Translated from *Uspekhi Khimii*, 49, 879-902 (1980) U.D.C. 543.544.8

Chemospecific (Covalent) Chromatography of Biopolymers

V.I.Lozinskii and S.V.Rogozhin

The literature on the applications of one of the modern methods for the isolation and thorough purification of biopolymers —chemospecific (covalent) chromatography based on the chemical interaction of substances in solution and the chromatographic carrier—is reviewed.
The bibliography includes 164 references.

CONTENTS

I. Introduction 460
II. General scheme for the process. Terminology 460
III. Chemical principles of the method 461
IV. Carriers for the chromatographic process 464
V. Applications of chemospecific chromatography 466
VI. Certain methodological problems of the chromatographic process 468

I. INTRODUCTION

Modern biochemical methods make it possible to isolate in a pure state from a wide variety of sources and to investigate the structure, properties, and the mechanism of action of virtually all classes of biopolymers present in the tissue and liquids of organisms even in extremely low amounts. However, the schemes developed for the isolation and purification of the required substance are sometimes too cumbersome and laborious. The success of new not unduly laborious but much more effective methods such as biospecific (affinity) chromatography, affinity electrophoresis, etc. is not therefore surprising. They have significantly simplified and reduced the cost (which is also important) of the processes involving the isolation and purification of peptides, proteins, enzymes, nucleic acids, etc.

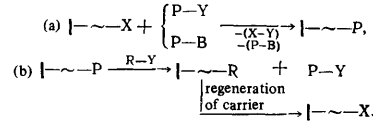
As will be shown below, there exist at the same time a number of problems which are difficult and sometimes simply impossible to solve on the basis of the physical properties of the substances alone or the principles of biological activity, since they require the "chemical involvement" of particular functional groups of the biomolecules. The processes in the isolation and purification of natural products, based on reversible chemical reactions between an insoluble carrier and the substances to be isolated, have come to be referred to as "covalent chromatography".

This review describes the main achievements of this method for the separation of substances and its applications and discusses some of its specific problems.

II. GENERAL SCHEME FOR THE PROCESS. TERMINOLOGY

"Covalent chromatography" is a comparatively new method for the isolation and purification of biopolymers. The first publication in this field refers to 1963 [1] and in the subsequent ten years only a few studies appeared[2-8]; subsequently, starting from 1973, interest in this method increased appreciably and the main bulk of the publications took place in the last five years. It is noteworthy that there are virtually no reviews of the method in the literature.

The general scheme for covalent chromatography includes the following sequence of operations†:



A mixture of biopolymers, one or several components of which are the required products of the separation (indicated by P—Y in the scheme above), is brought into contact with an insoluble carrier having a chemically active "anchor" group X capable of reacting selectively solely with group Y; this results in the immobilisaton of material carrying accessible Y residues and the products which have not been bound covalently (P—B) are washed off. The carrier–biomolecule covalent bond (hence the name of the method—covalent chromatography) is stable in the absence of a specific reagent (R—Y), but readily decomposes when such a reagent is introduced into the system in the next, deimmobilisation stage (b), as a result of which the required product of the separation passes to the solution. The carrier is regenerated as far as possible and is used again.

Several names have been proposed for the scheme given above: "covalent affinity chromatography"[7,8], "covalent chromatography"[9], and "reversible immobilisation"[10]. A more exact name for the method would probably be "chemospecific chromatography", which reflects the main ideas of the process—specific reversible chemical interaction of substances with an insoluble carrier. In the description below we shall therefore adhere to the terminology proposed here.

† Here and henceforth the symbol | denotes the matrix of the chemospecific carrier and the symbol ~ denotes an intermediate group ("spacer").

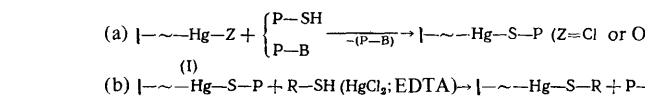
III. CHEMICAL PRINCIPLES OF THE METHOD

Since the main bulk of the publications in this field refer to studies on proteins and peptides, it is necessary to examine at the beginning the problem of which functional groups of protein molecules and which reversible chemical reactions involving them are used in the chemospecific chromatography (CSC) of proteins.

In the first place such groups must include the sulphydryl groups of the cysteine residues which form part of the composition of particular proteins. Whereas other groups (amino-, hydroxy-, phenoxy-, carboxy-, etc.) are present in other biopolymers (nucleic acids, polysaccharides) as well as proteins, the mercapto- and disulphide groups are almost exclusively a specific chemical feature of proteins as a class of biological macromolecules[11]. Numerous proteins with accessible SH groups are now known and the involvement of cysteine residues in enzyme catalysis by many enzymes (for example thiol proteases, creatine kinases, 3-phosphoglyceraldehyde dehydrogenases, etc.[12]) has been demonstrated. Reversible modifying agents for the mercapto-groups of proteins have also been thoroughly investigated[13]. It is not therefore surprising that reversible immobilisation via the SH groups of proteins was first used[1] for CSC and the majority of the publications on this method are in fact devoted to the isolation and purification of thiol-containing proteins and peptides on various carriers.

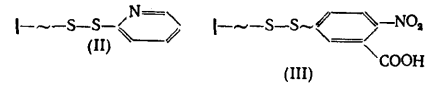
However, reactions other than those with SH groups can be used in the CSC of biopolymers. This has been demonstrated in a number of instances which will be discussed in detail below. Here one must emphasise that, in principle, it is possible to use the reversible reactions of any accessible groups in biomolecules subject to the only stipulation that the reactions must be highly specific and must occur under fairly mild conditions which do not decompose the biomolecules themselves or the carriers. Preliminary information concerning the effect of the low-molecular-weight reagent, whose insoluble analogue is to be employed, is therefore always necessary, i.e. CSC is based on data obtained in studies on the chemical modification of biopolymers. Thus this type of chromatography can also be regarded as the reversible modification of the functional groups of biological macromolecules by insoluble reagents.

As regards chemical processes involving SH groups, the formation reactions of either mercaptide (Hg—S), or disulphide (S—S), or thioester (CO—S) bonds have been used for CSC. The covalent bond between the mercury atom of the insoluble carrier and the mercapto-group of the protein or the peptide can be ruptured under mild conditions by low-molecular-weight thiols, soluble mercury salts, or complexones such as ethylenediaminetetra-acetic acid (EDTA):[1]

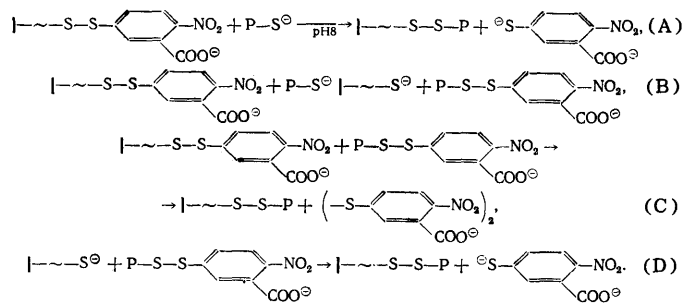


However, one should bear in mind that mercury-containing carriers must be used with a fair amount of caution, since the slow elution of the metal (partial decomposition of the carrier during manipulations and gradual hydrolysis of the carrier–ligand bonds), which always occurs, contaminates the protein, which is particularly undesirable if the biopolymer being isolated is to be introduced into some kind of organism. Furthermore, mercury carriers can retain also non-thiol proteins if the latter have intensely complex-forming groups capable of being incorporated in the coordination sphere around the mercury atom, which interferes with the isolation of the SH-products.

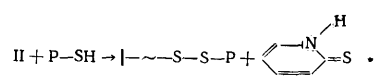
For CSC based on thiol–disulphide exchange reactions, insoluble analogues of reagents for SH groups have been used as the anchor groups of the carriers: di-(2-pyridyl)-disulphide (II) [14] and 5,5′-dithiobis-(2-nitrobenzoic acid) (III):[15]



The disulphide linkages of such carriers are activated by the electron-accepting effect of the aromatic system and readily enter into thiol–disulphide exchange reactions with the sulphydryl groups of the proteins. It has been noted[16] that, for carriers of type (III), the main reaction (A) via the CSC mechanism may be accompanied by other processes—(B), (C), and (D):



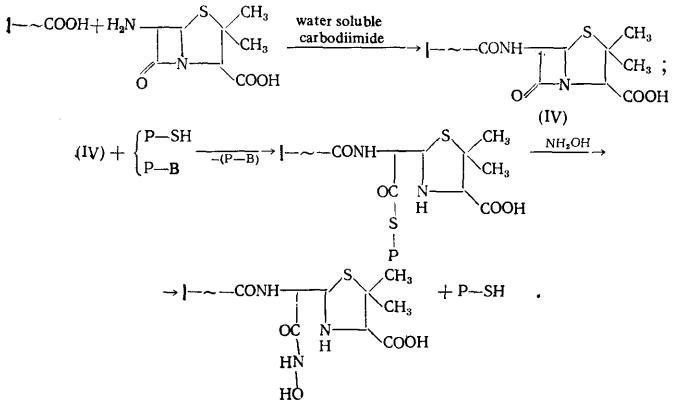
Such multiplicity of thiol–disulphide exchange reactions is missing in studies with carriers of type (II) containing dipyridyl disulphide active groups, since the 2-mercaptopyridine isolated immediately isomerises to 2-thiopyridone, which no longer takes part in the exchange processes and the reaction equilibrium is almost wholly displaced to the right[9,17,18]:



The deimmobilisation of the SH-protein occurs in the presence of an excess of a low-molecular-weight thiol (cysteine, 2-mercaptoethanol, and dithiothreitol).

