Bead cellulose and its use in biochemistry and biotechnology

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1. Introduction

In recent years, the significance of granular insoluble polymers, the action of which is based on interaction between their own functional groups and the compounds dissolved in the medium, has increased also in biochemistry and biotechnology. The so-called functional polymers are most frequently used as carriers of active compounds or groups, separation media, catalysts, adsorbents, *etc.*

In biochemical systems, hydrophilic polymers with expedient physical micro- and macrostructures have special significance with regard to the interacting substrates, particularly their mass, and to formation of optimal conditions for the interaction itself. Insoluble polysaccharides with porous structure and spherical shape of particles attract an extraordinary attention.

Cellulose has often been used as a carrier of functions in biochemistry, first of all due to its availability and good chemical reactivity. However, low porosity and nonspherical shape of the individual particles impeded its wider application. These disadvantages are eliminated with bead porous cellulose, prepared lately [1—4].

2. Bead cellulose

2.1. Methods of preparation

For preparation of spherical cellulose [3, 4] various procedures have been described. However, all involve the following three basic steps:

1. Liquefaction of the cellulose polymer;
2. Dispersion of the cellulose phase in a nonmiscible medium;
3. Solidification of drops and final modification of beads.

In the first stage the solutions of cellulose or its derivatives are prepared by procedures known for example in production of cellulose fibres, sheets and films. The starting material may be exceptionally a molten derivative, *e.g.* cellulose acetate.

In the second stage, drops of the liquid raw material are formed in an inert and nonmiscible medium. Dispersion is performed mostly by stirring, exceptionally by dropping or spraying.

In the third stage the conditions are set for sol/gel transition in drops, utilizing various mechanisms, such as chemical cross-linking, diminishing the solubility by chemical and physical effects, change of state by cooling, *etc.* Finally, the pure product is isolated, the required beads are sorted out and in some cases their chemical and physical structure is modified.

Bead cellulose prepared by the so-called TSGT (thermal sol-gel transition) process has been described most frequently [5, 1]. It is produced from technical viscose (*i.e.* from aqueous solution of cellulose xanthate) by dispersion with stirring in an organic solvent and thermal solidification without cross-linking through covalent bonds. In the course of solidification, the xanthate groups are decomposed and, consequently, the solubility in water disappears. Finally, decomposition is completed in alkaline medium and after washing a spherical, porous, regenerated cellulose is obtained.

2.2. Structure and properties

The chemical structure of bead celluloses does not differ from that of analogous nonspherical preparations. The difference in physical structure, particularly in porosity, accessibility, and shape of the individual particles is essential [6, 7].

The advantage of bead cellulose prepared by the TSGT process is chemical purity and the corresponding hydrophilicity. Natural celluloses contain hardly removable lipids which make them hydrophobic and bring about nonspecific adsorption in interactions with biochemical substrates. Spherical, regenerated cellulose contains essentially lower amount of lipids. TSGT bead cellulose has, similarly as other cellulose preparations, low content of carboxyl groups ($< 0.02\,\text{mmol}\,g^{-1}$), negligible content of sulfur ($< 0.03\,\%$), and its noncombustible portion decreases on washing with diluted mineral acids as well (from $< 0.15\,\%$ to $< 0.01\,\%$).

Porosity of never-dried TSGT bead cellulose, *i.e.* the volume % of water in the swollen mass may be as high as 90 % and by additional adjustments, especially by drying in various media [8] or partial drying [9] and reswelling, it is possible to obtain products with porosity values of 50 % to 90 %. By drying with nonpolar solvents (applying the solvent-exchange method) dry, highly porous materials with internal specific surface of about $300\,m^2\,g^{-1}$ [3] were obtained.

The porous structure of TSGT bead cellulose is typically heterogeneous, resembling rather a macroreticular structure than soft-gel polymer carriers. Insolubility is due to the existence of hydrogen bonds, cross-linking polymer chains. It leads to formation of rigid, oriented regions of quaternary structures, alternating with highly porous amorphous skeleton in accordance with the classical model of the cellulose structure. The result is a wide distribution of pore sizes, but also a relatively high mechanical strength, *i.e.* low deformability of particles. The never-dried TSGT bead cellulose is accessible to dextrans with relative molecular masses up to 0.5×10^6, however, known are also bead celluloses accessible to proteins with relative molecular masses of several millions [10]. TSGT bead cellulose has essentially higher mechanical strength than dextran or agarose gels and under flow-through in a column exhibits much lower pressure drop. First of all, its application is directed towards industrial scale, not only towards packing of laboratory columns for analytical or micropreparative use.

Common cellulose preparations consist mostly of native fibrous cellulose and thus, their granulometric composition is limited to fibres or fragments of fibres. Bead celluloses are available in various particle size from tens to thousands of micrometers for laboratory columns or industrial filters. Bead celluloses and

their derivatives can conveniently substitute common nonspherical cellulose materials, however, they are fully utilizable at industrial conditions only.

3. General applications

Bead cellulose and its derivatives were suggested to many various applications [3, 4, 11, 12]. It is a consequence of their typical properties, such as availability and low price, spherical shape, possibility to choose the particle size, mechanical strength (low pressure losses in columns, possibility to work as industrial filters), high porosity (accessibility for high-molecular-mass compounds), hydrophilicity (toleration of biological structures, fast kinetics), and chemical reactivity in derivatizations.

Majority of suggestions for applications of bead cellulose and its derivatives made till now concern biochemistry and biotechnology, mainly enzyme engineering. These suggestions are dealt with in the separate chapter 3.1. Besides these applications, the highest attention was paid to utilization of derivatives of bead cellulose in adsorption and ion-exchange chromatography of metal ions [4, 11, 12]. There were described many ion-exchange derivatives of bead cellulose, adsorbing metal ions selectively [4, 11, 13, 14]. Some of them are produced by United Chemical and Metallurgical Works, Ústí n/L, and supplied under the trademark Ostsorb [15]. Kinetic properties of these chelate-forming bead cellulose derivatives are very good [11, 13, 16—18]. The applications suggested concern the content of trace elements in surface waters [11—13] or removal of metal ions interfering with enzyme activity [12]. Further prospective applications of cellulose derivatives adsorbing metal ions selectively are dealt with in the special literature [19].

So far, bead celluloses have not belonged to commercial products with well-known and unlimited regions of use. The extent of possible applications is indicated by attempts to prepare new composite materials based on bead cellulose, suitable as carriers [20] or adsorbents [21] of biologically active compounds, and attempts to prepare bead charcoal [22] and cryoperlose, *i.e.* perlose saturated with a nonfreezing aqueous solution [23]. Perlose was proved suitable as packing material in protective filters used prior to adjustment of drinking-water by ion-exchange or reverse osmosis as well as in filtration of sodium chloride solution, used in analysis of particle size by a Coulter counter [24, 25]. Bead cellulose in dry form is one of the biopolymeric powders applied successfully in treatment of purulent, hard-healing wounds [9, 26] and is used also in cosmetic creams as ingredient [27]. Magnetic composite cellulose carrier in bead form [28] may introduce new elements into construction of immobilized bioreactors and chromatographic columns.

3.1. Applications in biochemistry and biotechnology

In the earliest years of development of enzyme engineering, solid-phase techniques found applications mostly in the fields of immobilization of enzymes, affinity chromatography and related techniques, sequential degradation and synthesis of proteins, enzymes inclusive [29]. With a vehement development of solid-phase techniques the requirements for the materials used, thus also for solid, water-insoluble carriers, increased. The list of basic requirements for an ideal carrier [29] contains many of the typical properties of bead cellulose presented in the parts 2.2. and 3. It is obvious that the material with such properties became in a short time attractive in many fields of biochemistry and biotechnology, where solid-phase techniques are used [30]. This has been partly reviewed already [3, 4, 31—34]. The present review deals with biochemical and biotechnological applications of bead cellulose in more detail.

In the fields utilizing bead cellulose and its derivatives up to the present most often, *i.e.* in adsorptions, liquid chromatographies, and heterogeneous biocatalysis, the problem of development of carriers is far from being the only one. Mathematical description of adsorption by time-concentration model and subsequent calculation of the parameters characterizing the nature of adsorption, *i.e.* stoichiometric *vs.* nonstoichiometric adsorption, and with this connected classification of chemisorption, biospecific and hydrophobic adsorption, confrontation of adsorption with analytical chromatography as methods enabling to establish the nature of the interaction between ligand and enzyme, are some of the problems typical of investigation of liquid chromatographies, accompanying applications of bead cellulose and its derivatives. Their solution aims at rationalization of the design of affinity chromatography.

