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Affinity Adsorbents Based on Carriers Activated by Epoxy-compounds

B.A.Klyashchitskii and P.V.Kuznetsov

The review is devoted to the synthesis and applications of affinity adsorbents based on carriers activated by epoxy-compounds. The methods for the introduction of epoxy-groups into carriers of different chemical types are discussed and conditions for the immobilisation of three-dimensional spacers and low-molecular-weight and polymeric ligands on carriers containing epoxy-groups are considered. Data are presented on the properties and applications of adsorbents of this type in affinity chromatography.
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I. INTRODUCTION

Extensive data have now accumulated on the synthesis and practical applications in affinity chromatography (AFC) of various types of adsorbents, which have been described systematically in a series of monographs[1-4] and reviews.[5-11] Reviews devoted to specific problems arising in the biospecific purification of individual classes and groups of biopolymers have appeared recently.[12-14]

The development of AFC would have been impossible without continual improvement of the chemical basis of this method, which includes the methods of synthesis and activation of solid carriers, the immobilisation of ligands, the analysis of affinity adsorbents, the study of their properties, etc. The chemical aspects of the AFC method have been the subject of discussion in the above monographs, individual reviews,[15,16] and conferences devoted to AFC.[17] A review of the synthesis and properties of adsorbents for covalent chromatography has been published.[18] It has apparently now become necessary to give a systematic account of specific chemical data on the methods of synthesis of affinity adsorbents of different types. The present review is devoted to the synthesis, properties, and applications of biospecific adsorbents of one of the most promising types, namely adsorbents based on epoxy-activated carriers (EAC).

Data on the affinity adsorbents based on EAC have been described in the literature extremely sketchily and in a fragmentary fashion. Nevertheless the application of adsorbents of this kind is organically linked to an urgent problem in AFC—the synthesis of stable materials resistant to hydrolysis, exhibiting a high biospecificity, and capable of functioning for a long time under various conditions. The employment of the classical method of the bromocyanogen activation of polysaccharide carriers[19] does not allow the preparation of adsorbents with a bond between the matrix and the spacer† (ligand) which is highly resistant to hydrolysis.[10-20] Despite a number of successful studies[21-24] using the polymeric spacers in accordance with the principle of multipoint addition, the attention of investigators has been attracted with increasing frequency by adsorbents based on EAC, including Sepharoses.

Apart from a high resistance to hydrolysis, the EAC possess a number of other important advantages—they are fairly hydrophilic and virtually lack charged groups, which reduces to a minimum the non-specific interactions between the biopolymer isolated and the adsorbent. These adsorbents make it possible to immobilise not only ligands with a primary amino-group but also (without additional modification) ligands with other functional groups, for example hydroxy-, carboxy-, and thiol groups. When EAC are used, it is frequently not necessary to introduce additional spacers. It is also quite important that, in the epoxy-activation of agarose carriers, a cross-link is formed between the agarose chains themselves, which imparts additional rigidity to adsorbents of the type of Sepharose and increases the mechanical and chemical stability of the carrier.

The present review gives a systematic account of the principal literature data on the synthesis and applications of adsorbents based on EAC for the purpose of AFC and also for the immobilisation of enzymes.

II. METHODS FOR THE ACTIVATION OF SOLID CARRIERS BY EPOXY-COMPOUNDS

The activation of solid carriers by epoxy-compounds was first proposed in the laboratory of Porath,[25-28] who has made a major contribution to the development of the methods for the separation of biopolymers in general and of AFC in particular.[29]

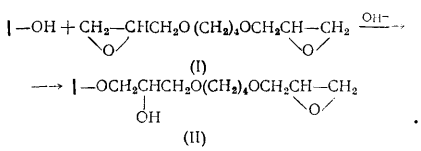
Sepharoses of various brands (2B, 4B, 6B, and CL), satisfying the majority of the requirements which must be met by carriers for the preparation of affinity adsorbents, are used most frequently in activation by epoxides and their derivatives. Celluloses, ultragel, Sephadexes, and other polysaccharide carriers are used more rarely. The employment of methacrylate, methacrylamide, and hydroxylalkyl methacrylate polymeric carriers,[30-33] as well as silica carriers[33,34] has been described.

†The term "spacer" used in the present review [the term "vstavka" or "insert" [10] was employed in the original Russian text (Ed.of Translation)] denotes a compound (or group of atoms) separating the ligand from the surface of the solid carrier. The terms "arms" and "leash" can also be encountered. Apart from "vstavka" mentioned above, the terms "prostranstvennaya gruppa" ("space group"), "speiser" (spacer), and "nozhka" (foot) are also used in the Russian scientific literature.

1. Activation of Polysaccharide Carriers

The methods for the activation of polysaccharide carriers by epoxy-compounds can be divided arbitrarily into two main groups: methods leading to the appearance of highly reactive terminal epoxy-groups on the surface of the carrier [the Sundberg–Porath method (1974) [27] and the method of Axen and co-workers (1975) [35]] and also methods making it possible to modify the surface of the carrier by various functional groups [Hjerten's method (1974) [36,37]].

(a) *The Sundberg–Porath method.* The method is based on the reaction of bisoxirans of the type of diglycidyl ether of butane-1,4-diol (I) with Sepharose in a highly alkaline medium (0.4–0.6 M sodium hydroxide)#:

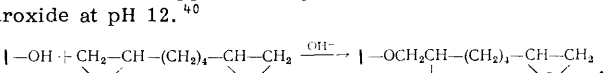


(I) (II)

The reaction time is 6–10 h and the temperature is 20 to 30 °C. The optimum concentration of the bisorixan (I) in the reaction mixture is approximately 30%.[27] The concentration of the epoxy-groups on adsorbent (II) was approximately 800 μmole per gram of the dry gel. According to the data of Sundberg and Porath, the stability of epoxy-activated gels is a maximum when they are stored in dimethyl sulphoxide at 4 °C or in 1 M sodium chloride at pH 6.8 and 4 °C. The epoxidation of CL-Sepharose by this method made it possible to employ the activated matrix for syntheses in non-aqueous media.[38]

The use of the bisoxiran (I) for the activation of cellulose has been recently reported.[39] The activation takes place in 0.6 M sodium hydroxide with added sodium tetrahydroborate (4 mg ml^{-1}) at approximately 20 °C for 18 h. The activity of epoxy-cellulose was fully retained for two weeks when it was stored in 0.1 M sodium hydroxide at 4 °C.

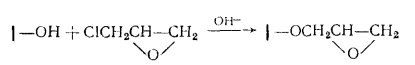
1,2:7,8-Diepoxyoctane (III) has also been used as the reagent for the activation of alginic acid. The epoxidation was carried out at approximately 20 °C for 3 h in 5 M sodium hydroxide at pH 12.[40]



(III)

The Sundberg–Porath method constitutes the basis of the synthesis of the commercial preparation "epoxy-activated Sepharose 6B" (EAS-6B) manufactured by Pharmacia Fine Chemicals (Sweden).

(b) *The Axen method.* This method of activation was first proposed to synthesise (via epoxy-derivatives) highly substituted thiol-containing gels for covalent chromatography.[35] The epoxidation of Sepharose by epichlorohydrin (1-chloro-2,3-epoxypropane) (IV) takes place fairly readily at 60 °C in the course of several hours in 1 M sodium hydroxide:



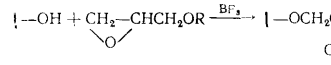
(IV) (V)

When 0.45 ml of compound (IV) was used for 3 g of the gel, the degree of substitution of approximately 600–700 μmoles of epoxy-groups per gram of dry gel (V) was attained. The optimisation of the epoxidation reaction conditions was subsequently investigated[41] and the following activation conditions were proposed: 30% of Sepharose, 5% of epichlorohydrin, and 0.4 M sodium hydroxide (2 h, 40 °C). It is of interest to note that the attempt to employ added organic solvents in the epoxidation reaction (25% solution of dimethyl sulphoxide or dioxan) did not increase appreciably the amount of epoxy-groups introduced. However, it was shown that in 25% dimethyl sulphoxide a degree of substitution of approximately 700 μmoles of epoxy-group per gram of the dry gel is attained after approximately 1 h, although the highest degree of substitution (800 μmole g^{-1}) is observed when the reaction is carried out in an aqueous medium for 2 h at 40 °C.

Other modifications of the method of Axen and co-workers have also been described. Thus Sepharose (IV) has been epoxidised with 0.48 M epichlorohydrin in 0.5 M sodium hydroxide at 30 °C in the course of 4 h.[42] According to other data,[43] the use of a solution of cetyltrimethylammonium bromide in the epoxidation reaction increased the quality of the gel and made it possible to reduce the activation time to 45 min at 45–50 °C. A method has been described recently[44] for the activation of Sepharose in 2 M sodium hydroxide with the gradual addition of epichlorohydrin to the reaction mixture over a period of 8–10 h at approximately 20 °C. The authors do not quote data for the degree of substitution of the gel by epoxy-groups, although the subsequent immobilisation of a number of enzymes (malate dehydrogenase, lactate dehydrogenase, and alkaline phosphatase) on the metal chelate carriers obtained was successful.

At the present time the method of Axen and co-workers is used to epoxidise Sepharose in order to obtain the commercial preparation "Thiopropylsepharose 6B" (Pharmacia Fine Chemicals). In addition activation of ultragel of type A4R with the aid of compound (IV) constitutes the basis of the preparation of commerical affinity adsorbents produced by the LKB Company (Sweden)—"acriflavin–A4R ultragel" and "heparin–A4R ultragel".

(c) *The Hjerten method.* This method was first developed by the authors in 1974 for the synthesis of neutral gels widely used in hydrophobic chromatography.[36,37] It is based on the reaction of Sepharose with a wide variety of epoxy-compounds in the presence of a catalyst—boron trifluoride:



A large number of hydrophobic adsorbents containing in their structure terminal alkyl and aryl groups, including those with alcoholic, carboxy-, methoxy-, cyano-, and other substituents, have been synthesised by this method.[45,46] The Sepharose gel:dioxan volume ratio in the reaction is 1:1; 1 ml of boron trifluoride etherate is used for 100 ml of the gel suspension. When 5 min have elapsed after mixing the reactants, the corresponding glycidyl ether is added and the reaction mixture is stirred for 40 min at 25 °C. The concentration of the ligands on adsorbents of this type was determined by NMR after acid hydrolysis. The capacity of the adsorbents with respect to the ligand reached 200 μmoles per gram of the dry gel.

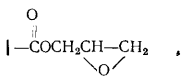
The method of Hjerten and co-workers is the basis of the preparation of the widely used stable hydrophobic commercial adsorbents "octyl-Sepharose CL-4B" and "phenyl-Sepharose CL-4B" (Pharmacia Fine Chemicals) whose capacity in relation

#Here and henceforth the bold line in the reaction schemes denotes the surface of the solid carrier.

to the binding of protein (serum albumin) reaches 15–20 mg per ml of the gel.

