-productive" complex (i.e. a complex which does not undergo a chemical transformation).

Resistance to enzyme action is important not only for the preservation of the sorption properties of the sorbent but also for the choice of the sorption procedure. The procedure can be static ("in a reservoir") or dynamic (chromatography). If a sorbent based on the substrate is employed, the sorption of the enzyme may be accompanied by a chemical transformation of the substrate with liberation of the reaction products to the surrounding solution. Since the reaction products also show affinity towards the enzyme and form fairly stable complexes with it, the result may be a competitive inhibition of the binding of the enzyme to the sorbent and the retention of part of the enzyme in solution. In those cases where the substrate reacts fairly rapidly and a static sorption procedure is employed, the inhibiting effect of soluble reaction products on the sorption of the enzyme can be very considerable. On the other hand, under dynamic conditions the products formed are continually removed by the stream of eluent flowing through the sorbent bed and have much less effect on the sorption of the enzyme. Therefore the static procedure can be used only with sorbents resistant to enzyme action or the sorption must be carried out under conditions where the enzyme is catalytically inactive; in other cases, the dynamic method is preferable.

The conditions governing the preparation and application of sorbents based on ligands discussed above apply in general also to sorbents based on enzymes. These are sometimes referred to as "enzymatic resins". Sorbents based on enzymes can be used to isolate and purify the corresponding ligands and also to investigate the binding of the ligand to the enzyme (although sorbents based on ligands are more suitable in this case). In view of the relative structural lability of enzymes, one must maintain conditions in the preparation and use of these sorbents which rule out irreversible denaturation of the enzyme.

The next section deals with studies based on using the affinity of the enzyme for the ligand, mainly for preparative purposes, and also data on the preparation of sorbents. A systematic account of the data described below may be based on the differences in the choice of the "sorbent–sorbate" system, which appears to be the most useful classification according to the general postulates concerning affinity sorption.

†In the operating cycle of the sorbent the author distinguishes its preparation for the experiment, the sorption and desorption of the enzyme, and sorbent regeneration (Editor).

III. THE USE OF ENZYME–LIGAND AFFINITY FOR PREPARATIVE PURPOSES

1. Natural Sorbents for Enzymes

The sorption of an enzyme on an insoluble substrate was first observed in 1910 by Starkenstein [5]—this was the sorption of liver amylase on potato starch. Later similar data were published by Ambard [6] for saliva amylase, by Chodat and Philia [7] for malt "diastase", and by Boekestein [8] for pancreatic amylase; in the last study, potato, rice, and wheat starches were tested as the sorbents.

Subsequently sorption on starch was widely used to isolate and purify amylases from various sources [9–20]. It was found that starch is a selective sorbent for α-amylase and does not sorb β-amylase. Thus Holmberg [9,10] discovered that malt α-amylase can be separated from β-amylase by sorption on starch from 50% ethanol solution in the presence of maltose; according to Holmberg's data, the sorbability of α-amylase increased as the concentration of alcohol was raised and the pH and temperature reduced. α-Amylase is an endoglycosidase, i.e. it cleaves the glycosidic linkages within the starch chain, in contrast to β-amylase, which splits off maltose residues from the non-reducing end of the carbohydrate chain. Therefore maltose, being a product of the activity of β-amylase, might have inhibited its binding to starch.

In a study of the sorbability of malt α-amylase on various starch and glycogen ("animal starch") specimens, Schwimmer and Balls [14] established that starch freshly precipitated with alcohol is the most effective sorbent; however, the sorbability of α-amylase on glycogen was highest. Bearing this in mind, Schwimmer and Balls [14] concluded that the efficiency of the sorption of α-amylase is determined by the area of the sorbent formed by maltoside chains with a length equivalent to 8 — 10 units and accessible to the enzyme in solution.

Schwimmer and Balls [15] purified malt α-amylase by chromatography on a column with wheat starch. The sorption was carried out at 5°C in the presence of 40% ethanol and calcium salts; the enzyme was desorbed with water saturated with calcium sulphate at room temperature. α-Amylase was isolated from the eluate in a crystalline state.

Thayer [17] separated the α- and β-amylases from *Pseudomonas saccharophila* by chromatography on a column with a mixture of potato starch and Celite. When the culture liquid was passed at 4°C through a column washed with water, only α-amylase was sorbed. Buffer solutions at different pH and with different calcium salt and maltose contents did not elute the sorbed enzyme. Elution was achieved using a concentration gradient of soluble starch and was very effective: the enzyme emerged in a sharp peak (3 ml of the eluate contained 75% of the total amount of the sorbed enzyme) and was concentrated by a factor of 100 compared with the initial solution. It is interesting to note that both amylases had the same pH activity optimum but differed sharply in their capacity for sorption on starch [17].

Markowitz et al. [18] reproduced Thayer's method [17] and obtained a highly purified crystalline preparation of α-amylase from *P. saccharophila*. A variant of this technique was employed by Neumann et al. [20] to isolate amylase from dog liver; the enzyme was purified by a factor of 23–147 with a yield of 45–75%.

Comparatively recently Vainer and Shul'man [21] published a report on the possibility of using starch to isolate amylase for practical purposes.

Leloir et al.[22] observed that insoluble starch can also be used as a sorbent for the purification of bean starch synthetase. Akazawa and Murata[23] and Tanaka and Akazawa[24] showed that starch synthetase is bound preferentially to amylose (a linear polysaccharide) rather than to the branched chain of amylopectin; the maximum sorption of starch synthetase on amylose occurred at a neutral pH and a low temperature but decreased sharply as the pH was reduced (to pH 5.0) and the temperature raised (above 25°C).

De la Haba[25] used sorption on starch for the purification of glycogen phosphorylase from rabbit muscle. When the whole muscle extract was passed through a column with potato starch at 3°C, the enzyme was completely bound to the sorbent; desorption was achieved by eluting the column with the soluble substrate (0.4% glycogen solution) and by raising the temperature simultaneously to 14°C. The enzyme was purified in this way by a factor of 33 with a yield of 100%; the specific activity of the resulting preparation corresponded to the activity of the doubly recrystallised enzyme. De la Haba[25] notes that the behaviour of glycogen phosphorylases *a* and *b* was identical under the conditions tested.