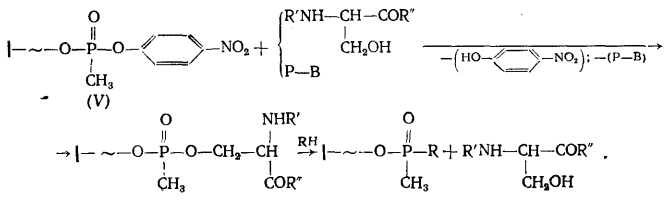
An interesting version of CSC has been achieved in the study of the penicillin-binding components of the membranes of *B. subtilis*[8,19,20]. As shown previously[21], these protein components are acylated by penicillin via the SH groups of the cysteine residues and the resulting thioester linkages can be ruptured either by hydroxylamine, or thiols, or hydrogen peroxide. A chromatographic process, carried out on carrier (IV) with immobilised 6-aminopennicillanic acid

(6-APA), has been developed on the basis of these data:



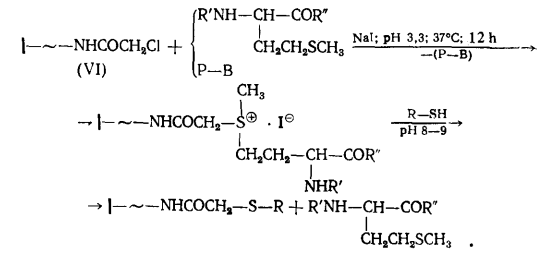
The above scheme is to some extent tentative, since the conditions governing the condensation of 6-APA with the COOH-carrier, quoted by the authors[8], do not exclude the formation in succession of several 6-APA molecules, linked by amide bonds, on the insoluble phase. It is therefore difficult to say which particular type of carrier is truly involved in the process—with monomeric or oligomeric penicillin. Another factor is important—this type of chemochromatographic approach can apparently prove useful in the study of the mechanism of the interaction of various medicinal drugs with cell components, since the formation of stable covalent ligand–protein bonds makes it possible to wash off thoroughly all the remaining substances and then to isolate the ligand receptors alone and to investigate them separately.

As already mentioned above, the CSC scheme need not be applied solely to SH-proteins. The reversible phosphorylation of the OH group of the serine residues has been used[7,22] in the isolation of enzymes containing this aminoacid at the active centre (acetylcholinesterase, α-chymotrypsin):



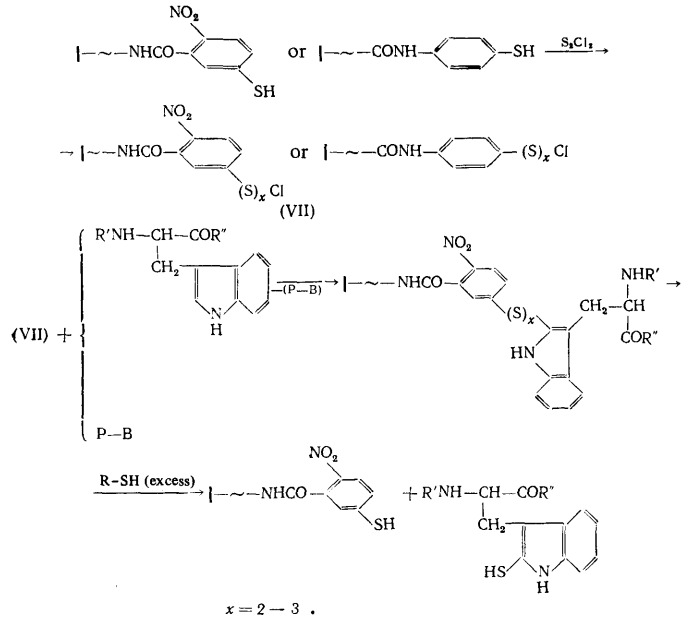
Here RH = 2-(hydroxyiminomethyl)-1-methylpyridinium iodide, 1,1′-trimethylenebis-(4-hydroxyiminomethyl)pyridinium dibromide, or hydroxylamine and R′ and R″ are polypeptide chains. The presence of serine in an activated form is apparently an essential condition for reaction with a carrier of type (V), since a protein not belonging to the class of serine enzymes but containing serine in its composition (serum albumin) is not retained by the carrier.

The formation of sulphonium derivatives on interaction of the thioester group of the methionine residue with α-halogenoacids in an acid medium and their subsequent decomposition with low-molecular-weight thiols has been used recently[23] in CSC involving methionine residues:



It is necessary that the methionine residue is not a C-terminal aminoacid, since otherwise it is converted into a homoserine residue and the protein or peptide is not fixed on the carrier. One should note that the fairly severe conditions required for the immobilisation of methionine-containing substances probably limit the applicability of this scheme to relatively stable proteins. For peptides, it is apparently not so significant if they do not contain labile linkages of the type Asp–pro.[24] There are also some doubts about the stability of the material of the carrier under the fixation conditions. Furthermore, the above scheme does not provide for the regeneration of the carrier.

The ability of sulphenyl chloride[25] and sulphur monochloride[26] to modify the tryptophan residues at the 2-position of the indole ring has been used[27] for the chemospecific isolation of tryptophan-containing compounds:



Ultimately the tryptophan residue is modified—an SH group appears in the indole ring. The introduction of this group, which is convenient for further modification, will apparently prove useful also in the study of the role of Trp residues in enzyme molecules. Since carriers of type (VII) bind covalently also mercapto-groups, it follows that, when it is necessary to carry out the process via the tryptophan residues alone, one must block appropriately the SH groups of the cysteine.

Table 1. Carriers for the chemospecific chromatography of biopolymers.

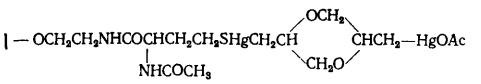
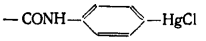
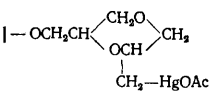
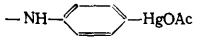
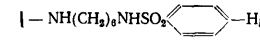
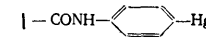
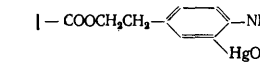
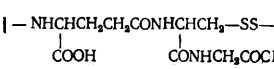
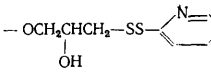
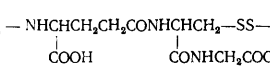
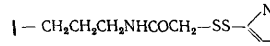
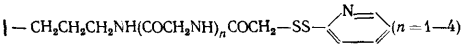
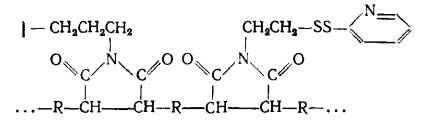
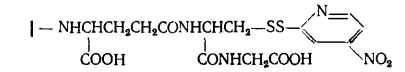
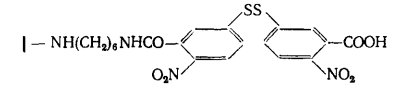
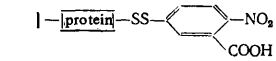
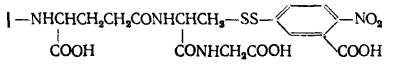
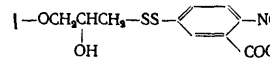
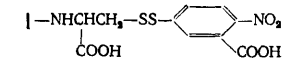
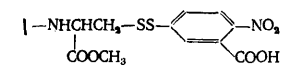
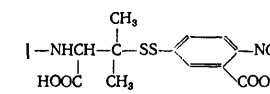
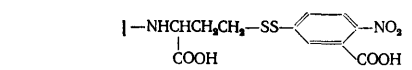
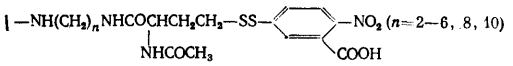
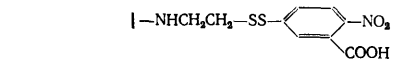
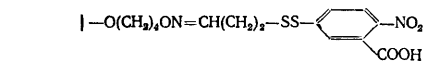
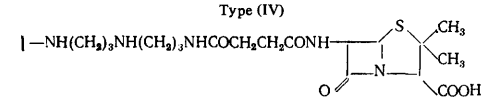
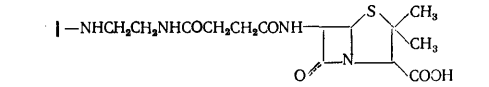
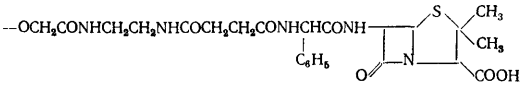
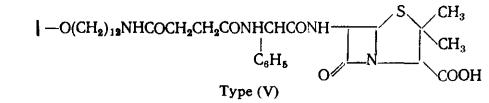
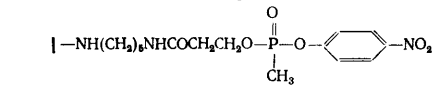
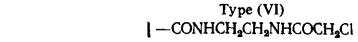
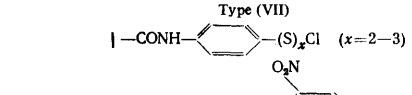
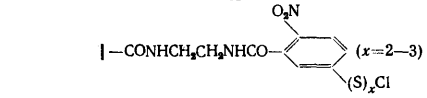
No.	Matrix	Chemospecific group with spacer	Capacity with respect to active groups	Refs.
		Type (I)		
1	Sephadex G-25		0.15 mmole g^{-1}	1
2*	Maleic anhydride–ethylene copolymer cross-linked by hexamethylenediamine		1.62 μmole g^{-1}	3
3	Cellulose		0.2—0.7 mmole g^{-1}	5
4	Sepharose 4B		0.2—0.5 mmole ml^{-1}	6
5	Ditto		0.2—1.2 μmole ml^{-1}	36, 82
6	»		1.2 μmole ml^{-1}	110
7a	Hydroxyalkyl methacrylate gel (Spheron)		0.03—0,10 mmole ml^{-1}	72
7b	Ditto		0,02—0,20 mmole ml^{-1}	72
		Type (II)		
8	Sepharose 4B		0.6—1.0 μmole ml^{-1}	9
9	Ditto		20 μmole ml^{-1}	42
10	Sepharose CL 4B		3.9 μmole ml^{-1}	41
11	Macroporous silica (Silochrome)		40 μmole g^{-1}	48—51
12	Ditto		10—20 μmole g^{-1}	51
13**	»		up to 160 μmole ml^{-1}	50,145
14	Sepharose 4B		3—10 μmole ml^{-1}	111
		Type (III)		
15	Sepharose 4B		0.14 mmole g^{-1}***	16
16****	Ditto		0.2—0.3 μmole ml^{-1}	83
17	»		0.95 μmole ml^{-1}	84
18	»		5.1 μmole ml^{-1}	33
19	»		0.048 μmole ml^{-1}	33
20	»		0.063 μmole ml^{-1}	33
21	»		0.033 μmole ml^{-1}	33