In heterogeneous biocatalysis, the subjects of interest have been the size--exclusion principle as one of the dominant factors governing the kinetics of heterogeneous biocatalysis, quantitative relations between the structure of the enzyme—carrier conjugates and their catalytic activity, mathematical models predicting kinetics of immobilized bioreactors, extended by considering the role of the carrier geometry, development of universal, *e.g.* thermochemical methods enabling to measure intrinsic kinetic constants of immobilized enzymes, while bead cellulose and its derivatives as porous and deformable carriers were parts of the models. The aim has been similar, *i.e.* to rationalize the development of immobilized bioreactors. Therefore, this last part of the paper has been compiled with purpose to present the individual results, parts, and chapters as a logical unit.

3.2. Functionalization of bead cellulose

Cellulose derivatives in insoluble bead form may be prepared in two fundamental ways. Either the respective derivative is prepared in soluble form, converted into the bead form, and fixed in a suitable way or, on the contrary, first the bead cellulose carrier skeleton of respective porosity is prepared and then functionalized by reactions known with other polymers. However, in practice, only the second method is used [34]. In functionalizations of the same starting material numerous experimental procedures on preparation of cellulose derivatives generally [35] and on preparation of cellulose adsorbents particularly may be utilized. When the porosity of the starting cellulose, stabilized by hydrogen bonds and disperse forces only, is to be preserved, it is necessary to pay an appropriate attention to the choice of the reaction conditions. Substantial decrease in porosity is brought about by direct evaporation of water or other polar solvents from macropores, but also by action of alkali. An alkali is often a reaction component in alkylation or acylation reactions and is also used in activation (mercerization) prior to reactions. It is, of course, necessary to avoid conditions at which the starting cellulose as well as the product of functionalization are soluble. The degree of substitution (D.S.) plays here an important role. Above its critical value (varying from 0.3 to 0.6 according to the substituent) the product becomes soluble. It may be prevented by cross-linking or, sometimes, only by change of conditions that, in heterogeneous reaction, lead to a different distribution of functional groups on the cellulose chain, though the average D.S. remains the same. For modification of bead cellulose in nonaqueous media, evaporation of water from the swelled starting material is avoided by successive solvent exchange [8] according to the WAN method (water—alcohol—nonpolar solvent), having no unfavourable effect on porosity.

Among bead cellulose adsorbents, the ion-exchange derivatives [36] occupy the most important place. They are prepared by treatment with classical [37—40] (for CM, DEAE, P, DEAHP) or in the case of bead cellulose for the first time used nontraditional (for SHP, TMAHP) alkylation reagents [41]. At present, seven basic types of ion exchangers in bead form are available on the market. They are reviewed in Table 1. There are described also others: ECTEOLA (product of the reaction of cellulose with epichlorhydrin and triethanolamine), aminoethyl, and sulfoethyl derivatives [37].

The morphology, depending on the method of preparation, is decisive [42]. The first studies on carboxymethylation of bead cellulose in water—acetone medium pointed to changes in content of carboxymethylated glucose units in amorphous and crystalline portions as well as in the position on the ring [43]. In the case of weak-base anion exchangers (DEAE and DEAHP) the method of

Table 1

Review of ion-exchange derivatives of bead cellulose

Designation	Functional group	Producer[a]
DEAE	—O—CH_2CH_2—$\overset{+}{N}H(C_2H_5)_2Cl^-$	1, 2, 3, 4
	—O—CH_2CH_2—$\overset{+}{N}(C_2H_5)_2$—CH_2CH_2—$\overset{+}{N}H(C_2H_5)_2Cl^-$ Cl^-	
DEAHP	—O—CH_2—CH(OH)—CH_2—$\overset{+}{N}H(C_2H_5)_2Cl^-$	3
TMAHP	—O—CH_2—CH(OH)—CH_2—$\overset{+}{N}(CH_3)_3Cl^-$	3
CM	—O—CH_2—COONa	2, 3, 4
SHP	—O—CH_2—CH(OH)—CH_2—SO_3Na	3
SE	—O—CH_2CH_2—SO_3Na	2
P	—O—$PO(ONa)_2$	2, 3

a) 1 — Pharmacia, 2 — Serva, 3 — United Chemical and Metallurgical Works, Ústí nad Labem, 4 — Amicon-Grace Co.

preparation influences also the quality of functional groups. Besides the generally given —CH_2—CH_2—$N(C_2H_5)_2$ groups, also varying amounts (as high as 57 %) of strongly basic quaternary groups are present. As it follows from the titration curves [43], their structure is probably: —CH_2—CH_2—$\overset{+}{N}(C_2H_5)_2$— —CH_2—CH_2—$N(C_2H_5)_2$ and its homologues.

Preparation of bead celluloses with coupled reactive dyes of the triazine or of vinyl-sulfone type for dye-ligand chromatography belongs to functionalizations affording the required derivative directly in a single reaction step. Mostly those dyes were used the group selectivity of which, after being bound to the skeleton, was known (Cibacron Blue F3GA [44, 45], Procion Red H3B [45], Remazol Yellow GGL [46], Remazol Brilliant Blue R [47, 48] and their Czechoslovak analogues). Somewhat different specificity has the adsorbent with combination of diethylaminoethyl (DEAE) groups and the blue dye [49]. By the reaction of intermediates for preparation of reactive dyes of the vinyl-sulfone

type, a material with primary aromatic amino groups [50] is prepared by using 4-aminophenyl 2-sulfatoxyethyl sulfone and commercial 4-aminophenyl--sulfonylethyl derivative (Ostsorb AV) [15, 51].

Diazotization of the amino groups results in very reactive diazonium groups (owing also to $—SO_2—$ in the *para* position). Through this group series of ligands containing reactive derivatives of phenol, imidazole, aromatic *tert*--amines, and also $—NH_2$, —SH may be bonded by copulation or other reactions. In this way were immobilized peptides [52] or proteins containing Tyr, His, Trp or chelating (8-hydroxyquinoline, salicylic acid) [50, 51] and other (tetracycline, anhydrotetracycline) [53] phenols and also DNA [54]. The chelating ion exchangers are reviewed in Table 2.

Table 2

Commercially available chelating bead cellulose ion exchangers[a]

Designation	Functional group	Adsorption capacity for Cu/(mmol g^{-1})
Ostsorb OXIN	8-Hydroxyquinoline	0.5
Ostsorb SALICYL	Salicylic acid	0.5
Ostsorb DETA	Diethylenetriamine	0.6
Ostsorb DTTA	Diethylenetriamine-tetraacetic acid	0.6

a) Producer: United Chemical and Metallurgical Works, Ústí nad Labem.

Reactivity and availability of hydroxyl groups of basic bead cellulose itself is insufficient for some modifications and interaction of products. Introduction of hydroxyethyl (or rather $—(CH_2—CH_2O)_n—H$, $n = 1—3$) or $—NH_2$ and/or —SH groups makes the situation better. Hydroxyethylation together with cross-linking enable also continuous change from macroporous- to gel-type porosity [55]. For preparation of the bead $—NH_2$ derivative, the reactions of alkylating cellulose derivatives (C-2, C-6) with ammonia [37] or diamines [56] are used. The products containing lower amounts of the amine (0.2 mmol g^{-1}) are utilized for example in immobilizations by the glutaraldehyde method [57]. Alkylation of diethylenetriamine affords a chelating ion exchanger [56] and its carboxymethylation results in another one of the "complexone-like" type [58]. By alkylation reactions also other ligands were bonded through the amino group (*e.g.* lysine and histidine [46], 4-aminomethylphenylboronic acid [52]).

Toluenesulfonate [37, 59], nitrate and nitrite [60], chlorodeoxy [59], and 3-chloro-2-hydroxypropyl [61, 62] derivatives are utilized as fundamental alkylating derivatives of bead cellulose. Their preparation and reactions proceed

with higher conversion and at lower temperature in the case of derivatives prepared from bead hydroxyethylcellulose. Alkylation reactions serve also in preparation of bead thiols of celluloses [59, 62] either using thiourea, xanthate or thiosulfate, and subsequent hydrolysis of these intermediates. The reaction of cellulose thiols with disulfide (*e.g.* 2,2′-dipyridyl disulfide) or the above-mentioned alkylation of thiosulfate [62] results in reactive cellulose disulfides.