2. Synthetic Polymeric Carriers containing Epoxy-Groups

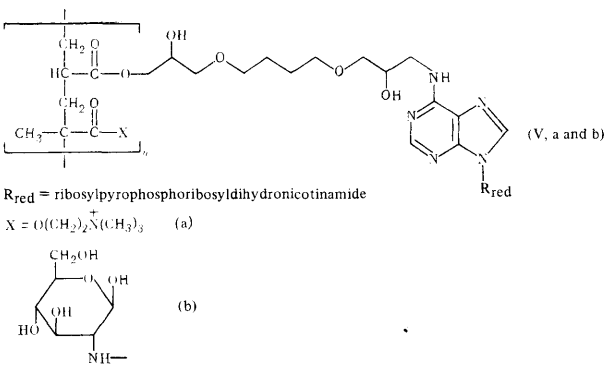
In the group of insoluble matrices obtained synthetically, glycidyl methacrylate gels (GMG), first synthesised by Czechoslovak investigators for the radical copolymerisation of ethylene methacrylate and glycidyl methacrylate, are of considerable interest.[32,33,47] The capacity of the carriers obtained with respect to the epoxy-groups was about 3.5 mmol g^{-1} and the epoxy-group was located on the surface of the carrier on a three-atom spacer:[47]



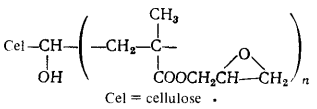
Turkova et al.[48] recently obtained hydroxyalkyl methacrylate gels (Separons H 300 and H 1000), activated by the epichlorohydrin, and, depending on the degree of activation, the content of the epoxy-groups was 140–1770 μmoles per gram of dry weight. It is of interest to note that the removal of the unreacted epoxy-group after the addition of the ligand, usually achieved by condensation with ethanolamine, was carried out by hydrolysis with 0.1 M perchloric acid.

Another example of EAC based on glycidyl methacrylate is provided by the "oxiran-acrylic carriers" manufactured by Pöhm Pharm (Federal Republic of Germany).[31] It is known that the second component in the copolymerisation reaction is allyl glycidyl ether, methacrylamide, or methylenebis-acrylamide. The carriers obtained have a macroporous structure, contain a large number of epoxy-groups (800 to 1000 μmoles per gram of the dry carrier), their swellability does not vary over a wide pH range (from 1 to 10), they have a high mechanical and hydrolytic stability, and they are resistant to microbes. The successful application of the oxiran-acrylic carriers to the immobilisation of proteins and enzymes has been demonstrated.[31] For example, the amount of bound albumin reached 140 mg and that of trypsin was equivalent to 600 activity units per gram of dry carrier. This type of carrier is apparently promising for employment in AFC.

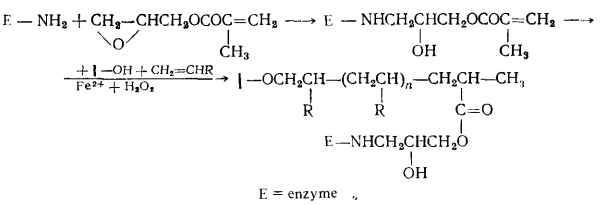
The synthesis of two water-soluble polymers on which NADH was subsequently immobilised, has been described.[30] The first was a copolymer of methacryloylcholine and 3-[4-(2,3-epoxypropoxy)butoxy]-2-hydroxypropyl acrylate. The immobilisation of NADH on this copolymer was achieved in three stages: alkylation of NAD at the N(1) atom, reduction of the nicotinamide component with dithionite, and Dimroth rearrangement involving the transfer of the alkyl bond from the N(1) atom to the exocyclic amino-group of the adenine component. The second polymer was a copolymer of the same epoxy-monomer and *N*-methacryloyl-2-glucosamine. NADH was added to this copolymer via the amino-group at the C(6) atom in a single stage. This resulted in the formation of the conjugates (V, a and b):



The copolymers of cellulose and poly(glycidyl methacrylate) having the following structure have also been obtained:

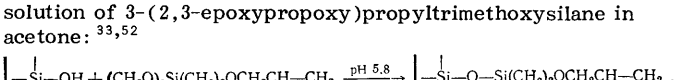


They have also been used to prepare immunosorbents[49] and for the immobilisation of certain enzymes.[50] A new type of polymeric material, consisting of a hydrophilic polysaccharide component and a synthetic hydrophobic polymer, has been obtained by graft polymerisation.[51] Sepharose 4B and the Sephadexes G-10 and G-200 were used as the polysaccharide matrices. The copolymerisation process with immobilisation of enzymes (α-amylase, chymotrypsin, and others) can be represented schematically as follows:



3. Activation of Silica Carriers by Epoxy-Compounds

The activation of carriers of this type has been inadequately investigated, although macroporous silica carriers are in fact important for the introduction of the AFC method into technological schemes for the purification of biopolymers. Porous glasses have been activated by treatment with a 5% solution of 3-(2,3-epoxypropoxy)propyltrimethoxysilane in acetone:[33,52]



A number of enzymes have been successfully immobilised on the carrier obtained and the capacity with respect to the protein was in the range 10–80 mg of the protein per gram of the carrier.[33] The same reagent in the form of a solution in toluene has been used to introduce the epoxy-groups into the silica carrier silochrome.[34] The activation of the "Fractosil-1000" (Merck) silica has been carried out analogously. The capacity of the resulting carrier with

respect to the protein was 20–30 mg per gram of the carrier.[33]

III. IMMOBILISATION OF LIGANDS ON CARRIERS CONTAINING EPOXY-GROUPS

One of the significant advantages of the EAC based on Sepharose (EAS-6B) is the absence of a special stage involving the introduction of a spacer in the synthesis of the affinity adsorbent. Indeed EAS-6B, obtained by the Sundberg–Porath method, has a fairly long hydrophilic spacer which permits the immediate immobilisation of the ligand. This simplifies the syntheses of adsorbents with low-molecular-weight ligands to the maximum extent and in a number of instances results in a process consisting of only one stage.

On the other hand, the application of activated Sepharose, obtained by the method of Axen and co-workers (1,2-EPS), as a rule requires the introduction of an additional spacer. The ligand immobilisation reactions will be considered below in relation to the above types of epoxy-Sepharose.

1. Adsorption of Low-Molecular-Weight Ligands or Spacers

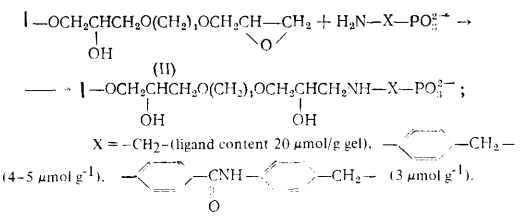
(a) *Adsorbents based on EAS-6B.* As a rule the immobilisation of ligands containing amino-, hydroxy-, or mercapto-groups takes place readily at pH from 7 to 13 at a temperature of 20–45 °C in the course of 10–65 h. These general conclusions are illustrated by specific examples in Table 1.

Table 1. Conditions for the immobilisation of low-molecular-weight ligands with amino-, hydroxy-, and mercapto-groups on EAS-6B.

Ligand	pH	Temperature, °C	Time, h	Refs.
		NH_2		
Trimethoprim analogue	9–10	—	16	[53]
Folic acid	10	30	16	[54]
8-Aminoxanthene	10–11	37	20	[55]
Acriflavin	11.5	20	8–10	[56]
S-Hexylglutathione	12	30	16	[57]
Galactosamine	13	30	20	[58]
		OH		
Ribose-5-phosphate	8.5	33	65	[59]
N-Acetylgalactosamine	11	40	16	[60]
Adenosine	11.2	37	24	[61]
Glycerylphosphorylcholine	13	45	20	[62]
		SH		
Reduced glutathione	7*	37	24	[63]

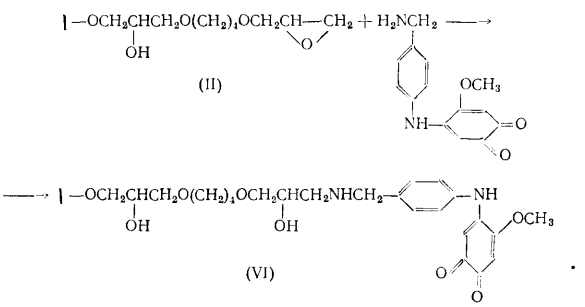
*The immobilisation was carried out in an atmosphere of nitrogen.

Under analogous conditions (0.1 M sodium hydroxide at pH 13), aminomethylphosphonic acid and derivatives of *p*-aminobenzylphosphonic acid were coupled to EAS-6B and the capacity of the adsorbent with the ligand containing the aliphatic amino-group proved to be approximately 4–5 times higher:[64]



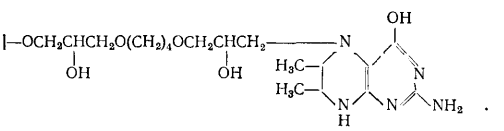
It is of interest that only adsorbents with an aromatic structure were capable of weak binding of alkaline phosphatase.

The use of amino-derivatives of 1,2-benzoquinone as ligands has been described.[65] The reaction was carried out at pH 10 in 0.07 M carbonate buffer solutions in a stream of nitrogen in the course of 5 h; the resulting capacity with respect to the ligand was entirely satisfactory—5.5 μmoles per ml of the packed volume of the gel:



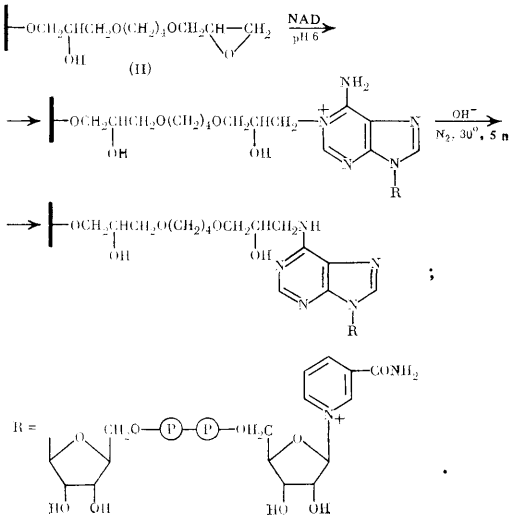
The adsorbent (VI) synthesised proved to be highly stable and was used for about a year for the isolation of menadione reductase.

A high capacity with respect to the ligand—approximately 280 μmoles per gram of gel—has been obtained following the immobilisation via the 5-position of the dimethyl derivative of 2-amino-4-hydroxytetrahydropteridine.[66] The reaction was carried out in deaerated 0.5 M sodium carbonate solution at pH 11.4:



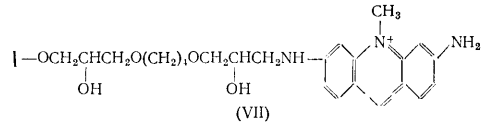
When the ligand contains carboxy-groups, it can be immobilised on the adsorbent as a result of the formation of ester linkages.[33,67] Ligand containing a phenolic hydroxyl also add effectively to EAS-6B in an acid medium. However, when the ligand contains both functional groups, the exact binding sites cannot be always established. Thus the exact structure of the adsorbents obtained from derivatives of auxin (5-hydroxyindolylacetic acid, 2-, 3-, and 4-hydroxyphenylacetic acids, etc.) and antiauxin (2-hydroxy-3,5-diiodobenzoic acid).[68] According to the authors, the adsorbents with indolylacetic acid as the ligand proved to be stable for only two weeks.