Table 1 (con'd).

No.	Matrix	Chemospecific group with spacer	Capacity with respect to active groups	Refs.
22	Sepharose 4B		0.19 μmole ml^{-1}	33
23	Ditto		—	59
24	»		0.045 μmole ml^{-1}	85
25	Cellulose		—	112
		Type (IV)		
26	Sepharose 4B		—	8
27	Ditto		—	8
28	»		—	113
29	Cellulose		—	114
		Type (V)		
30	Sepharose 4B		0.2-1.0 μmole ml^{-1}	7
		Type (VI)		
31	Cross-linked polyacrylamide		0.77 mmole g^{-1}***	22
		Type (VII)		
32	Cross-linked polyacrylamide		0.8 mmole g^{-1}***	27
33	Ditto		—	27

*Carrier exhibits an appreciable non-specific sorption with respect to the main proteins.
**The substituent R = *N*-vinylpyrrolidinone, ethyl vinyl ether, styrene, or ethylene.
***The capacity is quoted with respect to the dry weight of the carrier.
****The protein consists of the light κ-type immunoglobulin chain.

There have also been studies where a modified version of CSC, in which the anchor groups X were introduced into the biopolymer while the Y groups were contained in the carrier, has been used. For example, an SH-protein was treated with dipyridyl disulphide, the excess reagent was removed by gel filtration, the protein was hydrolysed by a proteolytic enzyme, and the solution of the resulting peptides was passed through a column with a carrier containing mercapto-groups[28]. In studies with polynucleotides both isolation of the mercapto-derivatives of nucleic acids on an Hg-carrier[29] and, conversely, the isolation of mercurated RNA and DNA on an SH-carrier have been used[30-32].

The choice of a chemical pathway for a chromatographic process is largely dictated by the specific conditions of each concrete problem. It is of course impossible to reach an unambiguous conclusion as to which version is best (with the anchor group on the carrier or on the biopolymer). One can only note that, from the standpoint of the stability of the carrier (in the case of insoluble thiol phases), preference should be given to chemosorbents with a protected SH group—in this form it is subject to much less risk of oxidation. The lower effectiveness of the fixation of the dithio-2-nitrobenzoate derivative of the protein on an SH-carrier compared with the reaction of an SH-protein and a type (III) carrier has also been demonstrated[33].

IV. CARRIERS FOR THE CHROMATOGRAPHIC PROCESS

Like any chromatographic process, chemospecific chromatography imposes requirements on the carrier: (1) chemical, physical, mechanical, and biological stabilities; (2) sufficient permeability to macromolecules ensuring their free approach to the active groups of the carrier; (3) hydrophilic properties of the carrier, permitting experiments in an aqueous medium; (4) homogeneity of shape—preferred use of

carriers in the form of spherical beads of identical size; (5) commercial availability of the material of the carrier matrix and as far as possible its low cost.

Apart from these properties of the matrices, which are generally speaking the same for all chromatographic processes involving biopolymers and which have been discussed in fair detail in specialist literature (see, for example, Klyashchitskii and Shapot[34] and Berezin et al.[35]), carriers with functional groups capable of undergoing readily reactions for the addition of anchor groups are necessary in CSC.

Table 1, which summarises the literature data for the carriers used in CSC, shows that chemosorbents based on agarose gels, which have proved useful for affinity chromatography and the immobilisation of biopolymers, have been used in most cases. Agarose matrices are available commercially, exhibit a non-specific sorption, and methods for the introduction of various groups into such carriers have been thoroughly developed[36–38]. However, such carriers are not free from a number of disadvantages either: namely their mechanical strength is low, the structure of the polysaccharide matrix breaks down under the influence of organic solvents and temperatures above 50–60 °C, and the rate of flow of the liquids through the column containing the gel is rather low even when a small excess pressure is applied, the carrier is subject to microbial infection, and, finally, it is fairly expensive. Cross-linked agarose gels of the type of Sepharose-CL,[39,40] which have improved physicomechanical properties, will probably prove very useful in this process, as shown[41] by the application of a chemosorbent based on this Sepharose derivative (No.10, Table 1). A similar cross-linked agarose carrier (Nos.9 and 18, Table 1) has been obtained[42] by treating a polysaccharide matrix with epichlorohydrin (for the introduction of epoxy-groups). However, since the degree of cross-linking was not controlled and the formation of cross-links in the gel was a concomitant process in the formation of the active groups on the carrier, it is not clear to what extent such cross linking took place.

Carriers based on cross-linked polyacrylamide gels have also been used in CSC (Nos.31–33, Table 1). They have been employed in studies with proteins of low molecular weight and peptides. Here the very matrix imposes limitations—biopolymers with high molecular weights (in excess of 100 000 for Bio-Gel P-100 and above 300 000 for Bio-Gel P-200, on the basis of which chemosorbents have been prepared[23,27]) cannot penetrate[43] into the particles of the swollen gel. Furthermore, the mechanical strength of weakly cross-linked polyacrylamide matrices is likewise low.

In recent years carriers on an inorganic base have been used increasingly in the immobilisation of various biologically active compounds[44,45] and also for the specific isolation of enzymes[46] and gene fragments[47] with the aid of affinity chromatography. Materials such as porous glass, macroporous silicas, metals, metal oxides, ceramics, etc. have a high mechanical strength and standardised characteristics, are resistant to microbial infection, and are readily available; any chaotropic agents, high salt concentrations, and organic solvents can be used in experiments with them.

We obtained[48–51] a number of carriers for the CSC of proteins and peptides (Nos.11–13, Table 1) on the basis of a macroporous silica—silochrome[52]. Carriers containing a polymer grafted to an inorganic surface (No.13, Table 1) (R = ethyl vinyl ether, *N*-vinylpyrrolidinone, etc.) are characterised by a non-specific sorption of proteins and an improved stability of the matrix in alkaline media, in addition to an ehanced capacity with respect to dipyridyl disulphide groups. We believe that inorganic carriers whose surface is coated by a hydrophilic polymer are promising for studies on biopolymeric phases, since they combine the useful physicomechanical properties of inorganic matrices with adsorption passivity (when a collect choice is made) of the polymer coating. The preparation and use of combined carriers of this type have been described in a number of publications on the immobilisation[53,54] and affinity chromatography[55–57] of enzymes.