Hanabusa and Kohno[26] described a variant of the de la Haba method[25] in which the enzyme was desorbed solely by increasing the column temperature; they found this procedure more convenient, since it yields the enzyme without an admixture of glycogen and further purification is not needed.

Selinger and Schramm[27] studied the binding of glycogen phosphorylase to glycogen. They found that at pH 6.8 and a temperature of about 0°C phosphorylase *a* forms an insoluble enzyme–substrate complex with glycogen (up to 62% of the enzyme was precipitated). A similar complex was not formed with dextran, mannan, and levan, which indicated the specificity of the binding of phosphorylase *a* to glycogen. When the pH was changed to 5.0 or 9.0, the complex remained stable but at pH 4.0 it decomposed with loss of enzyme activity. An increase of temperature promoted the dissociation of the complex. It is interesting that the addition of α-amylase to the insoluble complex even at 0°C resulted in its complete decomposition in several minutes. According to the authors[27], this implies that phosphorylase *a* forms an insoluble complex only with native glycogen but not with its amylase-catalysed hydrolysis products, among which there are high-molecular macrodextrins resistant to the action of amylase[28]. Under comparable conditions, phosphorylase *b* was precipitated only in the presence of magnesium salts[27]. (This finding can probably be used for the separation of phosphorylases *a* and *b*.)

Leloir and Goldemberg[29,30] used the selective coprecipitation of glycogen synthetase with glycogen for the purification of specimens of this enzyme obtained from different sources. Rat liver glycogen synthetase was purified in this way by a factor of 300 and contained as impurities only small amounts of glycogen phosphorylase and amylase; however, the yield of the enzyme was low (only 12%).[29]

Loyter and Schramm[31] found that glycogen is able to bind α-amylase and used this property to isolate and purify the enzyme. The maximum coprecipitation of α-amylase with glycogen occurred at pH 8.0, a temperature of 0–3°C, and after the addition of ethanol up to a concentration of 40%. Subsequently Schramm et al.[32] found that not only glycogen but also macrodextrins, which are produced by the amylase-catalysed hydrolysis of glycogen and are resistant to the further action of amylase[28], are also capable of forming an insoluble complex with amylase. It is interesting that calcium ions, required for the enzymatic activity of α-amylase, were also necessary for its binding to glycogen and macrodextrin. Thus, when calcium ions were removed from solution by ethylenediaminetetra-acetate, an insoluble complex was not formed. Strontium and barium ions showed a similar activating effect, but cadmium and magnesium ions lacked this capacity. Schramm et al.[32] suggested that calcium ions are required either to maintain the active conformation of the enzyme or are involved directly in the binding of the substrate.

The above data on the binding of enzymes active towards the α-(1 → 4)-glucosidic linkage (i.e. capable of cleaving or synthesising this linkage) to various α-(1 → 4)-polyglucosides show that such binding is extremely selective. For example, starch effectively sorbs α-amylase but not β-amylase; starch synthetase, which is bound to amylose but not to amylopectin, exhibits a definite selectivity towards the polysaccharide. On the other hand, the selectivity is not absolute. Thus, apart from α-amylase, starch sorbs effectively starch synthetase and glycogen phosphorylase; similarly, glycogen ("animal starch") binds glycogen synthetase and α-amylase in addition to glycogen phosphorylase.

One might have expected that all the enzymes acting on α-(1 → 4)-polyglucosides would exhibit similar functional properties in relation to binding of the substrates in view of the similarity of the chemical properties of the latter. Apparently this happens to some extent although the selectivity characteristic of enzymes, depending on the steric structure of the substrate molecules and possibly on supermolecular structural factors, is shown in many cases. Moreover, the conditions in the medium where optimal binding takes place are different for different enzymes, which is evidently due to the individual characteristics of the structure of their protein molecules. This factor probably makes it possible to isolate in a pure form any enzyme without admixtures (or with minimal amounts of admixtures) of other related enzymes.

Natural sorbents have been used to isolate and purify a number of other enzymes. Jeuniaux[33–35] isolated a chitinase from soil streptomycetes by selective sorption on chitin from whole culture liquid. After the chitinase had been bound, the chitin with the sorbed enzyme was extracted and washed. The enzyme was not desorbed; instead the chitin was subjected to total hydrolysis by the chitinase and the soluble hydrolysis products were removed by dialysis. The chitinase was purified in this way by a factor of 70 with a yield of 27%. The resulting preparation contained no proteases, cellulases, pigments, and reducing sugars and the amount of protein impurities without chitinolytic activity was less than 5%.

The study by Grant and Robins[36] on the purification of elastase from pig pancreas showed that not only the active enzyme but also zymogen (proelastase) are bound with equal efficiency to elastin; this observation enabled the authors to reach the very important conclusion that the substrate binding centre, specifically the catalytic centre in the elastase molecule, is structurally autonomous. Gertler and Birk[37] purified proelastase using its adsorption on elastin.

Orekhovich et al.[38] investigated the binding of certain proteases (pepsin, trypsin, chymotrypsin, and papain) to comminuted sheep wool and silk fibroin. The experiments were performed at two pH values (3.9 and 7.9). Under these conditions, pepsin and papain were not bound either to wool or to silk. Trypsin and chymotrypsin were

bound fairly satisfactorily to both sorbents and at both pH values (the amounts of bound trypsin and chymotrypsin reached 83 and 75% respectively). Trypsin was desorbed using a solution of cysteine in the presence of urea and chymotrypsin using a solution of histidine in the presence of sodium chloride. Probably the differences in the behaviour of the proteases investigated reflect differences in their specificity.

Ogawa and Toyama [39] used chromatography on a column with powdered cellulose to separate a commercial preparation of cellulase from *Trichoderma viride* into three active components, which differ in their affinities for the substrate.

Reese and Avigad [40] described a method for the purification of levansucrase based on a selective coprecipitation of the enzyme with levan. The enzyme was purified by a factor of more than 2000 with a yield of 31%. Other enzymes (cellulose, β-galactosidase, α-amylase, galactose oxidase, and ribonuclease) were not coprecipitated under these conditions, which confirms the specificity of the binding of levansucrase to levan. A similar procedure was employed by Bauer and Avigad [41] for the purification of fructan hydrolase.