An NAD-containing adsorbent has been synthesised using the Dimroth rearrangement in one of the stages—transfer from the N(1) position in the purine ring to the exocyclic amino-group—for the AFC of dehydrogenases:[69]

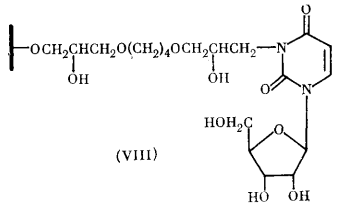


Thus the fairly ready alkylation of purines at the N(1) position in interaction with EAS-6B in weakly acid media (pH 5–6) can be regarded as an established fact; the subsequent Dimroth rearrangement leads to the immobilisation of the ligand at the exocyclic amino-group of the adenine ring.

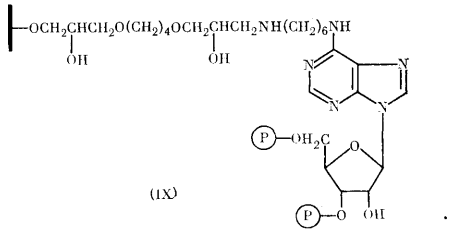
However, in a number of instances, particularly when the reaction is carried out in a strongly alkaline medium, the exact position in the polyfunctional ligand molecule at which the latter adds to EAS-6B is fairly difficult to determine. Thus, the adsorbent with adenosine immobilised on EAS-6B proved to be highly effective in the purification of deaminase.[61] The ligand was bound at pH 11.2 in 0.1 M sodium bicarbonate at 37 °C for 24 h and the authors believe that the ligand is most probably immobilised via the hydroxy-groups of ribose and not via the relatively unreactive amino-groups of adenine. Nevertheless, immobilisation via the exocyclic amino-group of the ligand is also apparently entirely feasible under these conditions—under similar conditions (Table 1) it has been possible to obtain the adsorbent with immobilised acriflavin (VII):[56]



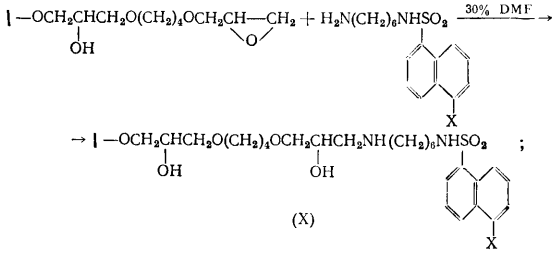
In similar cases, in the presence of several types of reactive groups in the ligand molecule, the proposed structures of the adsorbents probably cannot be regarded as finally established. Thus, Mitina et al.,[70,71] having established the covalent binding of uridine to EAS-6B at 37 °C in 0.1 M sodium hydroxide in the course of 16 h, describe only the postulated structure of the adsorbent (VIII) obtained:



More definite results were obtained after specific preliminary modification of the ligand molecule before immobilisation. For example, cAMP was initially converted into the C(8)-hydroxyethylthio-derivative, which was then readily coupled to EAS-6B at pH 11.[72] N(6)-ε-Amino-hexyl-3,5-ADP was analogously immobilised at pH 10 and 28 °C in the course of 20 h [73] and adsorbent (IX) was obtained:



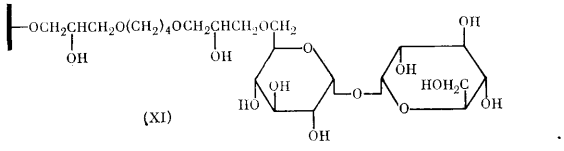
Derivatives of 1-sulphonaphthalene, which are calmodulin antagonists, have been immobilised at pH 9.8 in 30% dimethylformamide in the course of approximately 16 h at 40 °C via the amino-group of the hexamethylenediamine spacer, which had been previously introduced into the ligand molecule.[74] The adsorbent (X) synthesised contained approximately 20 μmoles of the ligand per ml of the gel.



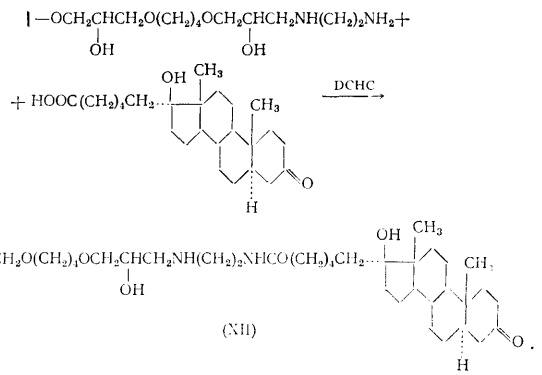
where X = H or Cl.

In connection with this synthesis, mention should be made of the organic solvent (dimethylformamide), which made it possible to obtain an adsorbent with a high capacity with respect to the ligand.

As stated above, carbohydrate ligands can be immobilised on EAS-6B without preliminary modification.[75,76] A detailed chemical study has been made[77] of the immobilisation of carbohydrates on EAS-6B. It was shown that the reaction proceeds mainly via the primary hydroxy-group and in the case of aminosugars it was found that the amino-group is six times more reactive in the reaction with the epoxy-group than the primary hydroxy-group. The preparation of a stable and effective adsorbent (XI) for the biospecific purification of proteins with a high affinity for non-reducing glucopyranosyl residues, by adding unprotected α,α'-trehalose to EAS-6B, has been described:[78]



The use of the short ethylenediamine spacer immobilised on EAS-6B for the subsequent coupling of the carrier to a carboxyl-containing steroid ligand by the carbodi-imide method has been described.[38] The adsorbent (XII) obtained was used for the isolation of steroid-binding serum proteins:



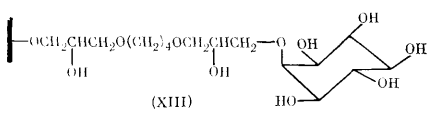
(XII)

An important feature of this synthesis was the use of CL-Sepharose, stable in non-aqueous media, because the ligand was immobilised in a 1 : 1 dioxan–dimethyl–formamide mixture with the aid of dicyclohexylcarbodi-imide (DCHC).

The use of EAS-6B for the synthesis of metal chelate adsorbents has been described.[79,80] The addition of the disodium salt of iminodiacetic acid has been achieved in 2 M sodium carbonate at 65 °C in the course of 24 h. The adsorbent obtained was used to purify nucleoside diphosphatase.[80]

S-Hexylglutathione–epoxy-Sepharose, in which the ligand was immobilised via its amino-group (pH 12, 30 °C), proved to be an interesting adsorbent based on EAS-6B. The unreactive epoxy-groups were blocked by 1 M ethanolamine in the course of 16 h. Several enzymes (glutathione-*S*-aryl transferase[81] and glyoxalase[57]) were purified on this adsorbent and in the latter case the yield of the enzyme was higher than on the adsorbent obtained by activating Sepharose with bromocyanogen.

According to Fazideen and Breitenbach,[82] it was possible to use EAS-6B in the immobilisation of the ligand in an organic solvent (dimethyl sulphoxide), although only in the course of a limited period (1 h at approximately 20 °C). The adsorbent (XIII) obtained was used for the purification of myoinositol oxygenase.

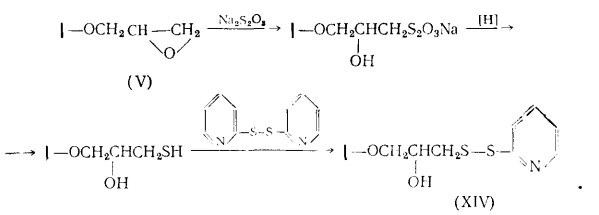


(XIII)

EAS-6B proved to be a convenient carrier for the immobilisation of *m*-aminophenylboronic acid.[83] The adsorbent synthesised bound effectively nucleotides, probably via the formation of a complex with the α-glycol group of ribose, and was investigated in this form by the authors with the aim of purifying several dehydrogenases.

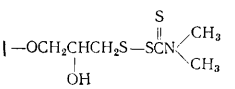
The covalent coupling to EAS-6B of other low-molecular-weight ligands, such as carbohydrate derivatives (*N*-acetyl-*D*-glactosamine,[60] *N*-acetyl-β-*D*-glucosamine,[41] tri-*N*-acetylchitotrioses,[41] cyclohepta-amyloses,[84] and β-*L*-fucosylamine[85]), an aminoalcohol (choline),[86] etc.

(b) *Adsorbents based on 1,2-EPS.* This type of epoxy-Sepharose[35] generally requires the introduction of an additional spacer in the immobilisation of the ligand. 1,2-EPS was first used to synthesise an adsorbent for covalent chromatography—"thiopropyl-Sepharose" (XIV),[35] which was obtained in accordance with the following scheme:



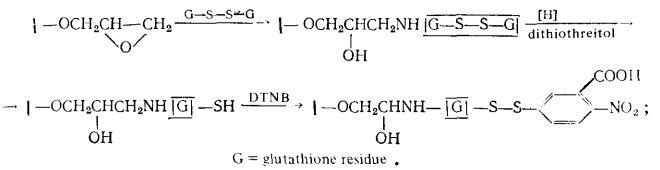
(XIV)

It is of interest to note that derivatives of di(2-pyridyl) disulphide with nitro- and carboxy-groups have also been investigated as ligands.[87] As expected, these derivatives of thiopropyl-Sepharose proved to be somewhat more active in thiol–disulphide exchange reactions and can be used in covalent chromatography together with the commerical adsorbent. The adsorbent (XV) with the dimethyldithiocarbamate residue as the ligand was obtained in the same study:



(XV)

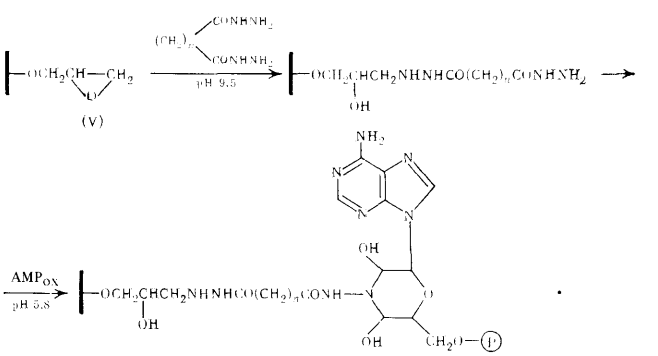
Epoxy-analogues of "activated thiol-Sepharose", the second commercially available adsorbent for covalent chromatography, have also been synthesised. Cystamine, cysteine, and glutathione residues were used as spacers and the ligand was the residue of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB).[43] The synthesis of the above adsorbents is indicated schematically below:



G = glutathione residue.