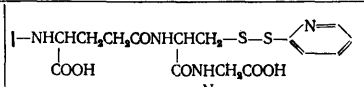
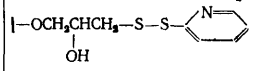
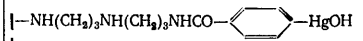
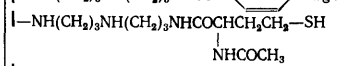
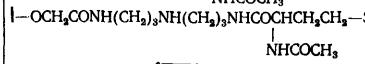
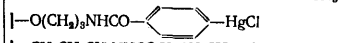
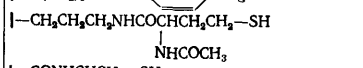
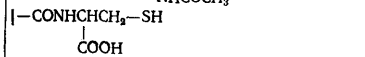
In the study of the properties of the carriers used, much attention has been devoted recently to the investigation of the influence of the distance of the immobilised affine ligand from the matrix on the efficiency of the chromatographic process and on the possibility of non-specific interactions. Such interactions (hydrophobic, electrostatic, sorption, etc.) usually interfere with the main procedure and its is therefore always desirable to eliminate them as far as possible. Summarising the results of these studies, one may conclude that the lengthening groups—the so called spacers—should undoubtedly by hydrophilic, simply because long polymethylene systems interact appreciably with the hydrophobic sections of protein molecules, as shown clearly, for example, by O'Carra et al.[58] Or, at least under the conditions employed (ionic strength of the solution, pH of the medium, and temperature), hydrophobic interactions should be minimal, which, however, must always be demonstrated in preliminary control experiments. It is also better if the spacer contains no ionogenic groups. Otherwise, there is, firstly, a superposition of ion exchange and, secondly, carriers of the gel type, containing charged groups, undergo an appreciable change in their volume and hence also there is a change in the size of the gel pores as the composition of the buffer is altered.

Although ionogenic groups do not in fact enter into the composition of the proposed spacer, the chosen chain of the chemical transformations for the immobilisation of the ligand frequently permits side reactions, which may "unexpectedly" give rise to an undesirable effect. For example, if the very popular method involving the attachment of amines to BrCN-activated agarose[37] is used, reactions via both NH_2 and SH groups can occur in the case of aminothiols[17]. This entails a decrease of the capacity of the carrier with respect to the mercapto-groups and introduces unnecessary ionogenic amino-groups. As regards the size of the spacer, the literature data are fairly contradictory. It is most likely that the optimum length is determined by the substance to be isolated and purified and by its structure and properties. It has been noted[34] that an unduly long spacer is relatively ineffective owing to its conformational bending, conversion into a helix, etc.

In CSC the influence of the length and structure of the spacer have so far been studied inadequately, although there are certain interesting data. Thus investigation of the kinetics of the deimmobilisation of lipoamide dehydrogenase with type (III) carriers (No.23, Table 1) established[59] that the enzyme dissolves under the influence of a reducing agent faster the longer the spacer, i.e. the approach of the cleaving agent to the covalent carrier–enzyme linkage is sterically facilitated. The chemical structure of the spacer near the active group is also important. It has been shown[33] that a carrier with the dithio-2-nitrobenzoate group at a quaternary carbon atom (No.21, Table 1) reacts to a very insignificant extent with SH-proteins—the shielding influence of the substituents has an effect, whereas branching at the next carbon atom of the main chain (Nos.17–20, Table 1) virtually no longer hinders the fixation of the protein. Thus, in the synthesis of active groups on carriers, it is necessary to take into account all the factors discussed above.

A number of carriers for CSC are now manufactured by certain companies. Table 2 presents the characteristics of

Table 2. Commercial carriers for chemospecific chromatography.

No.	Manufacturing company	Name of product	Matrix	Chemispecific group with spacer	Capacity with respect to active groups
1	Pharmacia Fine Chemicals (Sweden)	Activated Thiol-Sepharose	Sepharose 4B		1.0 μmole ml^{-1}
2	Ditto	Thiopropyl-Sepharose	Sepharose 6B		20 μmole ml^{-1}
3	Serva (Western Germany)	Servachrom-A-Hg	agarose		—
4	Ditto	Servachrom-A-SH	"		—
5	Bio-Rad Laboratories (USA)	Affi-Gel 401	"		6.0 μmole ml^{-1}
6	Ditto	Affi-Gel 501	"		3.0 μmole ml^{-1}
7	Pierce (USA)	Thiol/CPG	porous glass (d_{pore} = 550 A)		30 μmole g^{-1}
8	Koch-Light Ltd. (England)	Enzacryl-Polythiol	cross-linked polyacrylamide		—
9	Sigma (USA)	Cysteamine-agarose	agarose		0.1 μmole ml^{-1}
10	Ditto	*L*-Cysteine-agarose	"		2-10 μmole ml^{-1}

these chemosorbents based on the data supplied by the manufacturing companies (according to the 1977–1978 catalogues). Also included is information about certain commercially available SH-carriers, since they can be converted into derivatives with activated thiol functions by familiar reactions. However, one should note that all these carriers are fairly expensive, so that the development and practical employment of cheaper chemosorbents is an urgent problem whose solution will greatly extend the application of the CSC method not only in the laboratory but possibly also in biotechnology.

V. APPLICATIONS OF CHEMOSPECIFIC CHROMATOGRAPHY

The trends in studies on the CSC of biopolymers and the applications of this interesting highly selective method for the thorough purification of substances have now become clearly established. As already mentioned, most of the published data refer to studies with proteins or peptides. The use of CSC for the isolation of polynucleotides[30], gene fragments[29, 31,32], and low-molecular-weight cofactors[60] has been reported. Problems involving the isolation and purification of biopolymers, which have been solved with the aid of CSC, can be classified as follows.

1. Separation into fractions based on the chemical composition (for example, the separation of proteins with accessible SH groups from all the remaining mixture components).
2. Isolation of individual proteins and enzymes and isolation of isoenzymes.
3. Separation of chemically non-identical sub-units of oligomeric proteins.
4. Chemospecific isolation of peptides containing aminoacids with reactive side chains.
5. Purification of synthetic peptides.
6. Elimination of non-protein substances from proteins.
7. Isolation of modified nucleic acids and gene fragments.

Reversible immobilisation has also been used in the chemical modification of an enzyme temporarily fixed on an insoluble carrier followed by its dissolution[41,61]. This approach[41] makes it possible to avoid intermolecular cross-links if it is necessary to modify groups in the protein molecule in an isolated form by means of a bifunctional reagent.

Table 3 present a compilation of data on specific substances whose isolation or purification have been achieved with the aid of CSC. One should bear in mind that the chemospecific purification stage is hardly ever used in isolation. If there are simple methods for the preliminary fractionation and concentration of the desired product, then it is undoubtedly desirable to use them; this applies particularly to the removal of low-molecular-weight components, since they may contain substances which either interfere with the chemical fixation of the biopolymer on the carrier or are capable of cleaving the covalent carrier–biopolymer bond formed.

Table 3. Isolation and purification of biopolymers with the aid of CSC.

Substance isolated and purified	Source of biopolymer	Carrier		Refs.
		type	No. in Table 1	
1. Separation into fractions according to chemical composition				
SH-Protein fraction	rat liver homogenate	(I)	1	1
SH-Containing DNA nucleoproteins	rat liver cell nuclei	(I)	1	2
F3 histone fraction	calf thymus	(I)	5	82,86
Separation of SH-proteins from acid prolylcarboxypeptidase	ox adenohypophysis	(I)	6*	88
Band 3 polypeptide fraction	human erythrocyte membranes	(II)	1*	87
Penicillin-binding protein fraction	*B. subtilis* membranes	(IV)	26,27	8,19,20
Penicillin-binding proteins	*Salmonella typhimurium*	(IV)	28	113
2. Isolation of individual proteins and enzymes				
Haemoglobin albumin, human blood sera, glyceraldehyde-3-phosphate dehydrogenase, aldolase	separation of artificial mixture	(I)	1	1
Aldolase	rat muscle	(I)	1	1
Thiolated bovine γ-globulin	—	(I)	3	5
Mercaptopapain	preparation	(I)	4	6,89
"	"	(II)	8	9,17,18
"	"	(II)	9	115
"	dried papain latex	(II)	8	9,17,18
Mercaptoficin	*Ficus glabrata*	(I)	4	96
"	"	(II)	8	97
Cathepsin B_1	human liver	(I)	4	90
Ditto	ox spleen	(I)	4	91,92
Cathepsin B_2	ditto	(I)	4	92
Cathepsin H	human liver lysosomes	(I)	4	102
Cathepsin N	ox spleen	(I)	4	116
Streptococcal protease	—	(I)	4	93
Clostripain	*Clostridium*	(I)	4	103
SH-Protease	green bean plants	(I)	7	72
SH-Protease	*Phaseolus vulgaris*	(I)	7	104
SH-Protease of blood-sucking insects	*Phodnius prolixus*	(I)	4	117
Dipeptidylaminopeptidase I	ox spleen	(I)	4	118
L-pyrrolidonecarboxypeptidase	*B. amyloliquefaciens*	(I)	5	119
Thiolsubtilisin	—	(I)	4	68
Thioltrypsin	—	(I)	4	69
Oestradiol 17β-dehydrogenase	—	(I)	5	94
S-Adenosyl-L-methionine decarboxylase	rat liver	(I)	5	95
Ditto	sea urchin eggs	(I)	5	95
Creatine phosphokinase	chicken breast muscle	(I)	5	98
Ditto	human skeletal muscle	(I)	5	99
Cysteine-containing histone	calf thymus	(I)	5	100
ARE-histone	ditto	(I)	5	101
Factor XIII of blood clotting system	human blood plasma	(I)	6*	67
Ditto	human thrombocytes	(I)	6*	67
5-Methylthioadenosine nucleosidase	human placenta	(I)	6	120
Pyridoxamine(pyridoxin)-5-phosphate oxidase	baker's yeast	(I)	6*	121
Choline acetyltransferase	*Drosophila melanogaster*	(I)	5	122
"	mouse brain	(I)	5	123
Selenoprotein of the glycine reductase complex	*Clostridium sticklandii*	(I)	6*	124
Metallothionein	rat liver	SH–	–	125
Mercaptoalbumin	ox blood serum	(II)	8	105
"	ditto	(III)	15	16
Urease	*Canavalia ensiformis*	(II)	8	63,106
"	ditto	(II)	9	107
Collagen of type III	human and calf skin	(II)	1*	108
Phospholipase A_2	human blood plasma	(II)	1*	62
Ornithine decarboxylase	rat liver	(II)	1*	126
γ-Glutamylcyclotransferase	ditto	(II)	1*	127
Metallothionein	rat liver polysomes	(II)	1*	128
Copper-binding metallothionein	human liver	(II)	2*	129
Ferredoxin	*Lactuca sativa*	(II)	2*	130
α_1-Antitrypsin	human blood plasma	(III)	16	83,109
α_1-Antitrypsins of types M, S, and Z	ditto	(III)	16	131
Blood plasma SH-proteins (separated)	ditto	(III)	16–22	33
Human prealbumin	blood plasma	(III)	17	84
Ox prealbumin	ditto	(III)	17	132
F_1-ATPase	pig heart mitochondria	(III)	15	133
D-Alanine carboxypeptidase	*B. subtilis* membranes	(IV)	26,27	8,19,20
DD-Carboxypeptidase	*Proteus mirabilis* membranes	(IV)	29	114
Acetylcholinesterase	electric organ of the eel	(V)	30	7,22
α-Chymotrypsin	preparation	(V)	30	7,22