Cherkasov and coworkers [42-44] investigated the conditions governing the formation and decomposition of the enzyme–substrate complex of lysozyme with chitin using the chromatography of the enzyme on a column with the substrate. Two types of complexes, depending on the pH of the medium, were observed. One of the complexes predominates in a weakly acidic medium and is stable only in the presence of salts in solution, decomposing in a salt-free medium. The other complex predominates in a weakly alkaline medium, is stable also in the absence of salts, but decomposes when the solution is acidified. On this basis, two procedures were proposed for the isolation of lysozyme from various natural sources: (a) by sorption at pH ~ 5 in a salt solution followed by desorption with water and (b) sorption at pH 8–9 followed by washing the column with water and desorption with dilute acetic acid. Chitin obtained by standard methods usually contains free amino-groups owing to its partial deacetylation during purification. The presence of amino-groups imparts to such chitin the properties of an anion exchanger and leads to non-specific sorption of acid proteins (such as albumins), which are desorbed with acetic acid. Therefore, when ordinary chitin is employed, only the first procedure is applicable [45,46]. Treatment of ordinary chitin with dilute nitrous acid, which results in the replacement of the amino-groups by neutral hydroxy-groups, eliminates the non-specific sorption and permits the employment of the second procedure, which has the advantage that at pH 8–9 lysozyme does not exhibit a hydrolytic effect on chitin and the complex formed is more stable [47,48]. Lysozyme was isolated by this procedure from hen egg white and from aqueous extracts of horseradish and turnip in almost quantitative yields [45-48]. Chromatography on chitin was also successfully employed by Jensen and coworkers [49,50] for the purification of phage lysozyme. The authors note that the method made it possible to concentrate vanishingly small amounts of the enzyme and at the same time to free it from inhibitors which interfere with the determination of activity in the unpurified solution obtained by the lysis of phages. Thus the yield of lysozyme when isolated by this method was 300%, judging from the activity balance, which can only be explained by the liberation of the enzyme from inhibitors [50].

Among the studies discussed above, a typical adsorption technique was not always employed. For example, in the binding of enzymes to glycogen, the initial components were present in solution and yielded a precipitate only on interaction: this applies also to levansucrase and levan. However, the discussion of these investigations was useful not only because the method is similar to a variant of static adsorption but also because it is possible, in principle, to employ the data on the conditions governing the binding of the enzyme and substrate in experiments with synthetic adsorbents.

2. Synthetic Adsorbents for Enzymes

Erlanger [51] investigated the possibility of isolating and purifying chymotrypsin by binding it to a dipeptide inhibitor converted into a water-insoluble state: the benzyl ester of *N*-benzyloxycarbonyl-L-leucyl-D-phenylalanine (BECLP). It was rendered insoluble by introducing hydrophobic groups into the dipeptide molecule: the benzyl group (in the carboxy-group of phenylalanine) and the benzyloxycarbonyl group (in the amino-group of leucine). Bearing in mind the substrate specificity of chymotrypsin (chymotrypsin hydrolyses peptide bonds formed by the carboxy-groups of aromatic aminoacids), it might have been expected that BECLP would adsorb chymotrypsin. from solution. Indeed, Erlanger observed that chymotrypsin and chymotrypsinogen and pepsin were bound qualitatively to BECLP; trypsin and trypsinogen were also bound but less effectively. Other proteins tested (ribonuclease, human and bovine serum albumins, and β-lactoglobulin) were bound only in insignificant amounts. Erlanger believed that the approximately identical capacities of chymotrypsin and pepsin for binding to BECLP should have been expected in view of the similarity of their substrate specificities. However, the adsorption of trypsin (and trypsinogen) can hardly be due to a specific interaction, since this enzyme hydrolyses peptide bonds formed by the carboxy-groups of diamino-carboxylic acids (arginine and lysine). "Hydrophobic" ligands of the type employed by Erlanger have not found a wide application as sorbents.

Sorbents in which the ligand is linked covalently to an insoluble carrier acquired a greater practical importance for the sorption and chromatography of enzymes. Such sorbents were obtained for the first time by Lerman, who used them to isolate fungal tyrosinase [52]. Certain azophenols, known to inhibit tyrosinase, were chosen as ligands. They were linked to cellulose via an ether bond. Only sorbents whose ligands contain a *p*-hydroxyphenyl group at the free end, i.e. the group which is also present in tyrosine (the substrate of tyrosinase), showed a capacity for the selective sorption of tyrosinase:

1) cellulose —O—C_6H_3(OH)—N=N—C_6H_4—C_6H_4—N=N—C_6H_4—OH,
2) cellulose —O—C_6H_3(OH)—N=N—C_6H_4—N=N—C_6H_4—OH,
3) cellulose —O—CH_2—C_6H_4—N=N—C_6H_4—OH.

Sorbent (3), which was used for the chromatographic isolation of tyrosinase, proved to be the most effective. The enzyme could be purified by a factor of 61 with a yield of 56%.

Arsenis and McCormick [53] purified rat liver flavokinase by chromatography on flavin-substituted cellulose. 6-Amino-9-(1-D-ribityl)isoalloxazine, which is a substrate of flavokinase, and 7-amino-6,9-dimethylisoalloxazine, its

competitive inhibitor, were tested as ligands. In the reaction of these amines with chlorocarbonylmethylcellulose (the acid chloride of carboxymethylcellulose), the corresponding acetamide derivatives were obtained. Both adsorbents were found to be effective for the preparation of highly purified flavokinase.

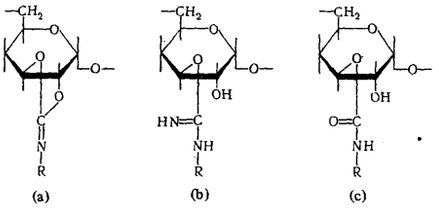
In another study, Arsenis and McCormick [54] investigated the possibility of purifying the apoenzymes of certain oxidoreductases containing flavin mononucleotide (riboflavin phosphate) as the coenzyme. The study was based on the observation that not only flavin mononucleotide but also its monomethyl ester can function effectively as the coenzyme of NADPH-cytochrome *C* reductase, pyridoxin (or pyridoxamine) phosphate oxidase, and glycolate oxidase. Bearing this in mind, the authors obtained esters of flavin mononucleotide with cellulose, phosphocellulose, and diethylaminoethylcellulose and tested them as adsorbents. It was found that, in column chromatography of the above apoenzymes on flavin-substituted cellulose, the enzymes are more firmly retained than on control specimens of celluloses, apparently due to the additional interaction between the apoenzyme and the coenzyme. Almost complete separation of the apoenzyme from impurities was achieved for apoglycollate oxidase; it was purified by a factor of more than 400 in a quantitative yield [54].