Series of methods for activation of bead cellulose and procedures for binding known with other hydroxyl carriers were modified or newly developed. Regarding the stability of bonds and easy performance of the reaction, the already mentioned diazotization of 2-(4-aminophenylsulfonyl)ethyl derivative, the activation with 2,4,6-trichlorotriazine [63], the oxidation with periodate [64—66] and quinone [67—70], and some variants of the glutaraldehyde method are considered most suitable. The classical activation with cyanogen bromide [71, 72] or esters of chloroformic acid (ethyl [63, 73], nitrophenyl [54, 73], *N*-oxysuccinimidyl [73]), which then bind ligands as derivatives of carbonic acid, is, due to lower hydrolytic stability of these bonds, less suitable. For binding of ligands with amino groups (combined with the $NaBH_4$ reduction), reactive aldehyde groups were introduced into bead cellulose, beside periodate oxidation, also by treatment with 4-isothiocyanatobenzaldehyde [66, 74], bromoacetaldehyde diethyl acetal [75], or by series of reactions of 3-chloro-2-hydroxypropyl derivative [76]. On the contrary, for binding of ligands carrying reactive aldehyde groups (*e.g.* obtained also by oxidation of polysaccharides or glycoproteins), the procedure utilizing cellulose with $—NH_2$ [77] or $—CO—NH—NH_2$ [78, 79] groups is suitable. Activation of bead cellulose with diisocyanates [80] was performed as well. Curiously enough, the reactions of the amino groups and carboxyls (either on the carrier or on the ligand) with carbodiimides, performable in acid medium, have not been described. Majority of immobilizations described, however, have been carried out in alkaline or neutral media.

3.3. Column packing materials, adsorbents, chromatographic separations

Bead cellulose and its derivatives have been applied almost in all liquid chromatographies typical of biochemistry and biotechnology. In the literature, size-exclusion chromatography (SEC) and bioaffinity chromatography, both analytical and preparative, occur most frequently. In the case of other chromatographies (ion-exchange, covalent affinity, hydrophobic, immobilized metal affinity), more attention has been paid to the analytical version.

The main principles of preparation of bead cellulose derivatives applied as adsorbents and column packing materials in biochemistry and biotechnologies are summarized in the part 3.2. There is outlined also the importance of choice

of the mode of ligand binding for retaining the structure and geometry of pores of bead cellulose. In this respect, the reactive forms of the ligand are preferred because of their ability to react with the unmodified bead cellulose directly. In most cases, low substitution degree (D.S.) of bead cellulose is satisfactory, *e.g.* with dyed anthraquinone-triazine derivatives it is in the range of $0.001 \leq \text{D.S.} \leq 0.21$ [47, 48, 81, 82]. As long as the structure and geometry of pores of the bead cellulose derivative are retained, the separation effect is a combination of size-exclusion properties of bead cellulose and specific interaction of the ligate with the immobilized ligand. In the quantitative relationship for zonal monovalent competitive chromatography this moment has been respected [83]. It is at the same time an illustration how information on bead cellulose, obtained by SEC, is transferred to its derivatives utilized in other chromatographies.

3.3.1. Size-exclusion chromatography

The use of gel substances as column packing materials in SEC is becoming more important for separation techniques in industrial processes as well as in laboratories. Column packing materials for SEC have conventionally been made of cross-linked dextran, agarose or polyacrylamide. However, these products are generally too expensive to be used in industrial applications since they are made of rather uncommon natural substances or they require carefully controlled processes for preparation. Moreover, they have some disadvantages, *e.g.* some of them are so soft that they are susceptible to deformation and exhibit a high resistance to flow-through of fluids. In comparison with these conventional macroporous gels, the bead cellulose (the cellulose gel) has similar low adsorptivity and exclusion limit towards many substances, higher chemical stability, thermal resistance, and remarkably improved mechanical strength [6, 84, 85]. The bead cellulose possesses both sufficient mechanical strength and considerable porosity. The flow resistance is in a good agreement with the spherical shape of the particles, and the allowed flow stress lies within technically useful limits. The beads exhibit excellent permeability over a wide range of molecular masses of permeable substances [85]. The pore size of the beads ranges from about ten to several hundreds nanometers, depending on the concentration and molecular mass of the starting cellulose materials [6, 84, 85]. Desalting and fractionation of biopolymers *via* SEC on bead cellulose were effective [85]. The methods used to prepare the bead cellulose were different in each work mentioned above and the authors did not compare the obtained products. However, the authors agreed in the properties recommending the bead cellulose as column packing material for SEC [7, 86—89].

Bead cellulose is available by several distributors: Amicon-Grace Co., Chisso

Co., Pierce Chemical Co. (U.S.A.) [10, 90], Daicel Chemical Industries (Japan) [91], and North-Bohemian Chemical Works SECHEZA, Lovosice (Czechoslovakia) [92]. Amicon-Grace Co. and Chisso Co. recommend their Matrex Cellufine Media as medium-pressure gels ideally suited for a wide range of chromatographic separations. They demonstrate on individual examples the outstanding flow rates in several column sizes and thus fast separations, high resolution due to small particle sizes, high capacity allowing large sample loads, and high throughput due to fast flow capabilities [10]. Besides, they report minimal fines generation even with rough handling, minimal changes in swelling and no shrinking during operational changes, compatibility with all commonly--used chromatographic solvents and buffers as well, autoclavability, and low cost per unit separation capacity [10]. Pierce Chemical Co. supplies cross-linked bead cellulose, the so-called Excellulose, having relative molecular mass exclusion limit of 5×10^3. Desalting of macromolecules and fractionation of small molecules ($400 < M_r < 5000$) have been successful [90]. Daicel Chem. Ind. provides Cellulose Gels characterized by relative molecular mass exclusion limits ranging within 10^4—10^6 and differing in compressibility by the eluent flow [91]. The particle size typical of beads produced by all manufacturers mentioned above is 40—100 μm and 100—200 μm [10, 90, 91]. The data characterizing Perloza (SECHEZA, Lovosice) are continuously completed [92], however, complete data on Perloza as a column packing medium for SEC have not been published up to the present. For the bead cellulose available by the aforementioned manufacturers a similar statement holds as for the literature data [6, 7, 84—89]: The methods used for preparation are obviously different and comparison of the results achieved at similar conditions is not available. Contrary to Czechoslovak manufacturers, foreign ones use probably the procedure *via* cellulose triacetate, known hitherto by the Japanese manufacturer only [91].

3.3.2. Ion-exchange chromatography

The use of ion-exchange derivatives of bead cellulose in ion-exchange chromatography of biochemical and, particularly, biopolymeric systems does not need special comments. Indeed, there is an extensive literature concerning the use of traditional forms of ion-exchange cellulose derivatives (*e.g.* standard fibrous, powder, microgranular) in ion-exchange chromatography [93]. Development and production of these cellulose column packing materials in bead form have become a matter of course also for renowned companies in the world [10, 94, 95]. Despite of that the development of methods of preparation, characterization of new properties, and broadening the sortiment of ion-exchange celluloses in bead form have continued [37, 50, 85]. One of the interesting results is for example the increase in cation- and anion-exchange capacity achieved by

cross-linking of bead cellulose and the simultaneous manifold increase in porosity [85]. This trend has been accepted also by the Czechoslovak manufacturer supplying ion-exchange bead celluloses named Ostsorb [15]. Table 3 presents examples of use of those ion-exchange bead celluloses the preparation and properties of which have been described in the literature.

Table 3

Use of bead cellulose ion exchangers in isolation of proteins

Designation	Purified protein	Ref.
DEAE	Human plasma	[39]
DEAE-Cellufine AH	Human serum	[85]
	Glucose isomerase	[96]
Ostsorb P	endo-DNase	[97]
Ostsorb DEAE	Pectinesterase, polygalacturonase	[98]
Ostsorb DEAE	Calmodulin	[99]

3.3.3. Chemisorbents, covalent affinity chromatography

Chemisorbents of thiols and disulfides occupy a dominant position among chemisorbents in biochemistry and biotechnology. Derivatives of bead cellulose functioning as chemisorbents of thiols and disulfides are presented in Table 4. With regard to their use in biochemistry and biotechnology, the above-mentioned functional groups may be classified as synthetic ligands. Moderate conditions of adsorption and desorption, selectivity, and reactivity are some of their typical properties. This is valid for example in the case of pyridyl disulfide derivatives *XII*, *XIII* [59] which have been known for a long time as ligands in covalent chromatography of thiol proteins, enzymes inclusive.

For desorption at mild conditions it is necessary to use thiol as a displacing agent [59]. *S*-Alkyl thiosulfate derivatives, *e.g.* THPC *XIV* [62], can be used similarly (Table 4). Desorption of papain was accomplished by addition of thiol (cysteine) in a weak alkaline medium. The purity of papain increased twice. Combination of covalent affinity chromatography on *S*-alkyl thiosulfate derivative of bead cellulose (THPC, *XIV*) with ion-exchange and size-exclusion chromatography was utilized in isolation of low-molecular-mass thiols from yeasts extracts [62].