Table 3 (cont'd).

Substance isolated and purified	Carrier		Refs.
	type	No. in Table 1	
3. Separation of non-identical subunits of oligomeric proteins			
SH-subunit of lombricine kinase	(I)	4	64
SH-subunit of yeast phenylalanyl-tRNA synthetase	(I)	4	65
SH-subunit of the calf lens alpha crystallin	(I)	4	134
a-Subunit of factor XIII of the blood clotting system	(I)	6*	67
κ-Casein and αs-casein	(II)	1*	66
β-Chain of ox haemoglobin	(II)	11	49
4. Chemospecific isolation of peptides			
Cysteine-containing peptides of the pepsin insulin hydrolysate	(I)	2	4
Cysteine-containing peptide of the trypsin hydrolysate of the SH-subunit of lombricine kinase	(I)	4	64
Cysteine-containing peptides of the trypsin hydrolysate of "Tu" factor for the elongation of the polypeptide chain	(I)	5	71
Cysteine-containing peptide of the chymotrypsin hydrolysate of human blood serum albumin	(I)	7	72
Cysteine-containing peptide of the BrCN-hydrolysate of yeast 3-phosphoglycerate kinase	(I)	4	135
Cysteine-containing peptides of pepsin hydrolysates: of hake muscle parvalbumin, ox serum albumin, and human ceruloplasmin	(II)	1*	74
Cysteine-containing peptides obtained by different types of hydrolysis of the β-chain of ox haemoglobin	(II)	11	49
Cysteine-containing peptides of chicken heart cytoplasmic aspartate aminotransferase	(II)	1*	76–78
Cysteine-containing peptides of di-2-pyridyl disulphide derivatives of albumin and ribonuclease	SH–	–	28
Cysteine-containing peptide of the BrCN-hydrolysate of ox serum albumin	(III)	15	16
Methione-containing peptides of the trypsin hydrolysate of lysozyme	(VI)	31	23
Tryptophan-containing peptides of the trypsin hydrolysates of glucagon and human blood serum albumin	(VII)	33	27
5. Purification of synthetic peptides			
Synthetic peptides of the 111–124 sequence of ribonuclease and the 1–37 sequence of histone H4	(I)	5	79
Synthetic peptide of the 33–48 sequence of allergen M	(II)	2*	73,136
Cbz-Trp-Ala	(VII)	33	27
6. Elimination of non-protein substances from proteins			
Elimination of residual cell components from the overall protein of baker's yeast	(II)	11	80
Elimination of non-protein impurities from reduced lysozyne	(II)	11	48
Elimination of surfactants and lipid-like compounds from proteins	(II)	11	81
7. Isolation of modified nucleic acids and gene fragments			
2-Thiouracil-containing RNA	(I)	6*	29
6-Thioguanosine-containing RNA	(I)	3	137
Mercurated RNA from different sources (chemical mercuration)	SH–	–	30–32, 138–141
Mercuriuridine-containing RNA	SH–	–	142
Mercurated RNA (chemical mercuration)	SH–	–	30,138,143
8. Isolation of cofactors			
Flavin-adenine dinucleotide	(I)	4	60
Coenzyme A from *Sarcina lutea*	(I)	4	60

*The number refers to Table 2.

The combination of CSC with other selective methods yields satisfactory results when it is necessary to obtain, for example, a particularly pure and active enzyme preparation. Thus, in the isolation of phospholipase A_2 from human blood serum[62], several preliminary purification stages were followed by the consecutive application of three affinity columns: chromatography on concanavaline A-Sepharose—for the isolation of glycoproteins, which included also the required phospholipase; chromatography on a column with immobilised antiserum and γ-immunoglobulin (in the fraction of which activity was detected) for the separation of immunoglobulin; and finally CSC on an activated thiol-Sepharose (No.1, Table 2). This yielded an enzyme with more than a 10 000-fold

degree of purification. The above example refers to the isolation of a protein present in the initial material in extremely low amounts. On the other hand, when the required product is one of the main components (for example, papain in dried papain latex[9]), it can be isolated with the aid of CSC fairly simply and rapidly using a minimum number of stages. This is particularly convenient for the thorough purification of commercial protein preparations. For example, the isolation of mercaptopapain from a commercial papain[9,17] or the isolation of highly active urease from a commercial preparation of this enzyme[63] have been achieved rapidly and effectively on chemospecific carriers, while the isolation of the SH-components alone of the above proteins by the usual methods is laborious and leads to considerable losses of valuable enzyme material.

It has been shown that it is possible to separate rapidly non-identical subunits of oligomeric proteins with the aid of chemospecific sorbents, when some subunits contain and others do not contain reactive aminoacid residues. The SH-subunit of lombricine kinase[64], the α-subunit of yeast phenylalanyl-tRNA synthetase[65], κ-casein and αS-casein from whole casein[66], the β-chain of bovine haemoglobin[49], and the *a*-subunit of factor XIII of the blood clotting system[67] have been isolated in this way. In the last case the SH-*a*-subunit, immobilised reversibly on an Hg-carrier (No.6, Table 2), was used also to investigate its non-covalent interactions with the *b*-subunit.

In the study of the properties of "unusual" proteolytic enzymes—thiolsubtilisin[68] and thioltrypsin[69], obtained by the chemical transformation of the serine residue of the active centre into a cysteine residue by the method of Photaki and Bardakos[70]—CSC made it possible to isolate in a single stage the active modified protein.

The chemospecific isolation of substances in the determination of the primary sequence in proteins and particularly high-molecular-weight proteins, where the separation of complex peptide mixtures based on the differences between the charges, molecular sizes, hydrophobic properties, etc. frequently becomes relatively ineffective, gives rise to interesting prospects. There exist two versions of the chemospecific extraction of peptides for further structural studies. The first involves the interaction of the active carrier with the mixture of peptides obtained enzymatically or by chemical hydrolysis. Depending on the type of carrier and the amino-acid composition of the protein fragments, the reaction with the corresponding side groups of the peptides can proceed: the mercapto-group of cysteine[4,16,64,71-73], the thioester group of methionine[23], and the indole ring system of tryptophan[27]. After washing away the unreacted material, the peptides fixed covalently on the chemosorbent are dissolved by treatment with an appropriate cleaving agent.

The second method involves the fragmentation (enzymatic or chemical) of the reversibly immobilised protein directly on the carrier[49,74-78]. The carriers on an inorganic base (No.11, Table 1), which we proposed[48], proved useful in this instance[49], because the matrix consisting of macroporous silicon dioxide (silochrome) is stable under the conditions of different types of treatment of immobilised polypeptide chains such as pepsinolysis, trypsinolysis, cleavage with bromocyanogen, rupture of aspartyl–proline bonds (pH 2.2, 37 °C, 36 h), as well as the solid-state version of the determination of the peptide sequence by the Edman method. The fragmentation of the reversibly immobilised enzyme simplifies the isolation and identification of the peptides containing functionally important (essential) chemical groups in the active centres, provided that it is possible to select conditions ensuring the covalent fixation of only such groups of the active centre on the chemosorbent.

Generally speaking, it is not difficult to envisage the chemical separation of peptides on a series of carriers which selectively extract from the mixture the peptide (or peptides) containing a specific trifunctional aminoacid.