Lowe and Dean [55] purified oxidoreductases using adsorbents based on other coenzymes—nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) attached to cellulose. These adsorbents were found to be suitable for the isolation of glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and threonine dehydratase, one chromatographic stage being sufficient for the separation of the enzymes.

Mosolov and Lushnikova [56] described a method for the purification of certain peptidohydrolases based on their selective binding to natural inhibitors of protein origin fixed on cellulose. The adsorbents were prepared by introducing the inhibitors into the azo-coupling reaction with *p*-diazobenzoylcellulose (this method presupposes that the protein inhibitors contain aromatic groups which are not essential for their functioning). Adsorbents based on the soya-bean trypsin inhibitor and the inhibitor of potato tuber proteases were obtained in this way. The adsorbent based on the trypsin inhibitor bound selectively and completely trypsin from solution at 0–3°C at pH 7.7 (1/15 M phosphate buffer); the enzyme was desorbed with dilute hydrochloric acid at pH 2.3; under these conditions, the enzyme–inhibitor complex is fully dissociated and the enzyme itself shows the maximum stability and does not undergo autolysis. Other proteins tested (bovine serum albumin, egg albumin, and ribonuclease) were bound to an insignificant degree, which indicated the specificity of the adsorbent. The efficiency of this adsorbent for the purification of trypsin is indicated by the finding that the specific activity of the crystalline trypsin preparation increased by a factor of 1.5–2 after adsorption and desorption, the preparation being completely free from autolysis products. The sorbent based on the inhibitor of potato tuber proteases was used [56] to purify the total protease preparation from *Actinomices fradiae*. After sorption and desorption, the proteolytic activity of the preparation also increased by a factor of 1.5–2; however, the solution retained an appreciable percentage of the activity, since not all the fractions of the preparation showed a sufficiently high affinity for the inhibitor.

The method of preparation of sorbents proposed by Porath and coworkers [57–60] has recently become most widely used. It consists of covalent binding of the ligand‡ to an insoluble polysaccharide carrier with the aid of cyanogen halides, for example bromocyanogen, and consists of two stages: (a) treatment of the polysaccharide with an aqueous alkaline solution of the cyanogen halide with formation of an intermediate reactive compound and (b) the coupling of the intermediate to the ligand in a neutral or weakly alkaline medium. The carriers can be starch, cellulose, and commercial products–Sephadex (cross-linked dextrin) and Sepharose (cross-linked agarose)—usually employed as molecular sieves. According to Porath and coworkers, the best carrier is Sepharose, which in aqueous solutions forms grains of an extremely porous gel so that not only its surface but also the inner space within the grains is accessible to enzyme molecules. As a result, adsorbents with a high capacity may be obtained on the basis of Sepharose.

An essential condition in the method of Porath and coworkers is the presence in the ligand of a free primary (aliphatic or aromatic) amino-group not required for binding to the enzyme, whereby the ligand can be attached to the carrier; if this group is absent, it must be specially introduced. The mechanisms of the reaction of cyanogen halide with the polysaccharide and of the subsequent stage where the primary amine is attached to the activated polysaccharide have not been finally elucidated. It has only been shown that, when a cyanogen halide reacts with the polysaccharide, a product containing additional nitrogen but not the halogen is formed; on the other hand, when this intermediate interacts with the amine, ammonia is liberated. Porath [59] suggests that this may involve the formation of the following structures:



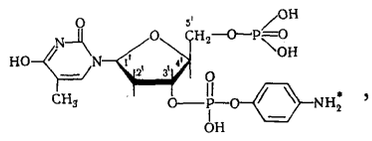
The adsorbents obtained by the method of Porath and coworkers are quite stable in the pH range between 2 and 12, which includes the region of stability of almost all enzymes. In 0.1 *N* hydrochloric acid, only 1.81% of the attached ligand groups are split off from the carrier in 4 days, while in 0.1 *N* sodium hydroxide 42.7% of these groups are removed during the same period; in the latter case the losses probably occur owing to the decomposition of the carrier itself [60].

Despite certain limitations, the method of Porath and coworkers generally makes it possible to obtain a fairly wide and varied selection of starting materials for the coupling of the ligand to the carrier and in this sense it can be regarded as one of the most general methods for preparing affinity sorbents. The preparation and applications of sorbents by this method are discussed below [57–60].

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‡The method is used to attach to the sorbent not only the ligand but also the enzyme. In particular, it is used for the affinity chromatography of inhibitors, transfer RNA, and others—see the fourth International Fermentation Symposium, March, 1972, Kyoto, Japan, Abstracts, pp. 64–65 (Editor).

Cuatrecasas et al. [61] were the first to use this procedure to prepare sorbents for enzymes: the nuclease from *Staphylococcus aureus*, α-chymotrypsin, and carboxypeptidase A. The substrate of the nuclease is di-(*p*-nitrophenyl)thymidine 3′,5′-diphosphate. The nuclease readily splits off *p*-nitrophenyl phosphate from the 5′-position but has only a slight effect on the same group in the 3′-position. On the other hand, compounds with a free (not esterified) phosphate group in the 5′-position are powerful inhibitors of the nuclease. Bearing in mind these findings 3′-(*p*-aminophenylphosphoryl)deoxythymidine 5′-phosphate,

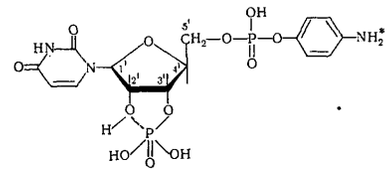


was chosen as the ligand for coupling with Sepharose. The inhibition constant for this substance, determined from kinetic data, is extremely low (10^{-6} M), which ensured a stable binding of the nuclease; the substance itself is stable in the pH range from 5 to 10; the p*K* of the amino-group is sufficiently low for effective interaction with Sepharose activated by bromocyanogen and the amino-group is sufficiently remote from the part of the ligand bound to the enzyme§. Nuclease from the whole culture liquid was isolated in quantitative yields in one stage by column chromatography using the sorbent obtained. The sorption was carried out at pH 8.0 (0.05 M borate buffer with added 0.01 M calcium chloride) and, after washing the column with the same buffer solution, the enzyme was desorbed with 0.01 M acetic acid.