Adsorption of thiols from water onto *O*-[*N*-(4-formylphenyl)thioureidoethyl]cellulose (FP-cellulose, FPC) was chosen as a model simplified for mathematical elaboration of chemisorption kinetics because of its irreversible, selec-

Table 4

Review of bead cellulose derivatives applied as chemisorbents

Derivative	Schematic structure of chemisorbent	Abbreviation	Adsorptive	Ref.
I	cel—$O(CH_2)_2NHCSNHC_6H_4CHO$[a]	FP-cellulose (FPC)	Phenylmethane[^{35}S] thiol, cysteine, glutathione reduced	[74, 100]
II	cel—$NHCSNHC_6H_4CHO$			
III	cel—$N(CH_2)_2NHCSNHC_6H_4CHO$			
IV	cel—OCH_2CH_2SH	ME-cellulose (MEC)	5,5′-Dithiobis(2-nitrobenzoic acid)	[51, 101, 102]
V	cel—$OCH_2CH(OH)CH_2SH$	MHP-cellulose (MHPC)		[51, 101]
VI	cel—$OCH_2CH_2SO_2C_6H_4SH$	MP-cellulose (MPC)		[51, 101, 102]
VII	cel—$OCH_2C_6H_4NHC_6H_4NHN{=}C(CN)_2$	PHPD-cellulose (PHPDC)[a]	Phenylmethane[^{35}S]thiol, dithiothreitol, cysteine, 2-mercaptoethanol, mercaptoacetic acid	[103]
VIII	cel—$O(CH_2)_2NHCH_2COC_6H_4NHN{=}C(CN)_2$			
IX	cel—$O(CH_2)_2NH(CH_2)_2COC_6H_4NHN{=}C(CN)_2$			
X	cel—$O(CH_2)_2N(C_2H_5)_2CH_2COC_6H_4NHN{=}C(CN)_2$			
XI	cel—$O(CH_2)_2N(C_2H_5)_2(CH_2)_2C_6H_4NHN{=}C(CN)_2$			
XII	cel—SSC_6H_4N		Cysteine, 2-mercaptoethanol, sodium sulfide	[59]
XIII	cel—$OCH_2CH(OH)CH_2SSC_6H_4N$			
XIV	cel—$OCH_2CH(OH)CH_2S_2O_3^-Na^+$	THP-cellulose (THPC)	[^{35}S]Cysteine, 2-mercaptoethanol, mercaptoacetic acid/papain	[62]

a) Prepared from powdery and fibrous celluloses Whatman (Maidstone) and Serva (Heidelberg).

tive, and stoichiometric character [74, 100]. Experimental data were fitted by empiric equation using linear [74, 100] and nonlinear [100] regression; both procedures provided similar course of adsorption kinetics but the latter revealed narrower intervals of confidence. Various experimental conditions and different structures of thiols [74] influence solely the rate of chemisorption and, consequently, the parameters $t_{0.5}$ and v_0, but exert no influence on the adsorption equilibrium state (parameter B_e).

At isothermal conditions parameters B_e, v_0, and $t_{0.5}$ have been found to be dependent on the initial concentration of the adsorptive only [51]. This enabled to express the total time and concentration dependence of the adsorption process by the equation

$$B = ct/(ct\bar{B}_e^{-1} + tc_B\bar{B}_e^{-1} + c\bar{v}_0^{-1} + c_v\bar{v}_0^{-1}) \qquad (1)$$

where B and $\bar{B}_e$ are the amount of substance adsorbed in time t and $t \to \infty$ from the solution with bulk concentration c and $c \to \infty$; $\bar{v}_0$ is the initial rate of adsorption at $c \to \infty$; c_B and c_v are the concentrations of substance at $B_e = 0.5\,\bar{B}_e$ and $v_0 = 0.5\,\bar{v}_0$; where B_e and v_0 represent adsorption at equilibrium and the initial rate of adsorption from bulk concentration c. The validity of eqn (*1*) was verified *via* chemisorption of an aromatic disulfide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) on three thiol derivatives of bead cellulose: MEC (*IV*), MHPC (*V*), and MPC (*VI*) [101] (Table 5).

Table 5

Parameters of eqn (*1*) for chemisorption of DTNB onto different thiol derivatives of cellulose and statistical evaluation of the obtained results

Derivative	$\bar{B}_e^{-1}$ / g mmol^{-1}	$c_B\bar{B}_e^{-1}$ / g dm^{-3}	$\bar{v}_0^{-1}$ / g h mmol^{-1}	$c_v\bar{v}_0^{-1}$ / g h dm^{-3}	α/%[a]
IVa	5.88	0.46	0.68	0.30	6.29±0.71
IVb	2.56	0.62	0.81	0.30	3.89±0.69
V	4.10	0.82	1.20	0.68	5.48±0.72
VI	2.92	0.53	1.65	0.25	3.83±0.70

a) Mean values ± S.E.M. of relative differences between the computed data and the data obtained by direct measurements ($n = 36$).

The experimental data obtained by direct measurements were compared with those computed from eqn (*1*) using mean ±S.E.M. of relative differences [101]. The constants $\bar{B}_e$, c_B, $\bar{v}_0$, and c_v, obtained from eqn (*1*) for three thiol derivatives of bead cellulose (*IV*—*VI*) applied, may be established from the results in Table 5. The mean values ±S.E.M. of relative differences of the obtained results

Table 6

Parameters of the time—concentration model established with different types of adsorption

Adsorbent	Adsorptive	$\bar{B}_e \cdot 10^{-3}$ / mol g^{-1}	$\bar{v}_0 \cdot 10^{-3}$ / mol g^{-1} h^{-1}	$c_B \cdot 10^{-3}$ / mol dm^{-3}	$c_v \cdot 10^{-3}$ / mol dm^{-3}	R^b	$S_d{}^c$
MEC	DTNB	0.182	1.106	0.075	0.300	0.983	0.002
MPC	DTNB	0.351	1.068	0.164	0.451	0.959	0.015
PHPC[a]	Perphenazine	2.512	0.631	1.757	0.171	0.966	0.049

a) 3-Phenoxy-2-hydroxypropylcellulose; *b*) R — coefficient of determination; *c*) S_d — standard deviation of the function.

point out a good correlation between the data gained by direct measurements and those from eqn (*1*). The highest deviation found between the calculated and measured values was 7 % [71, 101]. The values of the determination coefficient in each case exceeded 0.95 and suggested a good conformity.

In agreement with theoretical considerations [102], the obtained ratio c_v/c_B for the chemisorption reaction, *e.g.* the reaction of DTNB with MEC and/or MPC (*IV*, *VI*; Table 6) was higher than 1. This indicates that in the case of MPC the introduction of the hydrophobic phenyl group into the cellulose structure has no influence on the nature of the interaction [102].

3.3.4. Hydrophobic adsorbents, hydrophobic chromatography

Conventional hydrophobic interaction chromatography has been carried out mainly on uncharged alkyl and aryl derivatives of agarose. It was found that retention and selectivity depend substantially on the type of ligand. Proteins are generally more retained on the supports with more hydrophobic ligands. If the ligand is too hydrophobic, it is difficult to elute proteins in native states from the column. In contrast, if the ligand is very hydrophilic, very high concentrations of salt are required to retain proteins. Therefore, the ligand should be moderately hydrophobic [104]. Phenoxyalkyl chains bound to porous cellulose beads might fulfil all requirements.

In a preliminary study of hydrophobic adsorption [105] it was pointed out that the ratio between the amount of adsorbate and its concentration in water under equilibrium conditions is constant and independent of bulk concentration. Hence, hydrophobicity of an adsorbent might be adequately characterized by the partition coefficient of the amphiphilic probe in a system amphiphilic adsorbent—water.

The above considerations were verified experimentally using the batch-wise adsorption of phenothiazine derivative (trivial name: perphenazine) and bovine serum albumin (BSA) onto bead 3-phenoxy-2-hydroxypropylcellulose (PHPC) [105]. A kinetic study of adsorption revealed that in equilibrium the partition law governs the behaviour. The partition coefficient p was proved to be dependent on the hydrophobe density, and independent of the bulk concentration of the adsorptive. On the basis of the results, the mechanism is considered to be the liquid—liquid partition (LLP). The essential part is the formation of "high surface coverage" producing a coherent liquid-like film around the cellulose beads. It was anticipated that the properties of such layers would depend on the hydrophobe chain length and the hydrophobe density [106].