The use of adsorbents for the isolation and purification of SH-containing peptides and proteins has been examined in detail in a review.[18]

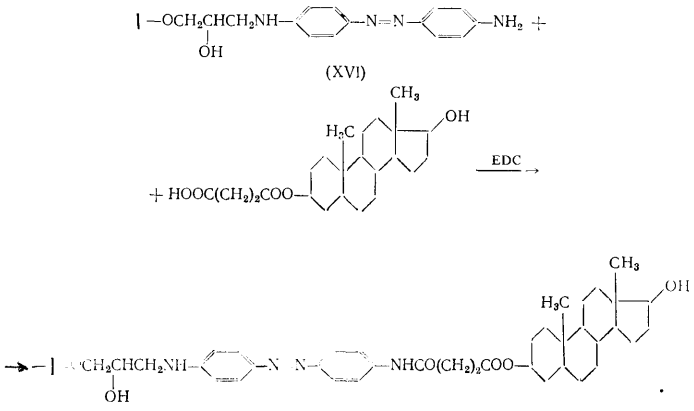
Using dicarboxylic acid hydrazides as spacers, a series of derivatives of hydrazidohydroxypropyl-Sepharose were obtained to which periodate-oxidised[88] nucleotides, for example, adenine and guanine nucleotides,[89] and NADP[43,90,91] were smoothly coupled. The dihydrazides are immobilised on 1,2-EPS at pH 9.5 at approximately 20 °C in the course of 10–16 h.[89] The subsequent addition of the oxidised nucleotides takes place via the ribose ring:



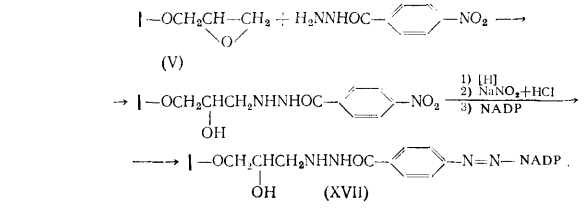
The hexamethylenediamine spacer was used for the immobilisation on 1,2-EPS of certain aminoacids[92] in the form of their *N*-hydroxysuccinimide esters. According to

Streikuviene et al.,[93] nonyloxypropyl-Sepharose proved to be most effective for the purification of phospholipase *D*.

The use of 4,4'-azodianiline as a spacer,[94] which was coupled to 1,2-EPS in 50% dimethylformamide in the course of 2 h, is of interest. The resulting coloured product (XVI) was coupled to the succinylated derivative of androstanediol in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC):

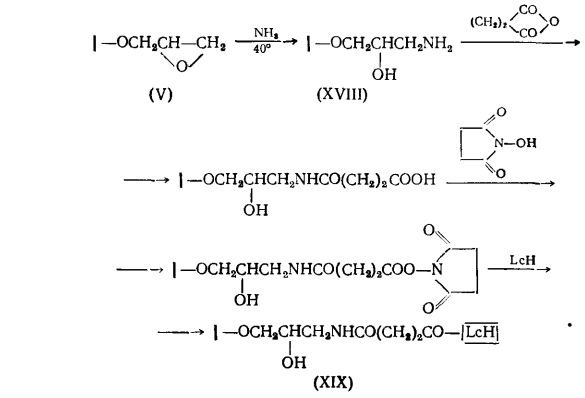


The syntheses of a series of adsorbents with an azo-linkage based on 1,2-EPS have been described. One of the examples of this kind is the preparation of NADP-azobenzo-hydrazidohydroxypropyl-Sepharose (XVII) in accordance with the following scheme:[95]



The spacer (*p*-nitrobenzohydrazide) immobilisation reaction on 1,2-EPS was carried out in aqueous dimethyl sulphoxide.

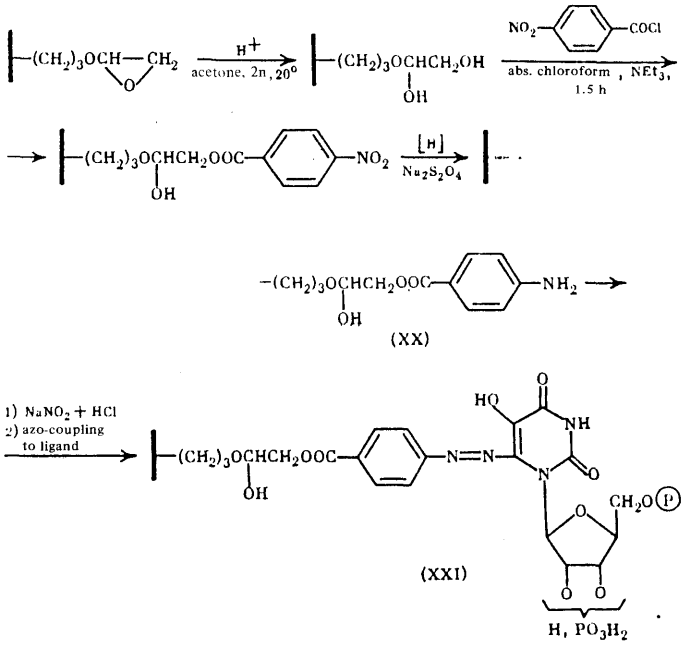
The amination of 1,2-EPS and EAS-6B has been achieved recently[96] in concentrated ammonia at 40 °C in the course of 2 h and amino-Sepharose (XVIII) and some of its derivatives were obtained for the first time. Thus, after the succinylation stage,[97] it was possible to obtain a highly stable affinity adsorbent (XIX) with hemagglutinin (LcH) as the ligand:



The amide and ester linkages in the adsorbent (XIX) proved stable at pH 9.8 (borate buffer solution). Nor are they ruptured when detergent solutions are used as eluents. Glucosamine-succinyloxypropyl-Sepharose has been obtained via an analogous scheme.[98]

The use of 3-amino-2-hydroxypropyl-Sepharose, obtained by the epoxidation of an agarose carrier with epichlorohydrin by means of a modified method[42] (25 °C, 4 h) followed by treatment with a 2 M ammonia solution for 16 h, for the immobilisation of a steroid ligand, namely 17β-hydroxy-3-oxo-5α-androstane-17α-(6-hexanoic acid) has been described.[99] The ligand was coupled to the amino-Sepharose in 70% aqueous dioxan in the presence of EDC.

(c) *Adsorbents based on silica carriers.* There have been no systematic studies of this group of EAC for the immobilisation of low-molecular-weight ligands. An investigation[34] of the immobilisation of 5-hydroxyuridine 2'(3'),5'-diphosphate on alusil in order to obtain an affinity adsorbent for the isolation of endonuclease A5 is of considerable interest. The azo-adsorbent based on epoxidised alusil was synthesised in accordance with the following scheme:



The content of amino-groups on the aminoaryl derivative (XX) was 133 μmol g^{-1} and the capacity of the azo-adsorbent (XXI) with respect to the nucleotide was 36.5 μmol g^{-1}. According to the authors' data, the adsorbent synthesised exhibited virtually no non-specific sorption and was extremely stable: after six isolation cycles, up to approximately 98.3% of the initial amount of ligand immobilised on the carrier remained. The possibility of employing in the syntheses of azo-adsorbents based on silicas of other spacers described [*the p-nitrobenzohydrazide spacer*[95] and spacers obtained on the basis of the hydrazides of *N*-(*p*-nitrobenzoyl)-ε-aminocaproic acid and *N*-(*p*-nitrobenzoyl)-glycylglycine] must be emphasised.[100]

2. Immobilisation of Polymeric Ligands

The immobilisation of polymers and biopolymers of different classes on EAC has not been studied in detail, although the necessity for such investigations is evident. The available literature data on this question are extremely scanty and scattered. EAS-6B has been usually employed for this purpose because of the presence in a carrier of this type of a long spacer, which ensures a fairly high reactivity of the carrier in reactions with biopolymers and the necessary steric accessibility of the immobilised ligand.

Lipopolysaccharides,[101,102] antibodies to human leukocytic interferon,[103] glycoproteins,[96] cellular material,[104] inhibitors of trypsin and trypsin-like enzymes,[27] polysaccharides,[76] and polynucleotides[105,106] have been covalently coupled to EAC as ligands. After the preliminary modification of its active centre with citraconic anhydride, ovomucoid was coupled to EAS-6B at pH 10 in the course of 16 h at 40 °C.[107] The percentage binding of the trypsin-like enzymes by this epoxy-adsorbent proved to be 92% compared with 30% for Sepharose activated with cyanogen bromide.

According to the data of Ustav et al.,[105] the periodate-oxidised t-RNA has been successfully immobilised on hydrazidoadipoyl-EAS-6B.

A simple and effective method of attaching DNA to epoxycellulose has been proposed.[39] The biopolymer was immobilised in 0.1 M sodium hydroxide at 20 °C in the course of 2 h. The DNA content in the resulting adsorbent was approximately 40 mg per ml of the gel. The adsorbent was distinguished by high stability with respect to organic solvents—ethanol, dimethylformamide, dioxan, toluene, etc., and also 2 M sodium chloride or 0.1 M sodium hydroxide in the course of 24 h at approximately 20 °C. The efficiency of the binding of polynucleotides varied in the sequence poly(dT) > poly(dC) = poly(dA) > poly(dG).

Studies of the conditions governing the immobilisation of peptides and proteins on EAC are of definite interest. Thus the optimum conditions for the attachment of glycyl-leucine to EAS-6B (pH 11, 50 °C in the course of 15 h) have been investigated.[27] The optimum binding conditions for the inhibitor of soya bean trypsin involve treatment in 0.5 M carbonate buffer solution at pH 9.5 and 25 °C for 24 h. The cell membrane proteins were immobilised on EAS-6B under similar conditions in the preparation of immunoadsorbents:[104] 0.05 M borate buffer solution at pH 9 in the course of 16 h at approximately 20 °C.

The data presented above suggest that biopolymers are immobilised on EAC under approximately the same conditions (alkaline medium, temperature 20–30 °C) as the coupling of low-molecular-weight ligands. The adsorbents obtained are as a rule highly stable.

A more detailed study of the processes involving the coupling of peptides and proteins to EAC, in particular GMG§, has been undertaken.[33,47] The author showed that a peptide of the type of glycyl-leucine is bound preferentially to GMG via the α-amino-group and the maximum degree of binding is observed after 72–96 h at 25–37 °C. The process proved to depend greatly on the pH with an optimum at pH 9.7. The capacity with respect to the ligand was approximately 20 μmol g^{-1}. The bond between the matrix and the ligand proved to be extremely strong: even acid hydrolysis under severe conditions led to the appearance of only trace amounts of glycine. On the other hand, the binding of the epoxy-groups of the carrier to the carboxy-groups of the ligand was independent of the pH. The ester linkage formed was hydrolysed in the usual way.

§Glycidyl methacrylate gel (Ed.of Translation).

In the immobilisation of serum albumin and trypsin on GMG, the maximum degree of binding of these proteins was found in an acid medium at pH 3, which suggested the important role of carboxy-groups in the coupling processes. The involvement of the lysine ε-amino-groups in the proteins investigated in the binding processes is manifested in the pH range 7–11. According to the authors, the immobilisation via the ε-amino-group is independent of the pH. The capacity with respect to the ligand of the adsorbents obtained at pH 8–11 was 3–4 times lower than for the adsorbents synthesised at acid pH. Apart from trypsin and albumin, it proved possible to immobilise also amino-glucosidase on GMG in an acid medium.[106] Subsequently it was shown that polymers with indole and phenol groups were effectively bound in an acid medium.[67] The optimum binding of indole-containing derivatives was observed at pH 3.85, the binding efficiency diminishing with increase of pH. Imidazole-containing derivatives had a binding optimum at pH 8.15.[33] For derivatives containing a primary amino-group, the binding optimum was detected at pH 9.55.