One of the problems in the solid-phase peptide synthesis is the elimination from a peptide with a specified sequence of the so-called "false" peptides, which are formed in the course of the synthesis mainly in consequence of the failure of the reactions to reach completion at various stages of the multistage process. A procedure has been proposed[79] for the isolation of the desired peptide by CSC. The essential feature of the method is that, after the completion of the synthesis of the required sequence, the peptide is augmented by the Cys–Met fragment (either consecutively, one residue at a time, or by adding the dipeptide at once) and then all the peptides obtained are separated from the insoluble support with simultaneous removal of the protective groups (for example, by treatment with HF). The treatment of this sum total of peptides by a sorbent chemospecific in relation to the SH groups removes the Cys–Met peptides from solution. All the remaining products are washed off. Peptides are removed from the chemosorbent by a low-molecular-weight thiol, the excess reductant being eliminated by gel filtration. Next follows the cleavage of the N-terminal dipeptide with bromocyanogen and repeated gel filtration. As a result, it is possible to separate many "false" peptides, particularly those whose chain growth was arrested during the synthesis for various reasons (*N*-acylation, the formation of a pyroglutamic derivative, the inaccessibility of the growing chain to the reactants in consequence of steric hindrance, etc.). Synthetic peptides on an Hg-containing carrier[79] and on a dipyridyl disulphide carrier[73] have been purified in this way. Clearly peptides containing methionine residues in the main sequence cannot be isolated in this way; in this case it is necessary to employ another selectively cleaved terminal fragment.

It has been shown recently[80,81] that CSC can be used for the elimination of non-protein substances from proteins and peptides. A carrier on an inorganic base has been used, which made it possible to use solvents and concentrated urea solutions to wash surfactants and lipid-like compounds out of the temporarily immobilised protein[81].

VI. CERTAIN METHODOLOGICAL PROBLEMS OF THE CHROMATOGRAPHIC PROCESS

When the two main stages of CSC, namely (a) covalent fixation of substances from solution on the carrier followed by (b) their deimmobilisation are carried out, it is necessary to remember that chemical reactions, at least those on the carriers known hitherto, do not take place instantaneously—a certain amount of time is required to complete the reactions. The stream of liquid through the material of the packing in the column must not therefore be very rapid, particularly in the column version of the process; the use of recirculation gives rise to satisfactory results in stage (a). There are few literature data for the gradient supply of the cleaving agent for the removal of the immobilised biomolecules from carriers. Nevertheless this deimmobilisation procedure apparently greatly improves the resolving capacity of the method as a whole.

Elution with stepwise changes in the strength of the cleaving agent has been used already in the first studies on CSC.[1,2] A model mixture of SH-proteins (mercaptoalbumin, glyceraldehyde-3-phosphate dehydrogenase, haemoglobin, and aldolase) have been separated by this method on a mercury-containing carrier (No.1, Table 1).[1] In order to rupture the mercaptide carrier–protein bonds, a reducing

agent (mercaptoethanol or dithiothreitol) was applied to the column, ensuring a linear concentration gradient, which made it possible to separate the required SH compound (factor XIII in the blood clotting system) from other thiol proteins linked to the chemosorbent, "by virtue of their different reactivities with respect to the cleaving agent"[67]. Strictly speaking, this version of the process actually constitutes a chromatographic separation of the substances and not simply a sequence of two operations: chemospecific sorption and desorption. It may be hoped that the separation of biopolymers by CSC using a cleaving agent gradient will be employed on an increasing scale.

Our data[80] show that the presence of a reductant concentration gradient in experiments with carriers of type (II) makes it possible to wash the protein off the insoluble phase with the minimum amount of the low-molecular-weight thiol required for this purpose, which greatly diminishes the risk of the intramolecular disulphide bonds of the protein itself being affected.

One should note a disadvantage which is encountered when carriers of types (II) and (II) are used. When the SH-containing product, initially fixed on the insoluble phase, is removed with a thiol, it is difficult to detect the biopolymer at the outlet from the column, since the residual "unused" active groups of the carrier are also cleaved by the reductant and are washed off together with the required products, which makes the ultraviolet detection of the eluted protein or peptide with the aid of the usual flow-type photometers virtually impossible owing to the intense absorption by 2-thiopyridone or 2-nitro-5-thiobenzoate. It is therefore necessary to analyse the material by some other method (for example by the gel electrophoresis of the fractions, by measuring, whenever possible, the enzyme activity, by chromatography—for peptides, etc.). It is thought that, at least for proteins and polypeptides, this disadvantage can be eliminated by employing a membrane eluate concentrator (model CEC1, Amicon, Holland) beyond the column, which cuts off the low-molecular-weight substances in the stream. The only important factor is that the change in the salt composition of the buffer occurring under these conditions does not cause a sharp impairment of the solubility of the biopolymers.

Finally we come to the last remark concerning experiments with SH-biopolymers or sulphur-containing carriers; it is always necessary to use deaerated buffer solutions; likewise it is extremely undesirable that even traces of heavy metal ions should be present. In order to bind such ions, EDTA is therefore usually introduced into the solution. These precautions guarantee the stability of the mercapto- and disulphide groups in the test compounds.

---o0o---

In conclusion one should note that chemospecific chromatography has now found an honourable place among other highly selective methods for the isolation and purification of biologically active compounds; it is developing vigorously, is being improved, and is being applied on an increasing scale to a wide variety of test objects.

During the preparation of the review for publication, reports have been published, describing the syntheses of certain chemosorbents[144-148] belonging to types (I)–(IV) described above and the isolation by the CSC method of peptides[149], proteins[144,145,148-161], nucleic acids[147,162], and fragments of membranes[163], as well as studies of certain methodological problems of the process[157,164].

REFERENCES

1. L.Eldjarn and E.Jellum, Acta Chem.Scand., **17**, 2610 (1963).
2. E.Jellum and L.Eldjarn, Biochim.Biophys.Acta, **100**, 144 (1965).
3. I.E.Liener, Arch.Biochem.Biophys., **121**, 67 (1967).
4. I.E.Liener and L.-P.Chao, Analyt.Biochem., **25**, 317 (1968).
5. J.R.Shainoff, J.Immunol., **100**, 187 (1968).
6. L.A.A.Sluyterman and J.Wijdenes, Biochim.Biophys. Acta, **200**, 593 (1970).
7. Y.Ashani and I.B.Wilson, Biochim.Biophys.Acta, **276**, 317 (1972).
8. P.M.Blumberg and J.L.Strominger, Proc.Nat.Acad.Sci. U.S.A., **69**, 3751 (1972).
9. K.Brocklehurst, J.Carlsson, M.P.J.Kierstan, and E.M.Crook, Biochem.J., **133**, 573 (1973).
10. J.Carlsson, R.Axén, and T.Unge, Europ.J.Biochem., **59**, 567 (1975).
11. Yu.M.Torchinskii, "Sera v Belkakh" (Sulphur in Proteins), Izd.Nauka, Moscow, 1977.
12. Yu.M.Torchinskii, "Sera v Belkakh" (Sulphur in Proteins), Izd.Nauka, Moscow, 1977, p.157.
13. Yu.M.Torchinskii, "Sera v Belkakh" (Sulphur in Proteins), Izd.Nauka, Moscow, 1977, p.118.
14. D.R.Grassetti and J.F.Murray, Arch.Biochem.Biophys., **119**, 41 (1967).
15. G.L.Ellman, Biochem.Biophys., **82**, 70 (1959).
16. L.J.Lin and J.F.Foster, Analyt.Biochem., **63**, 485 (1975).
17. K.Brocklehurst, J.Carlsson, M.P.J.Kierstan, and E.M.Crook, Meth.Enzymol., **34B**, 531 (1974).
18. J.Carlsson, Protides Biol.Fluides, **23**, 537 (1976).
19. P.M.Blumberg, Fed.Proc., **32**, 482 (1973).
20. P.M.Blumberg and J.L.Strominger, Meth.Enzymol., **34B**, 401 (1974).
21. P.J.Lawrencse and J.L.Strominger, J.Biol.Chem., **245**, 3653 (1970).
22. H.F.Voss, Y.Ashani, and I.B.Wilson, Meth.Enzymol., **34B**, 581 (1974).
23. Y.Shechter, M.Rubinstein, and A.Patchornik, Biochemistry, **16**, 1424 (1977).
24. D.Piszkiewicz, M.London, and E.L.Smith, Biochem. Biophys.Res.Comm., **40**, 1173 (1970).
25. E.Scoffone, A.Fontana, and R.Rocchi, Biochemistry, **7**, 971 (1968).
26. T.Wieland, O.Weinberg, and W.Dilger, Annalen, **592**, 69 (1955).
27. M.Rubinstein, Y.Schechter, and A.Patchornik, Biochem.Biophys.Res.Comm., **70**, 1257 (1976).
28. A.Svenson, J.Carlsson, and D.Eaker, FEBS Letters, **73**, 171 (1977).
29. M.Ono and M.Kawakami, J.Biochem., **81**, 1247 (1977).
30. R.M.K.Dale and D.C.Ward, Biochemistry, **14**, 2458 (1974).
31. M.M.Smith and R.C.C.Huang, Proc.Nat.Acad.Sci. U.S.A., **73**, 775 (1976).
32. G.F.Crouse, E.J.B.Fodor, and P.Doty, Proc.Nat.Acad. Sci.U.S.A., **73**, 1564 (1976).
33. C.-B.Laurell, E.Thulin, and R.P.Bywater, Analyt. Biochem., **81**, 336 (1977).
34. B.A.Klyashchitskii and V.S.Shapot, Uspekhi Biol. Khim., **17**, 234 (1976).
35. I.V.Berezin, V.K.Antonov, and K.Martinek (Editors), "Immobilizovannye Fermenty" (Immobilised Enzymes), Izd.Moskov.Gos.Univ., Moscow, 1976, Vol.1, p.68.
36. R.Axén, J.Porath, and S.Ernbäck, Nature, **214**, 1302 (1967).