In accordance with the above results, the increase in hydrophobe density was reflected in perphenazine and the BSA retention in the solid phase (Fig. 1). For

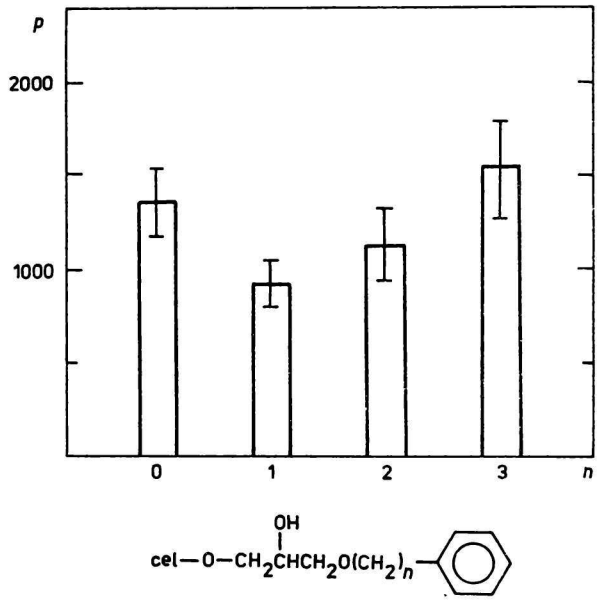


Fig. 1. The influence of hydrophobe chain length of hydrophobized celluloses on the perphenazine partition coefficient p in hydrophobized cellulose—water system. Hydrophobized cellulose ($n = 0$ and $n = 1$—3 (PHP $\approx$ 750 μmol g^{-1}) [106]) as a solid phase and 0.05 M borate buffer of pH = 9.0 as an aqueous phase were used. The values of p were expressed by the mean values ± confidence interval 95 % from 9 to 12 experiments.

BSA this function passed through a maximum (Fig. 2), and the hydrophobe chain length influenced the adsorption of solutes corresponding to a partition adsorption mechanism (Fig. 3). In such a case the presence of a liquid-like film composed of a hydrophobe—water binary mixture can be expected. This assumption was proved by perphenazine partition coefficient determination in immiscible liquids composed of an aqueous phase and binary mixtures of organic solvents. The change in binary mixture composition influenced the partition coefficient of perphenazine. This change, in the range of $1.9 \leq \varepsilon \leq 10.3$, was expressed as a linear function of Onsager's reaction field factor f. The solubility of perphenazine in organic solvents, water, and organic solvent—water binary mixtures was correlated with either dielectric constant or Hildebrand solubility parameter and in both cases the maxima were estimated. Both values of the dielectric constant ($\varepsilon \approx 30$) and the Hildebrand solubility parameter ($\delta \approx 26$) of the bonded hydrophobe phase in the region of perphenazine adsorption maxima were predicted. Phenoxyhydroxyalkyl chains bound to porous cellulose beads were used as the hydrophobes [106].

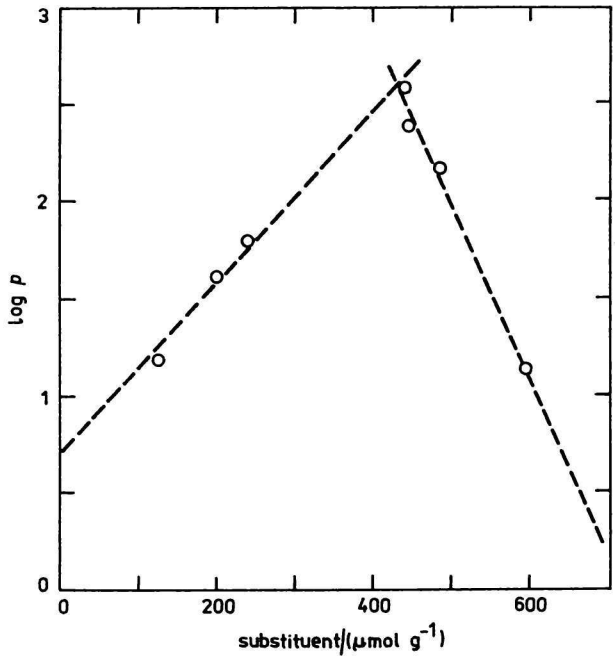


Fig. 2. The semilogarithmic plot of BSA partition coefficient p in PHPC—water system against the content of the hydrophobe. 0.05 M borate buffer of pH = 9.0 as an aqueous phase was used. The dashed lines mean the positive linear regression characterized by $r = 0.995$ and the negative linear regression characterized by $r = -0.994$.

The parameters $\bar{B}_e$, $\bar{v}_0$, c_B, and c_v (eqn (*1*)) valid for the adsorption of perphenazine onto bead PHPC were computed [102] and the c_v/c_B ratio was found to be less than 1. This substantiated the assumption that the c_v/c_B ratio may be used as an indicator of the hydrophobic nature of adsorption.

The nonstoichiometric model, suggested for adsorption of perphenazine and BSA on bead PHPC and considered equal to the partition mechanism, was verified by batch-wise adsorption of six proteins on bead PHPC [107]. It was found that the surface of the hydrophobic segment (PHP groups) of the adsorbent had to be sufficiently large so that the hydrophobic region of the protein surface might come into contact with it through multiple residues (Fig. 4). Fulfillment of this criterion should be a necessary prerequisite for predominance of partition of the protein between the hydrophobic segment in the form of a liquid-like film and the surrounding solution during adsorption. The absence of displacement phenomena in the zonal chromatography of six proteins on bead PHPC was observed. Displacing agents (water, PHP-poly(ethylene glycol)) and gradient elution (sodium chloride, ethylene glycol) had no effect on desorption [107]. With regard to high loading capacity ($B_e \leq 75$ mg IgG/g PHPC), it is

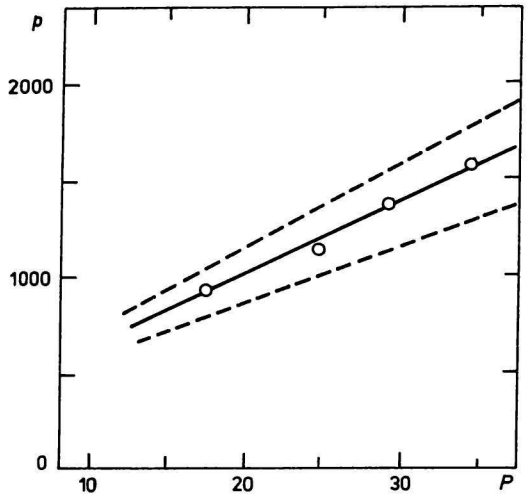


Fig. 3. The relationship between partition coefficient p of perphenazine in hydrophobized cellulose—water system and partition coefficient P of ω-phenyl-α-alcohols in 1-octanol—water system. The same hydrophobized celluloses as in Fig. 1 were used. In the direction from the left to the right investigated materials follow sequence on the axis of abscissa: phenylmethanol, 2-phenylethanol, phenol, 3-phenyl-1-propanol and that on the axis of ordinate: respective hydrophobized celluloses [106]. Full line represents the linear regression ($r = 0.992$) obeying the level of probability $p < 0.01$, dashed lines express 95 % confidence interval.

recommended to use bead PHPC for preconcentration, purification, and preseparation of substances and complex mixtures [108].

So far, only one example of use of PHPC for isolation of proteins has been reported in the literature [109]. The results have shown that bead PHPC may conveniently be used instead of Phenyl Sepharose* for routine isolation of

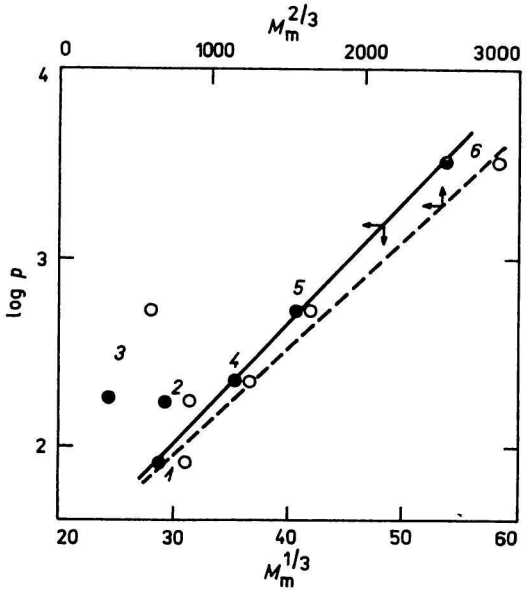


Fig. 4. The semilogarithmic dependence of protein partition coefficient p on relative molecular mass in terms of $M_m^{1/3}$ (●) and $M_m^{2/3}$ (○). Full line represents the results of linear regression of the values log p *vs.* $M_m^{1/3}$ ($\log p = 0.0639\, M_m^{1/3} + 0.09985$; $r = 0.9994$); dashed line represents the result of linear regression of the values log p *vs.* $M_m^{2/3}$ ($\log p = 7.55 \times 10^{-4}\, M_m^{2/3} + 1.379$; $r = 0.9927$); in both cases for $n = 4$, when the dots *2* (α-chymotrypsin) and *3* (lysozyme) were not included into correlation. *1.* Trypsin; *4.* ovalbumin; *5.* serum albumin; *6.* immunoglobulin G.