In the preparation of a sorbent specific to chymotrypsin [61], an enantiomeric analogue of the substrate—the methyl ester of D-tryptophan—was chosen as the ligand. However, the resulting sorbent was not altogether satisfactory, probably because the small dimensions of the ligand produced steric hindrance to the approach of the enzyme to the active surface of the sorbent. In view of this, another ligand—the methyl ester of ε-aminocaproyltryptophan—was tested. In this ligand the tryptophan residue bound to chymotrypsin is much more remote from the site of attachment to Sepharose, so that it is much more accessible to the enzyme in solution. The resulting sorbent proved to be extremely effective both for the purification of chymotrypsin itself and for the elimination of admixtures of this enzyme from preparations of other enzymes, in particular ribonuclease. The sorption was carried out in 0.5 M tris hydrochloride buffer at pH 8.0 and the enzyme was desorbed with 0.1 M acetic acid. The yield of the pure enzyme was more than 90%. The dissociation constant of the chymotrypsin complex with this ligand is about 10^{-4} M, i.e. higher by 2 powers of 10 than the value for the enzyme—inhibitor complex of nuclease. Nevertheless, in this case too the adsorbability of the enzyme was sufficiently complete and stable. Thus the requirement for a very high stability of the enzyme—ligand complex is not strictly obligatory.

In the preparation of the sorbent for carboxypeptidase A, the dipeptide L-tyrosyl-D-tryptophan was employed as a ligand [61]. The sorption was carried out in 0.005 M tris-HCl buffer at pH 8.0 with sodium chloride added up to a concentration of 0.3 M (evidently in order to prevent the non-specific sorption of impurities by the free carboxy-groups of tryptophan residues). The enzyme was desorbed with 0.1 M acetic acid. The sorption of carboxypeptidase A (within the limits of the working capacity of the sorbent) and its desorption were quantitative. The specificity of the sorbent was demonstrated by the absence of binding of carboxypeptidase B under the same conditions.

Wilchek and Gorecki [62] described the preparation and application of a sorbent for ribonuclease. The ribonuclease substrates are ribonucleic acid and pyrimidine 2′ : 3′-cyclic phosphates; compounds such as cytidine 2′-monophosphate, cytidine 3′-monophosphate, and uridine 2′-monophosphate are known to be competitive inhibitors of the enzyme. However, the attempt to attach cytidine 2′-monophosphate to Sepharose by the method of Porath and coworkers was unsuccessful, probably owing to the low nucleophilicity of the amino-group in the 4-position. Therefore another class of inhibitors, namely 2′(3′),5′-diphosphates, was tested: firstly, only the 2′(3′)-phosphate group is important for their inhibiting activity and the group in the 5′-position can be modified; secondly, inhibitors of this type are the most powerful known (the inhibition constant determined from kinetic data is approximately 10^{-5} M). Therefore 5′-(*p*-aminophenylphosphoryl)uridine 2′(3′)-phosphate was chosen as the ligand:



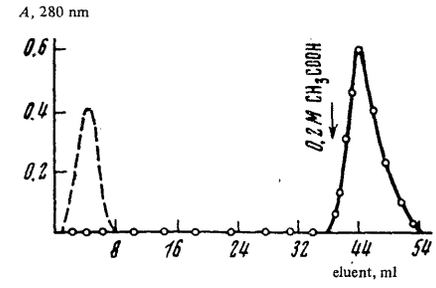


Figure 1. Affinity chromatography of ribonuclease A (after Wilchek and Gorecki [62]). The dashed line indicates the peak of ribonuclease inactivated by oxidation and reduction and also of enzymes with a different substrate specificity (trypsin and chymotrypsin). The arrow indicates the point where the eluent was changed. For details, see text.

The sorbent obtained firmly retained ribonuclease in 0.02 M acetate buffer at pH 5.2; the enzyme was desorbed with 0.2 M acetic acid (Fig. 1). With the aid of this sorbent, it was possible to free ribonuclease from various impurities, including phosphates of different types and also to eliminate selectively admixtures of ribonuclease from other preparations. The authors emphasise the importance of their method for the fractionation and purification of synthetic ribonuclease preparations [62].

§Here and henceforth the superscript* denotes the group attached to the carrier.

Porath and coworkers [63] synthesised a sorbent for asparaginase. The substrate of asparaginase is L-asparagine, while its enantiomeric form D-asparagine inhibits the enzyme. Therefore the ligand chosen was a product of the condensation of L(+)-β-chlorosuccinamic with hexamethylenediamine:

$$^{*}H_2N-(CH_2)_6-NH-\underset{\displaystyle COOH}{\underset{|}{CH}}-CH_2-CO-NH_2\ .$$

The latter played the role of an "insert" between the functional groups of the ligand and Sepharose in order to eliminate the steric hindrance in the sorption of the enzyme. Optimum sorption occurred in 0.05 M borate buffer at pH 8.6 with sodium chloride added to a concentration of 0.3 M; at a higher ionic strength the enzyme was more weakly bound to the sorbent, while reduction of the ionic strength resulted in an increase of the sorption of impurities. The enzyme was desorbed with 0.001 M solution of D-asparagine in the same buffer. The specific activity of the asparaginase preparation obtained exceeded the activity of the preparations produced by other methods. The resistance of the sorbent to the action of the enzyme was entirely satisfactory.

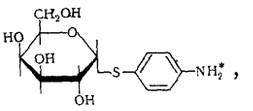
The sorbents obtained by the method of Porath and coworkers [57-60] have been used successfully for the isolation and purification of a number of other enzymes: chymotrypsin-like enzymes [64], plasminogen [65], wheat proteases [66], papain [67,68], β-galactosidase [69], β-xylosidase [70], DNA polymerase [71], 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase [72], lecithin : chloesterol acyltransferase [73], acetylcholinesterase [74,75], and chorismate mutase [76]. Kasche [77] and Feinstein [78] obtained by this method a sorbent based on an inhibitor of soya-bean trypsin and showed that it binds not only trypsin but also chymotrypsin (cf. Mosolov and Lushnikova [56]).