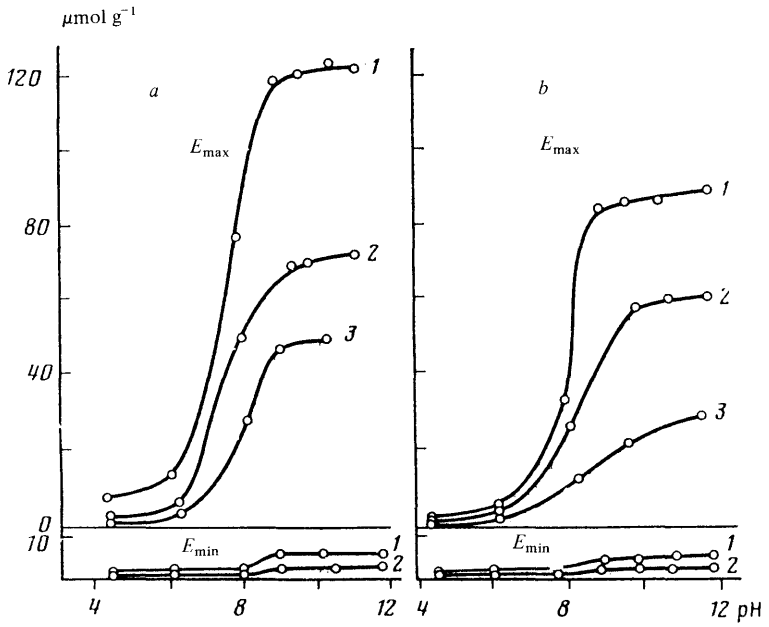
Data obtained in the study[33] of the influence of the nature of proteins on their immobilisation on EAC are of great interest. It was found that not only the binding conditions and the nature of the protein ligand but also the type of the polymer matrix are important in immobilisation. The results of these studies are presented in Table 2.

Table 2. Optimum pH for the immobilisation of certain proteins on various types of EAC.[33]

Immobilised protein*	Type of EAC**				
	porous glass	silica carrier	EAS-6B	GMG	Oxiranacrylic carrier
Serum albumin (4.9)	4 (45), 8.5 (20)	5 (50)	8 (300)	5 (20), 8 (10)	4—10 (50)
Papain (8.7)	8.5 (60)	8 (40)	9 (300)	7 (25)	—
Antilysin (10)	10 (80)	10 (20)	10 (300)	10 (15)	9.5 (100)
Trypsin (10.1)	8.5 (10)	3 (20)	11 (20)	3 (5)	10 (20)
Chymotrypsin	7—8 (50)	7—8 (20)	11 (40)	3 (10), 10 (5)	—

*The isoelectric point is indicated in brackets.
**The amount of immobilised protein in mg per gram of carrier is indicated in brackets.

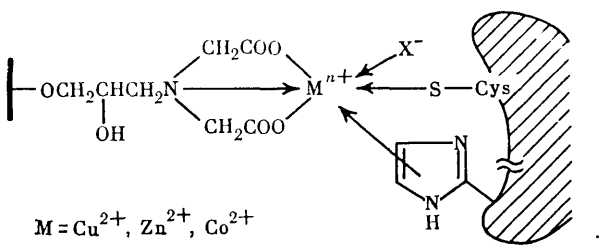
It follows from Table 2 that EAS-6B shows the maximum capacity with respect to the ligand and that only for this matrix was the immobilisation pH always in an alkaline region (pH 8–11). Such non-selectivity with respect to the nature of the immobilised protein should apparently be attributed to the effect of the spacer, which has the maximum length for the given type of EAC—12 carbon and oxygen atoms. Similar results have been obtained also for the oxiran-acrylic carrier. On the other hand, GMG and the adsorbent based on silica are the most sensitive to the nature of the immobilised protein and their capacity with respect to the ligand is low—for GMG it is lowest among all the EAC investigated. For certain proteins a clear-cut relation is observed between the isoelectric point and the optimum pH for immobilisation (for example, in the case of antilysin and to a lesser extent papain and albumin). According to the authors, the results for antilysin can be explained by the absence from this protein of tryptophan which tends to bind in an acid medium.



Dependence of the attachment of glycyl-D-phenylalanine to Separon H 300 (*a*) and Separon H 1000 (*b*), activated with epichlorohydrin, on the pH and reaction time; E_{max} = carriers activated to the maximum extent (1550 μmol g^{-1} for Separon H 300; 1700 μmol g^{-1} for Separon H 1000); E_{min} = carriers activated to the minimum extent (240 μmol g^{-1} for Separon H 300; 140 μmol g^{-1} for Separon H 1000); *1*) 48 h; *2*) 24 h; *3*) 12 h.

An analogous investigation has been carried out on epichlorohydrin-activated hydroxyalkyl methacrylate gels.[48] A study was made of the coupling of various amino-derivatives as a function of the pH, the reaction time, the ligand concentration, the degree of epoxidation of the carrier, and the chemical structure of the ligand. The Figure illustrates the dependence of the coupling of glycyl-D-phenylalanine on various types of epoxy-activated Separons on the pH of the medium and the reaction time.[48]

The covalent immobilisation of albumin and NAD-pyrophoshorylase on EAS-6B took place at 4 °C in the course of 28 h.[109] A number of enzymes (peroxidase, α-amylase, α-chymotrypsin, and glucose isomerase) have been recently immobilised using graft-polymerisation.[51] The method of non-covalent immobilisation for certain enzymes containing imidazole and thio-groups on an epoxy-activated metal chelate carrier is of definite interest.[44] The authors proposed the following structure of the carrier–enzyme complex:



Alkaline phosphatase and malate and lactate dehydrogenases have been immobilised in this way.

IV. THE APPLICATION OF ADSORBENTS BASED ON CARRIERS ACTIVATED BY EPOXY-COMPOUNDS IN AFFINITY CHROMATOGRAPHY

One of the main advantages of EAC is the simplicity of the preparation of affinity of adsorbents on their basis, the process involving only one stage in many instances. The biospecific adsorbents synthesised are exceptionally stable and are hardly hydrolysed in an alkaline medium. Thus the adsorbent with cAMP, immobilised on EAS-6B via the alcoholic hydroxyl, lost only approximately 0.04% of the ligand after storage in borate buffer solution for six months at 4 °C.[72] Immunoadsorbents based on EAS-6B lost the radioactive ligand to the extent of only 0.04–0.02%, while the percentage loss of radioactivity for the analogue based on Sepharose activated with bromocyanogen was 0.6–1.1% under the same conditions.[103] The complete absence of the scission of the coenzyme during the storage of NAD-containing epoxy-adsorbents compared with the bromocyano- and azo-derivatives has been demonstrated.[69]

A second important advantage of the adsorbents based on EAC is their high biospecificity owing to the hydrophilic nature of the spacer and the absence of additional ionogenic groups. As a consequence of this there is a possibility of employing adsorbents of this kind at earlier stages in the isolation and purification of biopolymers. For example, the direct use of thyroxine-epoxy-Sepharose[110] for the AFC of the serum in the early stages of the purification proved successful in the isolation of thyroxin-binding globulin. A 1000-fold purification of the test protein with a yield of about 70% was achieved. A fairly high degree of purification in the AFC stage (by a factor of 600) was retained also after the preliminary chromatography of the serum on hydroxyapatite.

Epoxy-activated Separon 1000 with the methyl ester of L-phenylalanyl-D-phenylalanine attached as the ligand has been used for the AFC of pepsin and proteinases from *Aspergillus oryzae*.[48] The high mechanical stability of the adsorbent made it possible to carry out the analytical AFC of pepsin under high pressure.

8-Aminoxanthine-epoxy-Sepharose showed a high selectivity in the single-stage isolation of the enzyme urate oxidase.[55] The degree of purification was by a factor of 88, the yield of the enzyme reached 93%, and the eluent was a 5 mM solution of uric acid.

The maximum degree of purification of cholinephosphate cytidylyltransferase on an EAS-6B-based adsorbent was by a factor of approximately 250 in the AFC stage with an overall yield of 59%.[62] A similar degree of purification (by a factor of 200) has been observed in the isolation of glutathione reductase on NADP-hydrazidoadipoylhydroxypropyl-Sepharose.[111] It is important to note that the adsorbent employed in the above study proved to be highly selective with respect to glutathione reductase (the arsenic-resistant fraction) and hardly bound the thiol-disulphide enzymes of the arsenic-sensitive fraction. An effective express method has been developed with the aid of this adsorbent for the preparation of almost homogeneous glutathione reductase.[111] It is remarkable that in both cases the AFC was carried out directly with the supernatant isolated after fractionation with ammonium sulphate. A simple and rapid three-stage method for the purification of α-amylase from triticale included in the last stage AFC on cyclohepta-amylose-epoxy-Sepharose.[84] It resulted in a final purification of the enzyme by a factor of 143 with an overall yield of about 33%.

In a number of studies the AFC on epoxy-sorbents was used in the concluding stages of the purification. Thus the application of pteridine-epoxy-Sepharose in the last (fifth) stage of the isolation of phenylalanine hydroxylase from rat liver permitted a degree of purification of the enzyme in the AFC stage by a factor of about 30.[66] The overall degree of purification was by a factor of 510 and the yield was 98%. The final phenylalanine hydroxylase preparation contained an admixture with catalase activity. AFC on *S*-hexylglutathione-epoxy-Sepharose was used in one of the stages of the purification of glyoxalase[57] and chromatography on this adsorbent led to an enzyme with a higher specific activity than purification on the adsorbent based on the bromocyanogen-activated Sepharose.

The use of metal chelate adsorbents based on epoxy-Sepharose has been described.[80,112-114] The purification of nucleoside diphosphatase by a factor of 194 and with a yield of about 16% has been achieved on these adsorbents.[80] The eluent was a 15 mM solution of L-histidine in 10 mM maleate buffer solution.

It is known that adsorbents based on EAC are widely used also in hydrophobic chromatography. Studies of the relation between the chemical structure of the adsorbents and the characteristics of the adsorption of various proteins are of definite interest. Studies[45,46] of blood proteins established the following general features. Adsorbents with lengths of the alkyl chain ranging from C_1 to C_5 gave rise to a relatively low degree of adsorption of blood proteins, while an increase in the hydrophilicity and polarity of the ligand by the introduction of hydroxy-, methoxy-, or cyano-groups greatly reduced the degree of adsorption. The maximum decrease was observed after the introduction of the cyano-group. On the other hand, the presence of a positive charge in the ligand molecule (a quaternary ammonium group) increased the degrees of adsorption of all the proteins investigated. It must be emphasised that the optimum lengths of the hydrocarbon chain in the ligand are different for different proteins. Fibrinogen, thrombin, and globulin have a binding optimum on the ligand with a C_7 chain, while for albumin and immunoglobulin G the adsorption optimum is on the ligand with a C_8 chain. The presence of branches in the ligand decreases the degree of adsorption.