*Trade mark for 3-phenoxy-2-hydroxypropylagarose produced by Pharmacia Biotechnology (Uppsala).

calmodulin (CaM). Calmodulin from nerve tissue of cattle has been isolated using hydrophobic chromatography on PHPC, and became commercially available [99]. As tested with gel electrophoresis, the purity of CaM isolated on this carrier was $\geq 95\,\%$ [99].

3.3.5. Affinity adsorbents, bioaffinity chromatography

Affinity adsorbents may be considered a special type of chemisorbents. Their binding function is often based on biospecific interactions and utilized in bioaffinity chromatography.

Numerous examples of use of conjugates of bead cellulose as affinity adsorbents are known from the literature. As it follows from Table 7, majority of the

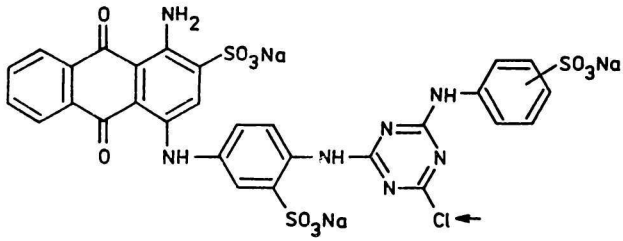
Table 7

Isolation and purification of biopolymers using derivatized bead cellulose as affinity adsorbent

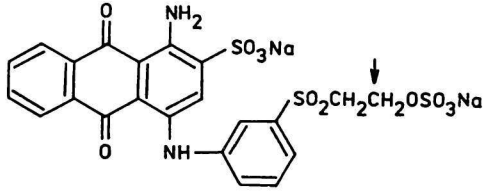
Covalently coupled ligand	Biopolymer purified	Ref.
Histamine	Methaemoglobin	[110]
DNA	DNase	[54]
Saccharides	Lectins	[3]
Trypsin inhibitor	Chymotrypsin	[69]
Aprotinine	Kallikreins	[111]
Bacitracin	Proteinases	[52]
Anhydrotetracycline	Anhydrotetracycline oxygenase	[112]
Anti-human IgG	Immunoglobulin(s)	[113, 114]
Reactive dyes	Lactate dehydrogenase	[47, 81, 82, 115]
Reactive dyes	Albumin(s), serum	[46, 49]
Hydroxamic acids	Urease	[34]
Acetate	Proteinases	[116]

affinity ligands used are natural ones. The synthetic ligands, presented in this table, resemble them either in their structures or action (or in both). It is customary to call their interaction with proteins (biopolymers) as biomimetic [117] with various degree of biorecognition. The most extensive and most significant group of synthetic ligands are textile reactive dyes [33, 118—121], called "universal pseudoaffinity ligands" [122]. The immobilized dyes in many cases mimic the properties of natural biological molecules and bind with high specificity to the ligand-binding sites of proteins [123]. For example, the anthraquinone textile dyes, especially Cibacron Blue 3G-A, are known to interact with

representatives of almost every conceivable class of enzymes plus a host of other, seemingly unrelated proteins [117]. Table 7 presents the anthraquinone--triazine dyes Cibacron Blue 3G-A and Remazol Brilliant Blue R (Fig. 5).



Cibacron Blue 3G-A



Remazol Brilliant Blue R

Fig. 5. Structures of anthraquinone—triazine dyes. The sites of immobilization on cellulose and other polysaccharides are indicated by arrows.

Also dyed adsorbents prepared from bead cellulose using other reactive dyes of the triazine and vinyl-sulfone type, *e.g.* Procion Red HE-3 B [124] and Remazol Yellow GGL [46], exhibit group specificity towards proteins. Blue adsorbents (*e.g.* Cibacron Blue 3G-A and its Czechoslovak analogues) function on the basis of specific interaction with serum albumin (isolation and removal of albumin from blood serum) and nucleotide-dependent enzymes (lactate dehydrogenase, LDH). Also the red adsorbent has an increased affinity for nucleotide-dependent enzymes. Yellow cellulose has the ability of binding immunoglobulins [46], the blue DEAE-cellulose has not [49]. This is utilized in their isolation and purification.

A rational design of affinity chromatography processes involves both mathematical modelling and experimental measurements of several parameters. Any model of separation of enzymes by affinity chromatography must consider the equilibrium relationships between the respective enzyme and the immobilized ligand [123, 125]. The foregoing relationship may be easily characterized by the enzyme—ligand dissociation constant.

Determination of dissociation constants of the complexes of enzyme—dye immobilized on bead cellulose (immobilized ligand; K_{I-L}) and of those immobilized on a water-soluble polysaccharide (mobile ligand; K_{M-L}) revealed at the same time information about the bead cellulose as an affinity matrix. The methods applied are batch-wise adsorption and zonal analytical chromatography, and the enzyme often used for these purposes [118—122] is the well-known LDH [126, 127].

Besides determination of the dissociation constants of enzyme—ligand complex, batch-wise adsorption makes possible to establish also the nature of the interaction between the enzyme (LDH) and the immobilized ligand (dyed bead cellulose), when utilizing the time—concentration model of adsorption (eqn (*1*)) [81, 103]. In agreement with theoretical considerations, the ratio c_v/c_B may serve as an indicator of the type of the adsorption. Thus, biospecific adsorption will be characterized by the ratio $c_v/c_B > 1$, whereas for hydrophobic adsorption this ratio should be always below one [103]. Because of the respective physical meaning, c_B was taken as the dissociation constant K_{I-L} and $\bar{B}_e$ as the accessible concentration of the immobilized ligand (I − L). The ratio c_v/c_B calculated from the data presented in Table 8 pointed to differences in the mechanism of the interaction. The interaction of LDH with Cibacron Blue immobilized on bead cellulose is a boundary one, composed of biospecific and hydrophobic interactions, whereas the interaction of LDH with immobilized Remazol Blue is unambiguously hydrophobic, *i.e.* nonstoichiometric [81, 103]. From the $\bar{B}_e$ and c_B values the accessible concentration of the immobilized Cibacron Blue and the respective dissociation constant K_{I-L} have been calculated [81].

Table 8

Batch adsorption of LDH on dye-cellulose beads processed according to eqn (*1*)

Dye immobilized on cellulose beads	pH	$\bar{B}_e \cdot 10^3$ / μmol g^{-1}	$v_0 \cdot 10^3$ / μmol g^{-1} h^{-1}	c_B / μmol dm^{-3}	c_v / μmol dm^{-3}	R
Cibacron Blue[a]	7	280.0	181.0	2.64	1.53	0.992
Cibacron Blue[a]	8.5	279.8	181.1	2.40	2.66	0.987
Remazol Blue[b]	8.5	42.0	2.23	59.79	0.75	0.966

Analytical concentration of immobilized dye: *a*) 0.46 mmol dm^{-3}; *b*) 3.42 mmol dm^{-3}.

These results were confronted with those achieved by zonal analytical chromatography [81]. In the treatment of the experimental results the molecular retention model, describing a competitive monovalent interaction, was used [83].