37. P.Cuatrecasas, J.Biol.Chem., **245**, 3059 (1970).
38. Affinity Chromatography. Principles and Methods, "Pharmacia Fine Chemicals", Uppsala, Sweden, 1977.
39. T.Låås, Protides Biol.Fluids, **23**, 495 (1976).
40. Sepharose-CL for gel filtration and affinity chromatography, "Pharmacia Fine Chemicals", Uppsala, Sweden, 1975.
41. G.P.Royer, S.i.Ikeda, and K.Aso, FEBS Letters, **80**, 89 (1977).
42. R.Axén, H.Drevin, and J.Carlsson, Acta Chem.Scand., **B29**, 471 (1975).
43. H.Determann, "Gel Chromatography" (Translated into Russian), Izd.Mir, Moscow, 1970, p.52.
44. H.H.Weetall, Analyt.Chem., **46**, 602A (1974).
45. G.Baum and M.Lynn, Process Biochem., **10**, 14 (1975).
46. H.H.Weetall and A.M.Filbert, Meth.Enzymol., **34B**, 59 (1974).
47. K.G.Skryabin, T.V.Venkstern, V.N.Zaharyev, V.P.Varlamov, S.V.Rogozhin, and A.A.Bayev, Z.physiol.Chem., **357**, 337 (1976).
48. V.I.Lozinskii, B.Yu.Zaslavskii, Yu.A.Davidovich, and S.V.Rogozhin, USSR P.540 870 (1975); Byul.Izobret., No.48, 72 (1976).
49. Ts.A.Egorov, M.I.Shakhparonov, Yu.A.Davidovich, V.I.Lozinskii, B.Yu.Zaslavskii, and S.V.Rogozhin, Bioorg.Khim., **3**, 1111 (1977).
50. V.I.Lozinskii, A.V.Il'ina, Yu.A.Davidovich, and S.V.Rogozhin, Symposium, "II Vsesoyuznaya Konferentsiya po Metodam Polucheniya i Analiza Biokhimicheskikh Preparatov, g.Olanie, 1977" (The IInd All-Union Conference on the Methods for Obtaining and Analysis of Biochemical Preparations, Olaine, 1977), p.105.
51. V.I.Lozinskii, I.G.Tsoi, Yu.A.Davidovich, and S.V.Rogozhin, Izv.Akad.Nauk SSSR, Ser.Khim., 1358 (1979).
52. N.K.Bebris, A.V.Kiselev, and Yu.S.Nikitin, Kolloid. Zhur., **29**, 326 (1967).
53. G.P.Royer, Chem.Technol., **4**, 694 (1974).
54. G.P.Royer, G.M.Green, and B.K.Sinha, J.Macromol. Chem., **A10**, 289 (1976).
55. V.P.Varlamov, G.E.Bannikova, A.V.Vlasov, B.E.Gadas, B.L.Tsetlin, and S.V.Rogozhin, Symposium, "II Vsesoyuznoe Simpozium po Polucheniyu i Primeneniyu Immobilizovannykh Fermentov, g.Abovyan, 1977" (The IInd All-Union Symposium on the Preparation and Applications of Immobilised Enzymes, Abovyan, 1977), p.1.
56. G.P.Royer, F.A.Liberatore, G.M.Green, W.E.Schwartz, and W.E.Meyers, Polymer Preprints, **16**, 76 (1975).
57. P.A.Anderson and L.Jervis, Biochem.Soc.Trans., **5**, 728 (1977).
58. P.O'Carra, S.Barry, and T.Griffin, FEBS Letters, **43**, 169 (1974).
59. C.R.Lowe, Europ.J.Biochem., **76**, 411 (1977).
60. Y.Matuo, R.Sano, T.Tosa, and I.Chibata, Analyt. Biochem., **68**, 349 (1975).
61. S.-i.Ikeda and S.Fukui, Biochem.Biophys.Res.Comm., **52**, 482 (1973).
62. R.Scherer, W.Huber-Friedberg, A.Salem, and G.Ruchenstroth-Bauer, Z.physiol.Chem., **357**, 897 (1976).
63. R.Norris and K.Brocklehurst, Biochem.J., **159**, 245 (1976).
64. E.Der-Terrossian, L.-A.Pradel, R.Kassab, and G.Desvages, Europ.J.Biochem., **45**, 243 (1974).
65. A.Murayama, J.P.Raffin, P.Remy, and J.P.Ebel, FEBS Letters, 53, 23 (1975).
66. H.Nijhuis and H.Klostermeyer, Milchwissenschaft, **30**, 531 (1975).
67. J.McDonagh, W.C.Waggoner, E.G.Hamilton, B.Hindenach, and R.P.McDonagh, Biochim.Biophys.Acta, **446**, 345 (1976).
68. L.Polgàr, Acta Biochim.et Biophys.Acad.Sci.Hung., **11**, 81 (1976).
69. H.Yokosawa, S.Ojima, and S.Ishii, J.Biochem., **82**, 869 (1977).
70. J.Photaki and V.Bardakos, J.Amer.Chem.Soc., **87**, 3489 (1965).
71. S.Nakamura, K.-i.Arai, K.Takahashi, and Y.Kaziro, Biochem.Biophys.Res.Comm., **66**, 1069 (1975).
72. J.Turkovà, S.Vavreinovà, M.Krivàkovà, and J.Čoupek, Biochim.Biophys.Acta, **386**, 503 (1975).
73. "Thiopropyl-Sepharose 6B", "Pharmacia Fine Chemicals", Uppsala, Sweden, 1977.
74. T.A.Egorov, A.Svenson, L.Rydén, and J.Carlsson, Proc.Nat.Acad.Sci.U.S.A., **72**, 3029 (1975).
75. Ts.A.Egorov, M.I.Shakhparonov, and S.V.Rogozhin, Symposium, "Sovetsko-Amerikanskii Simpozium po Khimii i Fizike Belka, Riga, 1976" (The Soviet-American Symposium on the Chemistry and Physics of Proteins, Riga, 1976), p.59.
76. T.V.Demidkina, M.I.Shakhparonov, Ts.A.Egorov, and Yu.M.Torchinskii, Symposium, "IV Vsesoyuznyi Simpozium po Khimii Belkov i Peptidov, Minsk, 1977" (The IVth All-Union Symposium on the Chemistry of Proteins and Peptides, Minsk, 1977), p.100.
77. Ts.A.Egorov, M.I.Shakhparonov, and I.V.Nazimov, Symposium, "IV Vsesoyuznyi Simpozium po Khimii Belkov i Peptidov, Minsk, 1977" (The IVth All-Union Symposium on the Chemistry of Proteins and Peptides, Minsk, 1977), p.34.
78. Ts.A.Egorov, M.I.Shakhparonov, T.V.Demidkina, and Yu.M.Torchinskii, Biokhimiya, **42**, 2253 (1977).
79. D.E.Krieger, B.W.Erickson, and R.B.Merrifield, Proc. Nat.Acad.Sci.U.S.A., **73**, 3160 (1976).
80. V.I.Lozinskii, B.Yu.Zaslavskii, Yu.A.Davidovich, and S.V.Rogozhin, USSR P.533 631 (1975); Byul.Isobret., No.40, 70 (1976).
81. V.I.Lozinskii, Yu.A.Davidovich, B.Yu.Zaslavskii, Ts.A.Egorov, and S.V.Rogozhin, Biokhimiya, **43**, 257 (1978).
82. A.Ruiz-Carrillo and V.G.Allfrey, Arch.Biochem.Biophys., **154**, 185 (1973).
83. C.-B.Laurell, J.Pierce, U.Persson, and E.Thulin, Europ.J.Biochem., **57**, 107 (1975).
84. G.Fex, C.-B.Laurell, and E.Thulin, Europ.J.Biochem., **75**, 181 (1977).
85. W.W.-C.Chan and K.Mosbach, Biochemistry, **15**, 4215 (1976).
86. A.Ruiz-Carrillo, Meth.Enzymol., **34B**, 547 (1974).
87. A.Kahlenberg and C.Walker, Analyt.Biochem., **74**, 337 (1976).
88. L.P.Alekseenko and V.N.Orekhovich, Bioorg.Khim., **3**, 1387 (1977).
89. L.A.A.Sluyterman and J.Wijdenes, Meth.Enzymol., **34B**, 544 (1974).
90. A.J.Barrett, Biochem.J., **131**, 809 (1973).
91. H.Keilovà and V.Tomàsek, FEBS Letters, **29**, 335 (1973).
92. K.Otto and H.Riesenkönig, Biochim.Biophys.Acta, **379**, 462 (1975).
93. A.A.Kortt and T.Yu.Liu, Biochemistry, **12**, 320 (1973).
94. J.C.Nicolas, Meth.Enzymol., **34B**, 552 (1974).
95. C.-A.Manen and D.H.Russell, Biochemistry, **13**, 4729 (1974).