Inman and Dintzis [79] described a method for the preparation of affinity sorbents based on Biogel (cross-linked polyacrylamide), used, like Sepharose, as a molecular sieve. In this procedure the carboxamide group of Biogel is converted by treatment with hydrazine hydrate into the hydrazide group, which in turn is converted subsequently into an acyl azide group by treatment with nitrous acid; the acyl azide group readily reacts with primary aliphatic and aromatic amines to form amides:

$$B-CO-NH_2 \xrightarrow{N_2H_4} A-CO-NH-NH_2 \xrightarrow{HNO_2} B-CO-N_3 \xrightarrow{R-NH_2} B-CO-NH-R.$$

Thus the method of Inman and Dintzis, like that of Porath and coworkers, requires the presence in the ligand of a free amino-group not required for binding to the enzyme; the difference is that polycrylamide has a carbon skeleton and in this respect is more stable than Sepharose or Sephadex.

Continuing the development of the methods of Porath and coworkers and Inman and Dintzis, Cuatrecasas [80-82] described the syntheses of various derivatives of Sepharose and Biogel, such that ligands can be attached to these carriers not only via the amino-group but also via the carboxylic, thiol, phenolic, and imidazole groups; also it is possible to vary the distance between the ligand and the carrier by interposing between them aliphatic chains with different numbers of atoms ("arms"). As mentioned above, the length of the "arms" is in some cases of decisive importance, for example in the synthesis of the adsorbent for chymotrypsin [61]. Another example described by Cuatrecasas [69,80] refers to bacterial β-galactosidase. *p*-Aminophenyl-β-D-thiogalactopyranoside



was employed as the initial ligand. When it was linked directly to Sepharose, the enzyme was not sorbed. When it was combined with the bromoacetamidoethyl derivative of Sepharose, a sorbent was obtained which only weakly retained the galactosidase:

$$\text{Sepharose}\ -NH-CH_2-CH_2-NH-CO-CH_2-NH-\ \text{ligand}\ .$$

On a sorbent with a longer "insert",

$$\text{Sepharose}\ -NH-(CH_2)_3-NH-(CH_2)_3-NH-CO-CH_2-CH_2-CO-NH-\ \text{ligand},$$

the enzyme was sorbed very strongly.

By comparing sorbents based on the same ligand but with different carriers, Cuatrecasas showed (using as an example the sorbents for nuclease [80] and β-galactosidase [80]), that the adsorbents based on Biogel have a lower capacity than those based on Sepharose, which is probably due to the lower porosity of Biogel.

3. Other Methods of Utilising the Affinity of the Enzyme for the Ligand in the Chromatography of Enzymes

Cavalieri and Caroll [83] showed that, by polymerising acrylamide in a solution containing DNA, it is possible to obtain a gel in which DNA is firmly incorporated and which is suitable for the isolation and purification of DNA polymerase.

In another method of employing ligands, a non-specific sorbent is saturated with a soluble ligand; for example, ligands containing basic or acid groups are attached to suitable ion exchangers. Such a procedure was described by Beers [84], who used it to purify polynucleotide phosphorylase on a column with polyadenylic acid attached to DEAE-cellulose. The properties of such sorbents have been hardly investigated and it is not clear whether the ligand molecules sorbed on the carrier retain the capacity for the selective binding of the enzyme. Bearing in mind the data of Cuatrecasas [61,69,80] concerning the role of the hydrocarbon "arm" between the ligand and the carrier, one must suppose that this capacity would be considerably weakened. In addition, it is evident that sorbents of this type are less stable than those with covalently bound ligands.

Yet another application of the affinity of the enzyme for the ligand in the chromatography of enzymes consists in a selective elution of the enzyme sorbed on a non-specific sorbent with a soluble ligand. Heppel [85] first showed that pyrophosphorylase sorbed on alumina gel is selectively and effectively eluted with a pyrophosphate solution. Subsequently this principle was used to purify fractose-1,6-diphosphatase [86-88], aldolase [87,89], malate dehydrogenase [90], nuclease [91], and pyruvate kinase [92]. Yoshida [93] isolated by this method glucose-6-phosphate dehydrogenase, pseudocholinesterase, lactate dehydrogenase, and alanine dehydrogenase. The chromatography of carboxypeptidase A on a DEAE-cellulose column in the presence of a competitive inhibitor (β-phenyl propionate) resulted in its resolution into five fractions differing in aminoacid composition and thermal stability [94].

IV. ANALYTICAL APPLICATIONS OF AFFINITY CHROMATOGRAPHY

An interesting analytical aspect of the application of affinity chromatography, described by Wilchek and coworkers [95], refers to the study of the active centres of enzymes by specific chemical modification. The specificity of the reaction in the region of the active centre (more precisely in the region of ligand binding) is achieved by employing in the reaction a ligand with a chemically reactive group. As a result, the ligand reacts with other groups of the enzyme in the region of ligand binding. Peptides obtained by the successive cleavage of the modified enzyme have been fractionated on a column with the native enzyme attached to an insoluble carrier¶. The column retains only the peptides which contain the modified amino acid residues—owing to the presence in them of the ligand group. By analysing these peptides, it is possible to locate the region of the active centre in the aminoacid sequence of the enzyme molecule. In the above study by Wilchek and coworkers [95], ribonuclease was azo-coupled with a diazotised inhibitor—5′-(*p*-aminophenylphosphoryl)uridine 2′(3′)-phosphate. This compound reacted with ribonuclease in molar proportions of 1 : 1. After the cleavage of the modified enzyme with trypsin, the mixture of peptides was fractionated on a column with native ribonuclease attached to Sepharose by the method of Porath and coworkers [57-60]. The chromatography was carried out at pH 5.0, which is optimal for the binding of the enzyme to the ligand [62]. The coloured product was firmly retained on the column and, after washing off the impurities, was desorbed with 0.8 M ammonium hydroxide solution. Analysis showed that the product is a single peptide incorporating aminoacid residues between 67 and 85 and containing the modified tyrosin residue. The authors note the rapidity and convenience of the method compared with ion-exchange chromatography and electrophoresis.