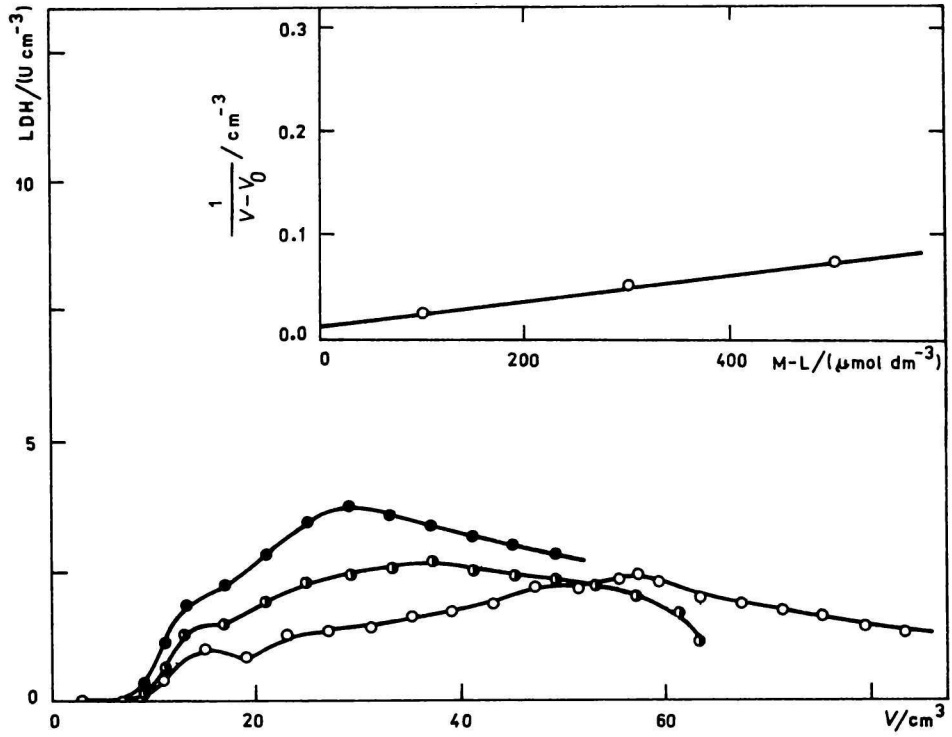


Fig. 6. Zonal chromatography of LDH on Cibacron Blue-cellulose beads with varying concentration of NADH in the eluting buffer, pH = 7. 200 U of LDH were applied to columns equilibrated with 100×10^{-6} M (○), 300×10^{-6} M (◑), and 500×10^{-6} M (●) NADH (mobile ligand) each in the buffer and eluted with these buffers. Inset: plot of $1/(V - V_0)$ *vs.* NADH concentration according to Ref. [81], correlation coefficient $r = 0.998$.

Fig. 6 demonstrates the elution profile of LDH when NADH was used as the mobile ligand [81]. From the linear dependence of the semireversed relationship it is evident that the immobilized dye (Cibacron Blue) interacted with the tetrameric enzyme (LDH) monovalently and competed with NADH for the only binding site. On the other hand, Remazol dye, by immobilization on cellulose and in the presence of NADH as the mobile ligand, has lost the ability to interact with LDH biospecifically (Fig. 7).

Competition of the immobilized and mobile Cibacron Blue for the binding site on LDH during zonal elution is projected to the elution profiles illustrated in Fig. 8. Cibacron Blue bound to dextran T 10 (CBD) has been used as the mobile ligand.

Table 9 summarizes the values of dissociation constants of the immobilized dye with LDH. Besides both methods mentioned above, the table presents also

Fig. 8. Zonal chromatography of LDH on Cibacron Blue-cellulose beads with varying concentration of Cibacron Blue-dextran T 10 conjugates in the eluting buffer A, pH = 7. 200 U of LDH were applied to columns equilibrated with 5×10^{-6} M (◑), 10×10^{-6} M (○), 20×10^{-6} M (●), and 60×10^{-6} M (◐) Cibacron Blue-dextran T 10 conjugates (mobile ligands) each in the buffer A and eluted with these buffers. Inset: plot of $1/(V - V_0)$ *vs.* concentration Cibacron Blue-dextran T 10 according to Ref. [81], correlation coefficient $r = 0.998$.

the results of batch adsorption, treated according to a model developed earlier [81]. These data should be completed by the results of kinetic determination of inhibition constants of CBD and Remazol Blue-dextran T 10 (RBD). For both dye—dextran conjugates the competitive type of inhibition towards NADH has been manifested. A difference of three orders of magnitude between the inhibitory power of CBD ($K_i = 0.054$ μmol dm^{-3}) and RBD ($K_i = 40.5$ μmol dm^{-3}) was found [81].

Recently, it has been concluded that the interaction between LDH and Cibacron Blue linked to the polysaccharide matrix decreased in the sequence dextran — hydroxyethylstarch — hydroxyethylcellulose [128, 129]. This fact has

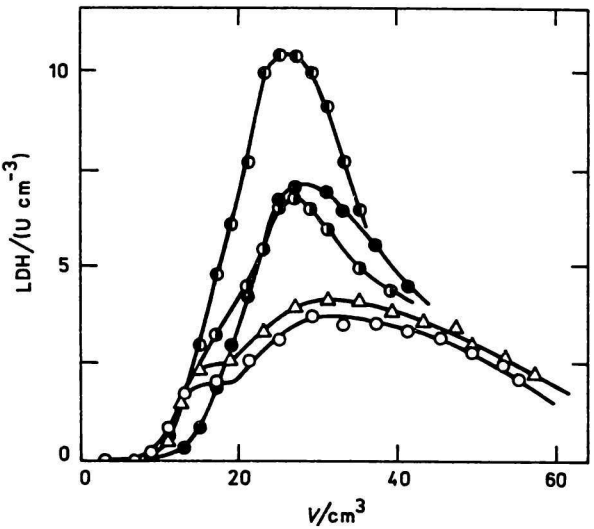


Fig. 7. Zonal chromatography of LDH on Remazol Blue-cellulose beads with varying concentration of NADH in the eluting phosphate buffers, pH = 7. 200 U of LDH were applied to columns equilibrated with 100×10^{-6} M (○), 200×10^{-6} M (△) NADH each in the buffer, 20×10^{-6} M (◑), 50×10^{-6} M (●), and 100×10^{-6} M (◐) NADH each in the 0.5 M phosphate buffer.

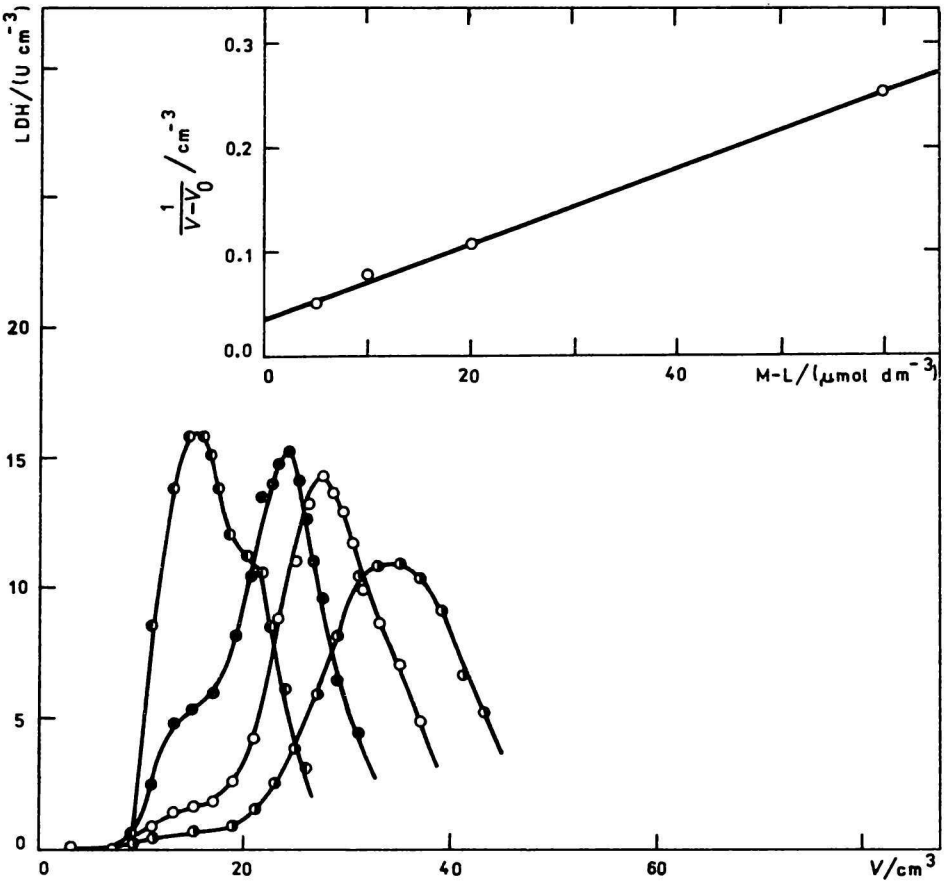


Table 9

Dissociation constants of Cibacron Blue-cellulose—LDH complex obtained by different procedures

Procedure	K_{I-L} / (μmol dm^{-3})	
	pH = 7	pH = 8.5
Batch adsorption	2.64	2.40
Batch adsorption[a]	1.71	1.21
Zonal chromatography[b]	0.56	1.78
Zonal chromatography[c]	1.92	2.20

a) The concentration dependence of the immobilized ligand; *b*) NADH used as the mobile ligand; *c*) Cibacron Blue-dextran T 10 used as the mobile ligand.

been ascribed to diverse conformations of polysaccharide in the solution, resulting both in differences in selective adsorption of LDH and different occlusion of the linked dye by the polysaccharide carrier [129]. The results obtained with batch adsorption manifested a similar phenomenon. Unlike dextran, cellulose in bead form diminishes the biospecificity of interaction of both linked dyes with LDH (Table 8) and, in the case of Remazol Blue, even the nature of the adsorbent—adsorbate interaction changes to a nonbiospecific, hydrophobic one. Zonal chromatographic experiments with NADH as the mobile ligand (Fig. 7) confirmed the previous conclusion.