96. C.D.Anderson and P.L.Hall, Analyt.Biochem., **60**, 417 (1974).
97. J.P.G.Malthouse and K.Brocklehurst, Biochem.J., **159**, 221 (1976).
98. R.J.Boegman, FEBS Letters, **53**, 99 (1975).
99. V.Madelian and W.A.Warren, Analyt.Biochem., **64**, 517 (1975).
100. K.Ishikawa and K.Iwai, J.Biochem., **77**, 391 (1975).
101. J.Bode and K.G.Wagner, Biochem.Biophys.Res. Comm., **62**, 868 (1975).
102. H.Kirschke, J.Langner, B.Wiederanders, S.Ansorge, P.Bohley, and H.Hanson, Acta Biol.Med.Germ., **36**, 185 (1977).
103. I.Ermöd and B.Keil, FEBS Letters, **77**, 51 (1977).
104. S.Vavreinovà and J.Turkovà, Biochim.Biophys.Acta, **403**, 506 (1975).
105. J.Carlsson and A.Svenson, FEBS Letters, **42**, 183 (1974).
106. J.Carlsson, R.Axén, K.Brocklehurst, and E.M.Crook, Europ.J.Biochem., **44**, 189 (1974).
107. J.Carlsson, I.Olsson, R.Axén, and H.Drevin, Acta Chem.Scand., **B30**, 180 (1976).
108. B.C.Sykes, FEBS Letters, **61**, 180 (1976).
109. C.-B.Laurell, Protides Biol.Fluids, **23**, 359 (1976).
110. R.Porta, C.Esposito, and G.Della Pietra, Int.J.Biochem., **8**, 347 (1977).
111. J.Grunwald and L.J.Berliner, Biochim.Biophys.Acta, **523**, 53 (1978).
112. A.A.Nedospasov and R.M.Khomutov, "Tezisy Vsesoyuznoi Konferentsii po Metodam Polucheniya Vysokoochishchennykh Fermentov, Vil'nyus, 1978" (Abstracts of the All-Union Conference on Methods for the Preparation of Highly Purified Enzymes, Vilna, 1978), p.89.
113. S.T.Shepherd, H.A.Chase, and P.E.Reynolds, Europ. J.Biochem., **78**, 521 (1977).
114. H.H.Martin, W.Schilf, and C.Maskos, Europ.J.Biochem., **76**, 585 (1976).
115. T.Stuchbury, M.Shipton, R.Norris, J.P.G.Malthouse, K.Brocklehurst, J.A.L.Herbert, and H.Suschitzky, Biochem.J., **151**, 417 (1975).
116. A.Ducastaing and D.J.Etherington, Biochem.Soc.Trans., **6**, 938 (1978).
117. E.S.Garcia, J.A.Guimarâes, and J.L.Prado, Arch. Biochem.Biophys., **188**, 315 (1978).
118. R.M.Metrione, Biochim.Biophys.Acta, **526**, 531 (1978).
119. D.Tsuru, K.Fujiwaka, and K.Kado, J.Biochem., **84**, 467 (1978).
120. G.Cacciapuoti, A.Oliva, and V.Zappia, Int.J.Biochem., **9**, 35 (1978).
121. T.Tsuge, K.Sen-Maru, and K.Ohashi, FEBS Letters, **93**, 331 (1978).
122. W.J.Driskell, B.H.Weber, and E.Roberts, J.Neurochem., **30**, 1135 (1978).
123. L.-P.Chao, Neurochem.Res., **3**, 549 (1978).
124. J.E.Cone, R.M.Del Rio, and T.C.Stadtman, J.Biol. Chem., **252**, 5337 (1977).
125. S.G.Shapiro, K.S.Squibb, L.A.Markowitz, and R.J.Cousins, Biochem.J., **175**, 833 (1978).
126. M.F.Obenrader and W.F.Prouty, J.Biol.Chem., **252**, 2860 (1977).
127. N.Taniguchi and A.Meister, J.Biol.Chem., **253**, 1799 (1978).
128. K.S.Squibb and R.J.Cousins, Biochem.Biophys.Res. Comm., **75**, 806 (1977).
129. L.Rydén and H.F.Deutsch, J.Biol.Chem., **253**, 519 (1978).
130. A.de la Torre, A.Chueca, and J.L.Gorgé, Phytochemistry, **17**, 35 (1978).
131. J.-O.Jeppsson, C.-B.Laurell, and M.Fagerhol, Europ. J.Biochem., **83**, 143 (1978).
132. G.Fez and R.Lindgren, Biochim.Biophys.Acta, **493**, 410 (1977).
133. C.Godinot, A.DiPietro, B.Blanchy, F.Penin, and D.C.Gautheron, J.Bioenerg.Biomembr., **9**, 255 (1977).
134. K.Malinowski and W.Manski, Immunochemistry, **14**, 603 (1977).
135. A.Fattoum, C.Roustan, J.Feinberg, G.Desvages, and C.-A.Pradel, Europ.J.Biochem., **82**, 161 (1978).
136. G.Lindeberg, J.Tengborn, H.Bennich, and U.Ragnarsson, J.Chromatogr., **156**, 366 (1978).
137. W.T.Melvin and H.M.Keir, Biochem.J., **168**, 595 (1977).
138. R.M.K.Dale, E.Martin, D.C.Livingston, and D.C.Ward, Biochemistry, **14**, 2447 (1974).
139. H.Biessmann, R.A.Gjerset, D.Lewy, and B.J.McCarthy, Biochemistry, **15**, 4356 (1976).
140. A.R.Thomason and F.M.Rottman, Anal.Biochem., **89**, 501 (1978).
141. D.J.Goss and L.J.Parkhurst, J.Biol.Chem., **253**, 7804 (1978).
142. H.C.Towle, J.-J.Tsai, S.Y.Tsai, and B.W.O'Malley, J.Biol.Chem., **252**, 2396 (1977).
143. G.P.Georgiev, Yu.A.Illyin, A.P.Ryskov, N.A.Tchurikov, G.N.Yenikolopov, V.A.Gvozdev, and E.A.Ananiev, Science, **195**, 394 (1977).
144. J.Coyette, J.-M.Ghuysen, and R.Fontana, Europ.J. Biochem., **88**, 297 (1978).
145. A.V.Il'ina, V.I.Lozinskii, Yu.A.Davidovich, and S.V.Rogozhin, USSR P.687 081 (1977); Byul.Izobret., No.35, 109 (1979).
146. V.I.Lozinskii and S.V.Rogozhin, Symposium, "I Vsesoyuznyi Simpozium po Molekulyarnoi Zhidkostnoi Khromatografii, g.Dzerzhinsk, 1979" (The Ist All-Union Symposium on Molecular Liquid Chromatography, Dzerzhinsk, 1979), p.13.
147. W.T.Melvin and H.M.Keir, Analyt.Biochem., **92**, 324 (1979).
148. H.Horitsu, Y.Banno, S.Kimura, H.Shimizu, and M.Tomoyeda, Agric.Biol.Chem., **43**, 663 (1979).
149. M.Fukuda, Y.Eshdat, G.Tarone, and V.T.Marchesi, J.Biol.Chem., **253**, 2419 (1978).
150. H.Singh and G.Kalnitsky, J.Biol.Chem., **253**, 4319 (1978).
151. C.-B.Laurell, J.Chromatog., **159**, 25 (1978).
152. L.Polgár and P.Halász, Europ.J.Biochem., **88**, 513 (1978).
153. N.Ninamiura and K.T.Yasunobu, Biochem.Pharmacol., **27**, 2737 (1978).
154. K.Hosoi, S.Kobayashi, and T.Ueda, Biochem.Biophys. Res.Comm., **85**, 558 (1978).
155. I.R.Cottingham and C.I.Ragan, Biochem.Soc.Trans., **6**, 1307 (1978).
156. J.H.Keen and W.B.Jakoby, Analyt.Biochem., **90**, 136 (1978).
157. E.Noguchi, M.Nishikimi, and K.Yagi, Analyt.Biochem., **91**, 367 (1978).
158. P.D.Chantler and W.B.Gratzer, Analyt.Biochem., **91**, 626 (1978).
159. J.Carlsson, Hindustan, Antibiot.Bull., **20**, 105 (1976–1978).
160. D.Prigent, C.Geoffroy, and J.E.Alouf, Compt.rend., Ser.D, **287**, 951 (1978).
161. M.Shipton and K.Brocklehurst, Biochem.J., **171**, 385 (1978).

162. D.J.Goss and L.J.Parkhurst, J.Biol.Chem., **253**, 7804 (1978).
163. Y.Eshdat and A.Prujansky-Jakobovits, FEBS Letters, **101**, 43 (1979).
164. J.Lasch and R.Koelsch, Europ.J.Biochem., **82**, 181 (1978).

Institute of Organoelemental Compounds,
USSR Academy of Sciences, Moscow