The same method, which has been called the method of "affinity labelling", was used by Wilchek [97] and Cuatrecasas [98] for the isolation of a modified peptide from staphylococcal nuclease. An affinity label was applied also to acetylcholinesterase [99], aspartate aminotransferase [100], lysozyme [101], and *β*-galactosidase [102].

Kravchenko and coworkers [103,104] used affinity chromatography to separate the products of the photochemical oxidation of lysozyme in the presence of Methylene Blue under the conditions of preferential oxidation of tryptophan residues. Chromatography on Amberlite CG-50 ion-exchange resin made possible only the separation of the overall photochemical oxidation products from the unchanged native enzyme (Fig. 2). Bearing in mind that the modification of tryptophan residues in lysozyme might affect its affinity for the substrate [1-4], the lysozyme was chromatographed on chitin [42,43]. The results of such chromatography are presented in Fig. 3. [103] The chromatogram shows clearly three peaks (*X*, *Y*, and *Z*), the *Z* peak emerging almost simultaneously with the front of the eluent. Since the peaks emerging with the front can have a complex composition, the *Z* peak was subjected to further analysis. When it was rechromatographed under conditions increasing the affinity of lysozyme for chitin (a higher ionic strength of the medium [43]), it was possible to separate it into several further fractions [104]. Kravchenko and Lapuk [105] showed that the main fraction of the *Z* peak consists of an individual modification product in which the tryptophan residue 28 was oxidised. It is interesting to note that the tryptophan residue 28 is remote from the active region of the enzyme and is not directly involved in the binding of the substrate [1-4]. Nevertheless, experiments showed that its oxidation leads to a significant decrease of affinity. Hence it follows that this aspect of affinity chromatography is not restricted to cases where the active region of the enzyme is modified.

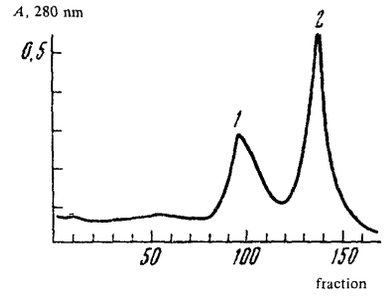


Figure 2. Chromatography of photochemically oxidised lysozyme on Amberlite CG-50 cation-exchange resin columns (after Kravchenko et al. [103]); 2.7 × 28.8 cm column; 0.2 M sodium phosphate buffer, pH 6.72; rate of elution 35 ml h^{-1}: 1) mixture of photochemical oxidation products; 2) native lysozyme. 10 ml fractions.

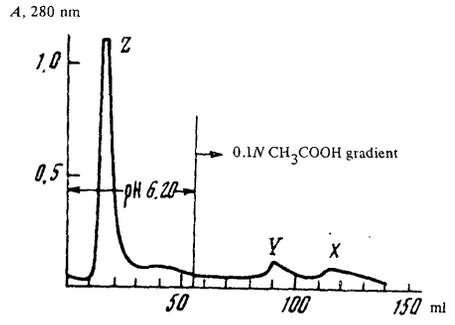


Figure 3. Chromatography of a mixture of products of the photochemical oxidation of lysozyme (peak *1* in Fig. 2) on chitin (after Kravchenko et al. [103]); 0.9 × 22.5 cm column with chitin dispersed to a 50 μm grain size; initial conditions: 1/15 M phosphate buffer, pH 6.20; volume of mixer for gradient elution 200 ml; rate of elution 15 ml h^{-1}.

A similar approach was employed by Kasche [77] to fractionate and characterise the functional properties of the products of the modification of heat-treated and *γ*-irradiated chymotrypsin on a column containing an inhibitor of soyabean trypsin attached to Sepharose by the method of

¶There is an extensive literature on sorbents containing enzymes attached to insoluble carriers. The methods of their preparation and various aspects of their applications have been described in the reviews by Silman and Katchalski [96], Gryszkiewicz [141], and Goldman et al. [142].

Porath and coworkers (Feinstein also reported [78] that, apart from trypsin, this type of sorbent is capable of specific binding of chymotrypsin). Native chymotrypsin showed an appreciable affinity for the inhibitor and lagged appreciably behind the eluent front. After heat treatment in solution, the enzyme was resolved into two fractions one of which emerged with the front and was completely inactive and the other corresponded to native chymotrypsin. After γ-irradiation, both in solution and in a dry state, two fractions were also obtained, one of which emerged with the front and was inactive. However, the second peak occupied an intermediate position on the chromatogram between the inactive and native enzymes. Measurements on the fractions showed that, as the enzyme emerges from the column, its enzymatic activity increases smoothly from 0 to 100%.

The use of affinity chromatography for analytical purposes is not as yet very common although this section has shown that there is a fairly broad base for such investigation and the few examples quoted demonstrate great possibilities of this method.

V. CHROMATOGRAPHIC CHARACTERISTICS OF THE PHYSICOCHEMICAL PROPERTIES OF THE ENZYME–LIGAND COMPLEX

Chromatography is one of the most direct and simplest methods for the investigation of the thermodynamics of sorption and complex formation [106–108].

Cherkasov and Kravchenko [109,110] were the first to employ this method for the investigation of the thermodynamic properties of the enzyme–substrate complex of lysozyme and chitin. Preliminary studies [42,43] showed that, depending on the pH of the medium, lysozyme forms two types of complex with chitin, with different conditions for their stability, as mentioned above (p. 895). Since the existence of two types of complex was not revealed by other methods, the chromatographic approach was employed for this purpose [109,110]. The equilibrium constant was found from the relation between the retention volume and the molar concentration of lysozyme at its peak on the output curve and the heat of complex formation was estimated from the relation between the retention volume and column temperature [106–108]. The determinations were made at pH 4.7 and 8.4, which correspond to the most favourable conditions for the two types of complex (at intermediate pH values lysozyme is distributed between the two complexes). The results obtained are listed in the Table which includes for comparison also data obtained by other methods at pH ~ 5 (there are no published quantitative data referring to pH 8–9; only graphical relations between the stability of the complex and pH, obtained by differential ultraviolet spectrophotometry [113] and fluorescence measurements [114,115], are available.