The isolation of prephenate dehydratase has been achieved using adsorbents with a hexamethylenediamine spacer and certain aminoacids as ligands.[92] *N*-Acetylated derivatives of these adsorbents lost their capacity to bind the enzyme. At the same time the enzyme could be desorbed only from the aminohexyl and glycylaminohexyl analogues of 1,2-EPS (in 0.5 M sodium chloride or 140 mM hexamethylenediamine).

A study[115] undertaken to screen the affinity adsorbents for the isolation of inorganic pyrophosphatase[115] showed that hexylepoxy-Sepharose with a high ligand concentration (40 μmol ml^{-1}) strongly bound the enzyme with a complete retention of its pyrophosphatase activity. The protein could not be eluted (1 M sodium chloride, substrate). On the other hand, the bromocyanogen analogues of the adsorbent (admittedly with a ligand concentration lower by a factor of 4–7) bound the enzyme much less strongly. It was readily eluted from these adsorbents with 0.05–0.1 M sodium chloride solution.

A number of studies have been published recently concerning the use of commerical Octyl-Sepharose and phenyl-Sepharose for the hydrophobic chromatography of many biopolymers, for example, choline acetyltransferase,[116] specific endonucleases,[117] alkaline phosphatase, and 5'-AMP nucleosidase,[118] and restriction endonuclease REcoCK,[119] and also for the separation of the phospholipid and protein components of complex III of the respiratory chain of mitochondria,[120] for the immobilisation of trypsin with preliminary modification of the enzyme,[121] for the isolation of microsomal cytochrome P-450,[122] for the investigation of cobalamin-binding proteins,[123] for the purification of calmodulin,[124] etc.

It has been noted that in certain cases where adsorbents based on Sepharose activated with cyanogen bromide are used the ligand undergoes appreciable bioinactivation, while the analogous epoxy-adsorbent retains a satisfactory affinity for the biopolymer isolated.[107] Thus in the AFC of a trypsin-like enzyme the highest percentage binding was achieved for the adsorbent based on EAS-6B (92%).

Thiopropyl-Sepharose is one of the adsorbents widely used in covalent chromatography.[35] The spectrum of its applications is fairly wide, encompassing the isolation of thiol-containing proteins,[125,126] thiol-containing peptides,[127] and mercurated polynucleotides.[128] The possibility of separating two similar thiol-disulphide oxidoreductases, protein disulphide isomerase, and glutathione-insulin transhydrogenase by covalent chromatography on thiopropyl-Sepharose has been demonstrated.[129] The supernatant was subjected to preliminary purification by fractionation with ammonium sulphate, gel-filtration on Sephadex G-200, and ion-exchange chromatography. The eluents for covalent chromatography were thiols with an increasing reducing capacity—20 mM L-cysteine, 50 mM reduced glutathione, and 20 mM dithiothreitol. The employment of thiopropyl-Sepharose made it possible[130] to separate highly reactive thiols, for example, cysteine proteinase from *Actinidia chinensis*, from the remaining thiol-containing components. The application of reduced thiopropyl-Sepharose in the purification of cytoplasmic aldehyde dehydrogenase involving the removal of an admixture of mitochondrial proteins has been described.[131] The same adsorbent modified with 2-hydroxy-5-nitrobenzyl bromide was used for the immobilisation of α-chymotrypsin

and β-amylase.[132] The problems of the application of covalent adsorbents are specifically considered in a review.[18]

The principal studies dealing with the applications of adsorbents based on EAC in AFC are listed in Table 3.

Table 3. The application of adsorbents based on EAC for the isolation and purification of biopolymers.

Ligand (spacer)	Biopolymer isolated source)	Refs.
	EAS-6B	
Inhibitor of osya bean trypsin	Trypsin (commercial preparation)	[27]
Ovomucoid	Trypsin-like enzyme (from *Streptomyces erethreus*)	[107]
Myoinositol	Myoinositol oxygenase (from rat kidneys) myoinositol kinase (from *Lemna gibba*)	[82]
Adenosine 3′,5′-diphosphate (hexamethylenediamine)	Succinate thiokinase (from pig heart)	[73]
Choline	Choline and enthanolamine kinases (from rat liver)	[86]
8-Hydroxyethyl-cAMP	Regulatory subunit of protein kinase	[72]
S-Hexylglutathione	Glutathione S-transferase (from human placenta)	[81]
Reduced glutathione	Glutathione S-transferase (from human liver)	[63]
Glycerylphosphoryl choline	Cholinephosphate cytidylyltransferase (from rat liver)	[62]
Ribose-5-phosphate, neopyrithiamine, thiamine pyrophosphate, hydroxythiamine	Transketolase (from *Candida utilis* and baker's yeast)	[59]
Adenosine	Adenosine deaminase (from human erythrocytes)	[61]
S-Hexylglutathione	Glyoxalase I (from rabbit liver)	[57]
2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine	Phenylalanine hydroxylase (from rat liver)	[66]
Trimethoprim and its analogues	Dihydrofolate reductase (from *E.coli*)	[53]
Aminomethyl-4-anilino-5-methoxy-1,2-benzoquinone	Menadione reductase (from rat liver)	[65]
8-Aminoxanthene	Urate oxidases (from ox liver, rat liver, and yeast)	[55]
Folic acid	Ribonuclease A (from ox pancreas)	[54]
Ditto	Endonucleases EcoRI (from *E.coli*) and BglII (from *Bacillus globiggii*)	[117]
NAD^+ or NADH	Alcohol and lactate dehydrogenases	[69]
3-Aminophenylboronic acid + nucleotides and coenzymes	Dehydrogenases	[83]
Cyclohepta-amylose	α-Amylase (from a hybrid of wheat and full flax)	[84]
Aminomethyl-, 4-aminobenzyl-, and *N*-(4-aminobenzyl)-4-aminobenzyl-phosphonic acids	Alkaline phosphatase (from ox small intestine)	[64]
Arabinan	α-L-arabinofuranosidase (from *Aspergillus niger*)	[76]
Iminodiacetic acid + Cu^{2+}	Nucleoside diphosphatase (from rat liver)	[80]
Ditto	Lactoferrin (from human milk)	[112]
Iminodiacetic acid + Zn^{2+}	Alpha$_2$-SH-glycoprotein (from human plasma)	[113]
D-Galactosamine	β-Galactosyl-binding lectin (tridacnin) from *Tridacna maxima*)	[58]
Trehalose	Concanavalin A (from *Canavalia ensiformis*)	[78]
N-Acetyl-D-galactosamine, *N*-acetyl-D-glucosamine	Lectins (from soya beans and wheat seedlings)	[60]
Tri-N-acetylchitotriose, methyl *N*-acetyl-β-D-glucosaminide	Agglutinin (from *Solanum tuberosum*)	[41]
17β-Hydroxy-3-oxo-5α-androstane-17β-(6-hexanoic acid) (diaminoethyl)	Steroid-binding protein (from human serum)	[38]
Auxin analogues	Auxin-receptor proteins	[68]
Thyroxin	Thyroxin-binding protein (from human serum)	[110]
N-(6-Aminohexyl)-5-chloronaphthalene-1-sulphonamide	Calmodulin and calcium-binding protein S-100 (from ox brain)	[74]
Agglutinin from *Ricinus communis*	Tyroglobulin	[41]
t-RNA (hydrazidoadipoyl)	Ribosomal proteins	[105]
Iminodiacetic acid + Zn^{2+}	Interferon (from human fibroblasts)	[114]
Interferon from human leukocytes	Corresponding antibodies	[103]
β-Ecdysone	Proteins of the corresponding antiserum	[133]
Lipopolysaccharides from *Shigella sonnei*	Homologous antiserum, bacteriophage SKVI	[101]
Membrane material from human lymphoblastoid cells	Corresponding antibodies	[104]
	EAS-6B and 1,2-EPS	
Haemagglutinin (succinyl)	Glycoproteins	[96]
	1,2-EPS	
2-Thio-4-(nitro, carboxy)pyridine; tetramethylthiuram disulphide residue	Cysteine peptides, mercaptalbumin, papain, urease, ox serum albumin, pancreatic ribonucleases	[87, 127]

Table 3 (cont'd.).

Ligand (spacer)	Biopolymer isolated (source)	Refs.
2-Nitro-5-thiobenzoic acid (aminoethyl, glutathione, cysteine)	Glutathione reductase (from rat liver)	[43]
NADP (*p*-azobenzohydrazide)	Ditto	[95]
Ditto or NADPH (hydrazidosuccinyl, hydrazido-adipoyl)	"	[90, 91, 111]
Aminoacids (hexamethylenediamine, succinyl)	Prefenate dehydratase	[92]
3-Hemisuccinate of 5α-androstane-3β, 17β-diol(4,4′-azodianiline)	Testosterone-oestradiol-binding globulin (from human plasma	[94]
Cortisol, 5α-dihydrotestosterone	Steroid-binding proteins	[134]
Adenine and guanine nucleotides (hydrazidoadipoly)	Elongation factor Tu (from *E.coli* MRE 600)	[89]
Alkyls, hydroxy(alkoxy)alkyls, and their cyano- and carboxy-derivatives	Albumin, globulin, fibrinogen, prothrombin	[45, 46]
Thiopropyl	Protein disulphide-isomerase (from ox liver), glutathione-insulin transhydrogenase (from ox liver)	[126, 129]
Ditto	Actinidin (from *Actinidia chinensis*)	[130]
"	Copper-binding proteins from human liver	[125]
	Amino-1,2-EPS	
Lactose, melibiose, maltose, *N*-acetylchitotetraose; glucosamine, galactosamine(succinyl)	Agglutinins (from soya, *Arachis hypogaea*, wheat seedlings, etc.)	[98]
17β-Hydroxy-3-oxo-5α-androstane-17α-(6-hexanoic acid)	Androgen-binding protein (from rat epididymis)	[99]
	Epoxycellulose	
Deoxyribonucleic acid	Polynucleotides, complementary m-RNA (from bird liver)	[39]
	GMG	
Trypsin	Trypsin inhibitors	[47]
	Epoxy-separon H 1000	
Methyl ester of L-phenylalanyl-D-phenylalanine (ε-aminocaproyl)	Pepsin and proteinases (from *Aspergillus oryzae*)	[48]
	Silica(alusil)	
5-Hydroxyuridine 2′(3′), 5′-diphosphate (*p*-azobenzoyl)	Endonuclease A5 (from actinomycetes)	[34]

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The data described above show that biospecific adsorbents based on EAC constitute one of the commonest types of adsorbents currently used in AFC and related methods. One sometimes encounters the view that AFC in a pure form does not exist at all and that the biospecific interaction of the biopolymer to be isolated with the sorbent in AFC is inevitably accompanied by a complicated set of interactions including non-specific electrostatic, hydrophobic, and other effects. Both AFC and associated methods, such as hydrophobic, covalent, and metal chelate chromatography are therefore incorporated in a single group of methods. This tendency carries the risk, both formal and purely theoretical, of an erroneous interpretation of the chromatographic results, particularly when the AFC method is used for the investigation of the mechanisms of biochemical reactions, for the determination of the constants for the association of biopolymers with low-molecular-weight ligands, etc. An arbitrary mixing of the methods, indicated above, for the isolation and purification of biopolymers can be caused, and this is not the least important cause, by the application in AFC, particularly for preparative purposes, of carriers such as porous glass, silochromes, and a number of others, which by their very nature can introduce into the purification process non-specific interactions which do not, however, prevent in many cases the attainment of a fairly high degree of purification of the biopolymer.