The Table shows that the results obtained by the chromatographic method at pH 4.7 agree very satisfactorily with those obtained at pH ~ 5 by other methods. Hence it follows that affinity chromatography is a fully valid method for the study of the thermodynamics of the binding between enzyme and substrate.

The data for the properties of the complex at pH 8–9 are contradictory. It follows from the work of Rupley et al. [113], Chipman et al. [114], and Lehrer and Fasman [115] that, on increase of pH from 5 to 8–9, the stability of the complex falls (the variation of other thermodynamic quantities with pH is not quoted in these reports). On the other hand, the results of the chromatographic determination show that the stability of the complex at pH 8.4 is higher almost by a factor of 10 than at pH 4.7 (cf. the values of ΔG° in the Table). The discrepancies are probably due to the inadequacy of the spectroscopic methods for the investigation of the complexes. Indeed these methods are indirect and are based on the detection of effects associated with changes in the conformation of lysozyme on formation of the enzyme–substrate complex.

Thermodynamic parameters of the formation of a lysozyme–substrate complex.

pH	$-\Delta F^\circ$, kcal mole^{-1}	$-\Delta H^\circ$, kcal mole^{-1}	$-\Delta S^\circ$, e.u.	Method	References
4.7	6.9	14.0	23.8	Chromatography	109, 110
5.0	7.0	12.8	19.7	Calorimetry	111
5.3	7.0	16.6	32.2	Kinetics	112
5.3	7.2	14.3	23.0	u.v. photometric	113
8.4	8.1	9.6	5.0	Chromatography	109, 110

On the other hand, analysis of the differences between the enthalpies and particularly the entropies of complex formation at pH 4.7 and 8.4 (see Table) shows that the conformation of lysozyme changes appreciably only when the complex is formed in an acid medium, but there is little alteration or none at all in an alkaline medium [110]. Thus, by virtue of the conformational properties of lysozyme, indirect methods evidently detect only the complex formed at pH ~ 5 and lead to the incorrect conclusion that the stability of the complex decreases as pH rises to 8–9. Similar situations can arise also in many other enzyme–substrate systems.

The applicability of kinetic methods for such purposes is limited to conditions where the enzyme exhibits its activity; for example, in the case of lysozyme and chitin these methods are applicable at ~pH 5 but not above pH 8.

Thus the direct method of affinity chromatography is more informative than the kinetic and spectroscopic methods now widely employed in this field.

The developments of recent years in the field of affinity chromatography show that the method can be fruitfully employed in a wide variety of different types of enzyme studies. On the whole, the present state of affinity chromatography shows it to be a promising analytical and preparative method in enzyme chemistry.

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During the preparation of the review for the press, reports appeared on the use of affinity chromatography for the purification of tyrosine hydroxylase [116], neuraminidase [117], dihydrofolate dehydrogenase [118–120], plasminogen [121–124], thrombin [125], pepsin [126], carboxypeptidase A [127,128], carboxypeptidase B [128,129], transaminases [130], trypsin-like enzymes [131], and certain proteases [132]. Figs. 4 and 5 present examples of the isolation and separation of carboxypeptidases A and B.

The method of "affinity labelling" of the active centre of the enzyme has been described for carboxypeptidase A [133,134] and aldolase [135].

Akanuma et al. [136] used affinity chromatography to investigate the properties of carboxypeptidase B complexes with various analogues of basic and aromatic amino-acids.

Wilchek et al. [137] reported certain changes in the functional properties of protein kinase in its chromatography on Sepharose to which cyclic adenosine monophosphate had been attached.

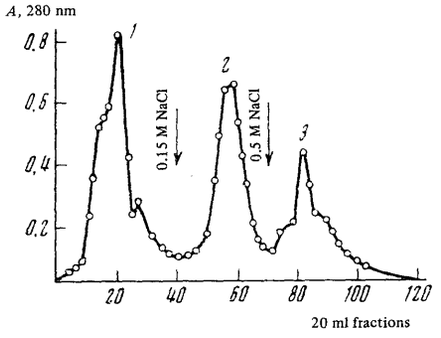


Figure 4. Separation of carboxypeptidases A and B by chromatography on Sepharose 4B with bound ε-aminocaproyl-D-tryptophan (after Reeck et al. [128]); initial eluent; 0.02 M tris-HCl buffer, pH 7.5, with added 0.05 M NaCl, followed by NaCl concentration gradient elution; the change of eluent is indicated by arrows: 1) impurities; 2) carboxypeptidase B; 3) carboxypeptidase A.

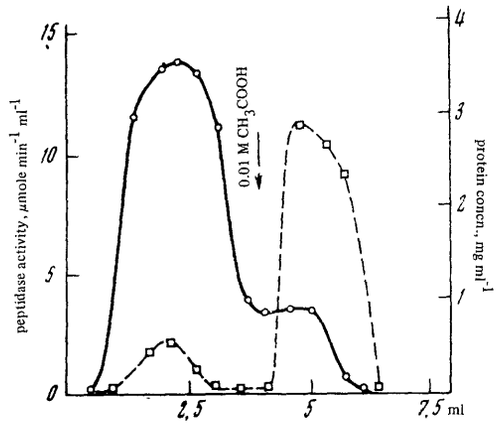


Figure 5. Isolation of carboxypeptidase A from a mixture of proteins by chromatography on porous glass with bound glycyl-D-phenylalanine (after Robinson et al. [127]). A column with granulated (200 mesh) porous glass (pore diameter 200 nm) was charged with a mixture of carboxypeptidase A, lysozyme, rubonuclease A, and bovine serum albumin. Initial eluent: 0.02 M tris-HCl buffer, pH 7.5, with added 0.2 M NaCl; the arrow indicates the change of eluent; continuous line—protein; dashed line—peptidase activity.

Mosbach et al. [138] and Weibel et al. [139] described the preparation of adsorbents for dehydrogenases with nicotinamide-adenine dinucleotide as the coenzyme.

The introduction of a new type of insoluble carrier—porous granulated glass—is noteworthy [127,139]. The porous glass is activated by treatment with aminosilanes, the amino-group introduced thereby being employed for the covalent binding of ligands.

Several reviews have been published on the affinity chromatography of enzymes and certain other bioorganic compounds [140,143,144].

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