The efforts of investigators concerned with the further development of the AFC method will undoubtedly be directed to the elimination or reduction to a minimum of non-specific

effects in the chromatographic process, because this will make it possible to demonstrate fully the unique possibilities of the AFC method. For a clear-cut formal separation of the AFC method from other purification procedures, the term "bioaffinity chromatography" has been proposed.[10,135,136] The new methods for the activation of polysaccharide matrices which have appeared recently have been chosen to obviate the disadvantages of the method of bromocyanogen activation, which is still most widely used. These include the modification (based on the study of the reaction mechanism) of the bromocyanogen method itself,[137,138] the employment of reactive sulphonyl chlorides[139,140] and carbonylating reagents,[141,142] *O*-succinylation,[143] the application of activated chloroformates,[144] and a number of others. Activation by epoxy-compounds occupies an honourable place among these methods. Its evident advantages, namely simplicity, chemical strength of the bond formed, hydrophilicity of the adsorbents obtained, and elimination or reduction to a minimum of the charged groups of the adsorbent, promote the maximum approach of the interaction between the biopolymer to be isolated and the immobilised ligand to processes occurring under physiological conditions, i.e. the achievement of the idea of the AFC method in a pure form. One may also expect further extensive application of adsorbents based on EAC for the effective purification of biopolymers and as a refined instrument for biochemical research.

REFERENCES

1. C.R.Lowe and P.D.G.Dean, "Affinity Chromatography", Wiley, London, 1974, p.272.
2. W.B.Jakoby and M.Wilchek (Editors), "Methods in Enzymology", Acad.Press, New York, 1974, p.810.
3. I.A.Bezvershenko, "Affinnaya Khromatografiya" (Affinity Chromatography), Izd.Naukova Dumka, Kiev, 1978, p.126.
4. J.Turkova, "Affinity Chromatography" (Translated into Russian), Izd.Mir, Moscow, 1980, p.471.
5. S.W.May and O.R.Zaborsky, "Separation and Purification Methods", 1974, Vol.3, p.1.
6. M.Wilchek, in "Immobilized Biochemicals and Affinity Chromatography" (Edited by R.R.Dunlap), Plenum Press, New York, 1974, p.15.
7. J.Porath and T.Kristiansen, in "The Proteins" (Edited by H.Neurath and R.L.Hill), Acad.Press, New York, 1975, Vol.1, p.95.
8. J.Porath, J.Macromol.Sci., Chem., 1976, **10A**, 1.
9. A.H.Nishikawa, P.Bailon, and A.H.Ramel, J.Macromol.Sci., Chem., 1976, **10A**, 149.
10. B.A.Klyashchitskii and V.S.Shapot, Uspekhi Biol. Khim., 1976, **17**, 234.
11. J.Porath and K.D.Caldwell, in "Biotechnological Application of Proteins and Enzymes" (Edited by Z.Bohak and N.Sharon), Acad.Press, New York, 1977, p.83.
12. B.A.Klyashchitskii, Bioorg.Khim., 1980, **6**, 485.
13. V.S.Vesa, Prikl.Biokhim.Mikrobiol., 1980, **16**, 629.
14. H.Lis and N.Sharon, J.Chromatog., 1981, **215**, 361.
15. H.Guilford, Chem.Soc.Rev., 1973, **2**, 249.
16. T.Miron and M.Wilchek, J.Chromatog., 1981, **215**, 55.
17. B.A.Klyashchitskii, "Tezisy Vsesoyuznoi Konferentsii po Perspektivam Ispol'zovaniya Biospetsificheskoi Khromatografii v Tekhnologii Proizvodstva Vysokoochishchennykh Fermentov, Vil'nyus, 1982" (Abstracts of Reports at the All-Union Conference on the Prospects for the Utilisation of Biospecific Chromatography in the Technology of the Manufacture of Highly Purified Enzymes, Vilna, 1982), p.10.
18. V.I.Lozinskii and S.V.Rogozhin, Uspekhi Khim., 1980, **49**, 879 [Russ.Chem.Rev., 1980(5)].
19. R.Axen, J.Porath, and S.Ernback, Nature, 1967, **214**, 1302.
20. J.Turkova, J.Chromatog., 1974, **91**, 267.
21. M.Wilchek, FEBS Letters, 1973, **33**, 70.
22. V.Sica, I.Parikh, G.A.Puca, and P.Cuatrecasas, J.Biol.Chem., 1973, **248**, 6543.
23. B.A.Klyashchitsky and V.Kh.Mitina, J.Chromatog., 1981, **210**, 55.
24. B.A.Klyashchitsky, V.Kh.Mitina, G.E.Morozevich, and R.I.Yakubovskaya, J.Chromatog., 1981, **210**, 67.
25. J.Porath, J.-C.Jonson, and T.Laas, J.Chromatog., 1971, **60**, 167.
26. J.Porath and L.Sundberg, Nature (New Biol.), 1972, **238**, 261.
27. L.Sundberg and J.Porath, J.Chromatog., 1974, **90**, 87.
28. J.Carlsson, R.Axen, and T.Unge, Europ.J.Biochem., 1975, **59**, 567.
29. J.Chromatog., 1981, **215**, 13, 19.
30. C.W.Fuller, J.R.Rubin, and H.J.Bright, Europ.J. Biochem., 1980, **103**, 421.
31. D.M.Krämer, K.Lehmann, H.Pennewiss, and and H.Plainer, Prepar.Short Comm.IUPAC, 1979(3), 1559.
32. J.Coupek, P.Gemeiner, V.Jirku, J.Kalal, V.Kubanek, L.Kuniak, J.Peska, L.Rexova, J.Stamberg, F.Svec, J.Turkova, B.Veruovic, and J.Lemek, Chem.Listy, 1981, **75**, 512.
33. I.Lemanova, J.Turkova, M.Capka, L.A.Nakhapetyan, F.Svec, and J.Kalal, Enzyme Microb.Technol., 1981, **3**, 229.
34. G.E.Bannikova, V.P.Varlamov, O.L.Samsonova, and S.V.Rogozhin, Bioorg.Khim., 1982, **8**, 212.
35. R.Axen, H.Drevin, and J.Carlsson, Acta Chem. Scand., 1975, **29B**, 471.
36. S.Hjerten, J.Rosengren, and S.Pahlmann, J.Chromatog., 1974, **101**, 281.
37. J.Rosengren, S.Pahlmann, M.Glad, and S.Hjerten, Biochim.Biophys.Acta, 1975, **412**, 51.
38. P.H.Pétra and J.Lewis, Anal.Biochem., 1980, **105**, 168.
39. L.G.Moss, J.P.Moore, and L.Chan, J.Biol.Chem., 1981, **256**, 12655.
40. R.W.Coughlin, M.Aizawa, and M.Charles, Biotechnol. Bioeng., 1976, **18**, 199.
41. I.Matsumoto, Y.Mizuno, and N.Seno, J.Biochem., 1979, **85**, 1091.
42. A.H.Nishikawa and P.Bailon, J.Solid-Phase Biochem., 1976, **1**, 33.
43. P.V.Kuznetsov, V.S.Bondar', and L.A.Likhacheva, "Tezisy Vsesoyuznoi Konferentsii po Perspektivam Ispol'zovaniya Biospetsificheskoi Khromatografii v Technologii Proizvodstva Vysokoochishchennykh Fermentov, Vil'nyus, 1982" (Abstracts of Reports at the All-Union Conference on the Prospects for the Utilisation of Biospecific Chromatography in the Technology of the Manufacture of Highly Purified Enzymes, Vilna, 1982), p.73.

44. P.R.Coulet, J.Carlsson, and J.Porath, Biotechnol. Bioeng., 1981, **23**, 663.
45. W.Kissing and R.H.Reiner, Chromatographia, 1978, **11**, 83.
46. W.Kissing and R.H.Reiner, J.Solid-Phase Biochem., 1979, **4**, 221.
47. J.Turkova, K.Blaha, M.Malanikova, D.Vancurova, F.Svec, and J.Kalal, Biochim.Biophys.Acta, 1978, **524**, 162.
48. J.Turkova, K.Blaha, J.Horacek, J.Vajcner, A.Frydrychova, and J.Coupek, J.Chromatog., 1981, **215**, 165.
49. G.E.Orlov, D.L.Yaglom, A.E.Gurvich, A.D.Virnik, and Z.A.Rogovin, Vop.Med.Khim., 1969, **15**, 287.
50. A.D.Virnik, R.I.Fesenko, Yu.V.Artemova, Z.A.Rogovin, and V.A.Yaklovlev, "Tezisy I Vsesoyuznogo Simpoziuma po Immobilizovannym Fermentam. Tallin, 1974" (Abstracts of Reports at the Ist All-Union Symposium on Immobilised Enzymes, Tallin, 1974), p.85.
51. L.D'Angiuro, P.Cremonesi, G.Mazzola, B.Focher, and G.Vecchio, Biotechnol.Bioeng., 1980, **22**, 2251.
52. S.H.Chang, K.N.Gooding, and F.E.Regnier, J.Chromatog., 1976, **120**, 321.
53. R.L.Then, Biochim.Biophys.Acta, 1980, **614**, 25.
54. F.Sawada, Anal.Biochem., 1979, **98**, 184.
55. T.Watanabe and T.Suga, Anal.Biochem., 1978, **86**, 357.
56. M.O.Mansson, B.Mattiasson, G.Gestrelius, and K.Mosbach, Biotechnol.Bioeng., 1976, **18**, 1145.
57. N.Elango, S.Janaki, and A.R.Rao, Biochem. Biophys.Res.Comm., 1978, **83**, 1388.
58. B.A.Baldo, W.H.Sawver, R.V.Stick, and G.Uhlenbruck, Biochem.J., 1978, **175**, 467.
59. T.Wood and S.Fletcher, Biochim.Biophys.Acta, 1978, **527**, 249.
60. P.Vretblad, Biochim.Biophys.Acta, 1976, **434**, 169.
61. W.P.Schrader, A.S.Stacy, and B.Pollara, J.Biol. Chem., 1976, **251**, 4026.
62. P.C.Choy and D.E.Vance, Biochem.Biophys.Res. Comm., 1976, **72**, 714.
63. P.S.Simons and D.L.Van der Jagt, Anal.Biochem., 1977, **82**, 334.
64. M.Landt, S.C.Boltz, and L.G.Butler, Biochemistry, 1978, **17**, 915.
65. E.P.Titovets, Biokhimiya, 1982, **47**, 276.
66. S.Webber, G.Harzer, and J.M.Whiteley, Anal.Biochem., 1980, **106**, 63.
67. J.Drobnik, J.Vlasak, J.Pilar, F.Svec, and J.Kalal, Enzyme Microb.Technol., 1979, **1**, 107.
68. B.Tappeser, D.Wellnitz, and D.Klamst, Z.Pflanzen-physiol., 1981, **101**, 295.
69. H.-L.Schmidt and G.Grenner, Europ.J.Biochem., 1976, **67**, 295.
70. V.Kh.Mitina, B.A.Klyshchitskii, I.G.Dubinina, and G.I.Vornovitskaya, Khim.Prirodn.Soed., 1980(1), 134.
71. V.Kh.Mitina, B.A.Klyshchitskii, I.G.Dubinina, and G.I.Vornovitskaya, Zhur.Obshch.Khim., 1981, **51**, 217.
72. W.Weber, C.-W.Vogel, and H.Hilz, FEBS Letters, 1979, **99**, 62.
73. S.Barry, P.Brodelius, and K.Mosbach, FEBS Letters, 1976, **70**, 261.
74. T.Endo, T.Tanaka, T.Isobe, H.Kasai, T.Okuyama, and H.Hidaka, J.Biol.Chem., 1981, **256**, 12485.
75. P.Vretblad, FEBS Letters, 1974, **47**, 86.
76. R.Waibel, R.Amado, and H.Neukom, J.Chromatog., 1980, **197**, 86.
77. R.Uy and F.Wold, J.Chromatog., 1977, **81**, 98.
78. B.A.Klyshchitskii, Khim.Prirodn.Soed., 1979, **5**, 709.
79. J.Porath, J.Carlsson, I.Olsson, and G.Belfroge, Nature, 1975, **258**, 598.
80. I.Ohkubo, T.Kondo, and N.Taniguchi, Biochim. Biophys.Acta, 1980, **616**, 89.
81. C.Guthenberg, K.Akerfeldt, and B.Mannervik, Acta Chem.Scand., 1979, **B33**, 595.
82. H.Fazldeen and M.Breitenbach, Monatsch.Chem., 1979, **110**, 823.
83. V.Bouriotis, I.J.Galpin, and P.D.G.Dean, J.Chromatog., 1981, **210**, 267.
84. M.P.Silvanovich and R.D.Hill, Anal.Biochem., 1976, **73**, 430.
85. E.M.Beier, B.A.Klyashchitskii, and G.Ya.Vidershain, Bioorg.Khim., 1979, **5**, 268.
86. P.J.Brophy and D.E.Vance, FEBS Letters, 1976, **62**, 123.
87. J.Carlsson, Hindustan Antibiot.Bull., 1978, **20**, 105.
88. M.Wilchek and R.Lamed, in "Methods in Enzymology" (Edited by W.B.Jakoby and M.Wilchek), Acad.Press, New York, 1974, Vol.34, p.475.
89. M.Ustav, J.Remme, and A.Lind, Bioorg.Khim., 1979, **5**, 365.
90. P.V.Kuznetsov and V.S.Bondar', "Tezisy Dokladov V Vsesoyuznogo Simpoziuma po Fizike i Khimii Belkov i Peptidov. Baku, 1980" (Abstracts of Reports at the Vth All-Union Symposium on the Physics and Chemistry of Proteins and Peptides, Baku, 1980), p.82.
91. V.S.Bondar', P.V.Kuznetsov, and L.S.Kolesnichenko, Symposium, "Regulyatornye Effekty i Obmen Monoaminov i Tsiklonukleotidov" (Regulatory Effects and Metabolism of Monoamines and Cyclonucleotides), Krasnoyarsk, 1981, No.3, p.71.
92. P.Campbell and G.I.Glover, J.Solid-Phase Biochem., 1978, **3**, 107.
93. I.Streikuviene, G.Nekrasaite, and V.Kuliene, "Tezisy Vsesoyuznoi Konferentsii po Perspektivam Ispol'zovaniya Biospetsificheskoi Khromatografii v Tekhnologii Proizvodstva Vysokoochishchennykh Fermentov, Vil'nyus, 1982" (Abstracts of Reports at the All-Union Conference on the Prospects for the Utilisation of Biospecific Chromatography in the Technology of the Manufacture of Highly Purified Enzymes, Vilna, 1982), p.60.
94. W.Rosner and R.N.Smith, Biochemistry, 1975, **14**, 4813.
95. V.S.Bondar', P.V.Kuznetsov, and V.V.Mezhevikin, "Trudy III Vsesoyuznoi Mezhuniversitetskoi Konferentsii po Fiziko-Khimicheskoi Biologii" (Transactions of the IIIrd All-Union Interuniversity Conference on Physicochemical Biology), Izd. Tbilissk.Univ., Tbilisi, 1982, p.62.
96. I.Matsumoto, N.Seno, A.M.Golovtchenko-Matsumoto, and T.Osawa, J.Biochem., 1980, **87**, 535.
97. P.Cuatrecasas and I.Parikh, Biochemistry, 1972, **11**, 2291.
98. I.Matsumoto, H.Kitagaki, Y.Akai, Y.Ito, and N.Seno, Anal.Biochem., 1981, **116**, 103.
99. F.Larrea, N.A.Musto, G.L.Gunsalus, J.P.Mather, and C.W.Bardin, J.Biol.Chem., 1981, **256**, 12566.
100. B.A.Klyashchitskii, V.F.Pozdnev, V.Kh.Mitina, A.I.Voskoboev, and I.B.Chernikevich, Bioorg. Khim., 1980, **6**, 572.
101. E.Romanowska, C.Lugowski, and M.Mulczyk, FEBS Letters, 1976, **66**, 82.
102. J.Fox and K.Hechemy, Infect.Immunol., 1978, **20**, 867.

103. K.Berg, I.Heron, and R.Hamilton, Scand.J. Immunol., 1978, **8**, 429.
104. H.J.Zola, Immunol.Methods, 1978, **21**, 51.
105. M.Ustav, R.Villems, and A.Lind, FEBS Letters, 1977, **82**, 259.
106. M.Ustav, A.Lind, M.Saarma, and R.Villems, Bioorg.Khim., 1977, **3**, 1699.
107. K.Nagata and N.Yoshida, J.Biochem., 1981, **89**, 1121.
108. F.Svec, J.Kalal, I.I.Menyailova, and L.A.Nakhapetyan, Biotechnol.Bioeng., 1978, **20**, 1319.
109. H.-H.Ting and W.J.D.Whish, Biochem.Soc.Trans., 1980, **8**, 635.
110. B.Kagedal and M.Kallberg, Clin.Chim.Acta, 1977, **78**, 103.
111. V.S.Bonbar', P.V.Kuznetsov, L.A.Likhacheva, and L.S.Kolesnichenko, "Tezisy Vsesoyuznoi Konferentsii po Perspektivam Ispol'zovaniya Biospetsificheskoi Khromatografii v Tekhnologii Proizvodstva Vysokoochishchennykh Fermentov, Vil'nyus, 1983" (Abstracts of the Reports at the All-Union Conference on the Prospects for the Utilisation of Biospecific Chromatography in the Technology of the Manufacture of Highly Purified Enzymes, Vilna, 1982), p.74.
112. B.Lonnerdal, J.Carlsson, and J.Porath, FEBS Letters, 1977, **75**, 89.
113. J.P.Lebreton, FEBS Letters, 1977, **80**, 351.
114. V.G.Edy, S.Billiau, and P.De Somer, J.Biol.Chem., 1977, **252**, 5934.
115. E.A.Plaksina, G.V.Kolomiichenko, V.A.Sklyankina, A.T.Mevkh, and S.M.Avaeva, Biokhimiya, 1982, **47**, 1483.
116. J.R.Slemmon, P.M.Salvaterra, G.D.Crawford, and E.Roberts, J.Biol.Chem., 1982, **257**, 3847.
117. O.I.Moroshnichenko, B.S.Naroditskii, S.N.Khil'ko, T.N.Platonova, I.M.Gruber, and T.I.Tikhonenko, Biokhimiya, 1982, **47**, 686.
118. D.R.Armant and C.L.Rutherford, J.Biol.Chem., 1981, **256**, 12710.
119. T.M.Uporova, S.N.Khil'ko, I.I.Nikol'skaya, and S.S.Debov, Vop.Med.Khim., 1982, **28**, 78.
120. Y.Shimomura and T.Ozawa, Biochem.Intern., 1980, **1**, 283.
121. H.-L.Wu and G.E.Means, Biotechnol.Bioeng., 1981, **23**, 855.
122. M.Pasanen and O.Pelkonen, Biochem.Biophys.Res. Comm., 1981, **103**, 1310.
123. J.A.Begley, S.M.Heckman, and C.A.Hall, Biochem. Biophys.Res.Comm., 1981, **103**, 434.
124. R.Gopalakrishna and W.B.Anderson, Biochem. Biophys.Res.Comm., 1982, **104**, 830.
125. L.Ryden and H.F.Deutsch., J.Biol.Chem., 1978, **253**, 519.
126. D.A.Hilsson and R.B.Freedman, Biochem.Soc. Trans., 1979, **7**, 573.
127. A.Svenson, J.Carlsson, and D.Eaker, FEBS Letters, 1977, **73**, 171.
128. R.M.K.Dale and D.S.Ward, Biochemistry, 1975, **14**, 2458.
129. D.A.Hillson and R.B.Freedman, Biochem.J., 1980, **191**, 389.
130. K.Brocklehurst, B.B.Baines, and J.P.G.Malthouse, Biochem.J., 1981, **197**, 739.
131. T.M.Kitson, J.Chromatog., 1982, **234**, 181.
132. K.D.Caldwell, R.Axen, and A.T.Mevkh, Bioorg. Khim., 1978, **4**, 707.
133. B.A.Sage and J.D.O'Connor, Anal.Biochem., 1976, **73**, 240.
134. A.G.Predko and O.V.Sviridov, "Tezisy Dokladov VII Konferentsii Molodykh Uchenykh 'Sintez i Issledovanie Biologicheskie Aktivnykh Soedinenii'" (Abstracts of Reports at the VIIth Conference of Young Scientists on 'The Synthesis and Investigation of Biologically Active Compounds'), Izd.Zinatne, Riga, 1981, p.105.
135. P.O'Carra, S.Barry, and T.Griffin, in "Methods in Enzymology" (Edited by W.B.Jakoby and M.Wilchek), Acad.Press, New York, 1974, Vol.34, p.108.
136. J.Porath, Biochimie, 1973, **55**, 943.
137. J.Kohn and M.Wilchek, Enzyme Microb.Technol., 1982, **4**, 161.
138. J.Kohn and M.Wilchek, Biochem.Biophys.Res. Comm., 1982, **107**, 878.
139. K.Nilsson and K.Mosbach, Europ.J.Biochem., 1980, **112**, 397.
140. K.Nilsson and K.Mosbach, Biochem.Biophys.Res. Comm., 1981, **102**, 449.
141. G.S.Bethell, J.S.Ayers, W.S.Hancock, and M.T.W.Hearn, J.Biol.Chem., 1979, **254**, 2574.
142. G.S.Bethell, J.S.Ayers, M.T.W.Hearn, and W.S.Hancock, J.Chromatog., 1981, **219**, 353.
143. J.L.Groff, R.Cherniak, and R.G.Jones, Carbohydr. Res., 1982, **101**, 168.
144. M.Wilchek and T.Miron, Biochem.Intern., 1982, **4**, 629.

Institute of Biological and Medical Chemistry,
Academy of Medical Sciences of the USSR,
Moscow

Kemerovo State Medical Institute