The dissociation constant values of the LDH—immobilized Cibacron Blue complex ($0.6 \leq K_{I-L} \leq 2.6\ \mu mol\ dm^{-3}$), established by both methods, indicated the presence of biospecific interactions. On the other hand, the differences in the values of dissociation constants K_{I-L} and K_{M-L} (Table 10) were ascribed to

Table 10

Dissociation constants obtained from zonal chromatography using equation valid for monovalent interaction [81, 83]

System		K_{M-L} / (μmol dm^{-3})		K_{I-L} / (μmol dm^{-3})	
Immobilized dye[a]	Mobile ligand	pH = 7	pH = 8.5	pH = 7	pH = 8.5
Cibacron Blue	NADH	93.94	200.0	0.56	1.78
Cibacron Blue	Cibacron Blue-dextran T 10	11.14	8.05	1.92	2.22

a) Analytical and accessible concentrations of immobilized dye were 138 μmol dm^{-3} and 6.66 μmol dm^{-3}, respectively.

nonspecific interactions with bead cellulose [81]. In order to minimize the effect of nonspecific interactions of LDH with the matrix and the effect of other specific proteins in the LDH preparation on the values of dissociation constants K_{I-L} and K_{M-L}, additional study was made [82]. For this purpose CB--bead cellulose (*XVa—XVd*) with the dye content of 1.7—14.6 μmol g^{-1} and two LDH preparations (a crude and a purified one) were used. For all combinations of both variables, the reciprocal plot of elution volume of LDH *vs.* concentration of the mobile ligand CBD was linear. Consequently, the values of the respective dissociation constants could be calculated according to the equation valid for a monovalent interaction (Table 11).

As concluded in both papers [81, 82], the nature of interactions between LDH and the dye-ligand immobilized on bead cellulose was biospecific, since inclusion of a natural ligand (NADH) brought about a competitive elution of LDH. In concomitance, the values of K_{I-L} were found to be lower than those

Table 11

Review of dissociation constants K_{M-L} and K_{I-L}

CB-cellulose	K_{M-L}/(μmol dm^{-3})		K_{I-L}/(μmol dm^{-3})		Correlation coefficient	
	1	2	1	2	1	2
XVa	9.1	3.1	1.6	1.2	0.992	0.998
XVb	4.4	2.9	1.55	1.9	0.998	0.994
	6.4		1.7		0.998	
XVc	8.1		1.9		0.986	
	12.2		2.0		0.975	
XVd	20.0		3.15		0.962	
	8.0		1.0		0.988	

Columns 1, 2 denote preparations of LDH (1 — crude and 2 — purified).

of K_{M-L}. This has been interpreted in terms of additional binding interaction due to the dye-ligand in immobilized form. The differences in K_{I-L} and K_{M-L} values may serve for estimation of the contribution of nonspecific interaction of the matrix to the total interaction between dye-ligand and enzyme. This contribution, reflected in practically constant values of K_{I-L}, remained unchanged in the studied concentration range of the immobilized dye and in the presence of other protein admixtures.

The crude LDH was purified by preparative dye-ligand chromatography on the bead Remazol Blue-cellulose column, followed by ultrafiltration, dialysis, and two-fold dilution with glycerol [81]. The purity of LDH was controlled by means of a fast-protein liquid chromatography [82]. The chromatograms of crude and purified LDH are presented in Fig. 9.

Also the nonderivatized cellulose may serve as a ligand, namely for β-glucanases, when suppressing its substrate properties and offering it to the enzyme as an imitation of the substrate. This is achieved mostly by cross-linking. It was done so in the case of cross-linked bead cellulose which was used as an affinity adsorbent for β-1,3-glucanases [130]. Only weak interaction was observed between the enzyme and the adsorbent.

The hydrophilic skeleton of bead cellulose has a favourable effect on kinetics of adsorption on chelating ion exchangers, *i.e.* on those with bonded 8-oxyquinoline, salicylic, chromotropic, and ethylenediaminetetraacetic acids, and diethylenetriamine [17]. The specificity of these and other ligands bonded on bead cellulose is known in analytical chemistry. These adsorbents, after adsorption of metal ions, were used in affinity chromatography, the so-called immobilized metal affinity (IMA) chromatography, *e.g.* of subtilisin [131].

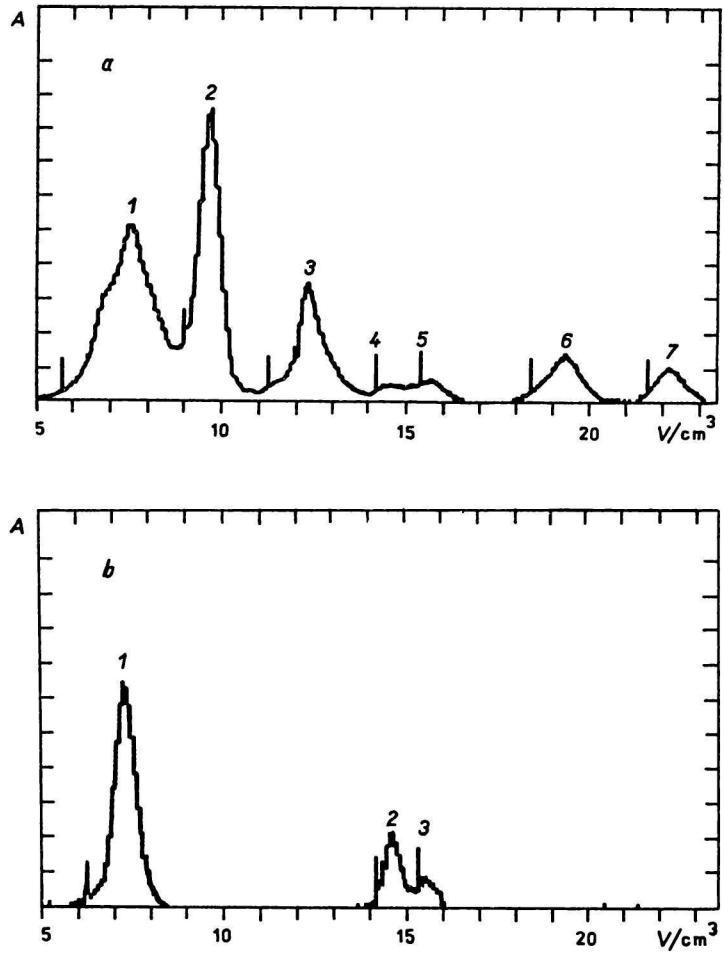


Fig. 9. Elution profiles of crude (*a*) and purified (*b*) LDH were recorded with the aid of the FPLC equipment. Effluent was monitored as absorbance at $\lambda = 280$ nm (axis of ordinate), numbers depicted on the axis of abscissa mean elution volume (cm^3) diminished for 5 cm^3. The column (Superose 12 HR 10/30) was loaded with 20 U of LDH per 0.3 cm^3

3.4. Heterogeneous biocatalysis

3.4.1. Immobilized enzymes and cells

The most frequent type of immobilization of enzymes on bead cellulose is the covalent immobilization. All three typical procedures may be found in the following examples:

1a. Reaction of the enzyme with the activated carrier (Table 12);

1b. Reaction of the enzyme with the carrier using a homobifunctional reagent (Table 13);

1c. Reaction of the enzyme with a heterobifunctional reagent and subsequent reaction of the activated enzyme with the carrier (Table 14).

Most examples are provided by the method *1a*. Among them, there are procedures universal for activation of natural and synthetic polymers (diazotization/copulation (*XIX*), formation of amides (*XXIV*) and hydrazides (*XXXV*)) as well as procedures typical of activation of natural polysaccharides (reactions of vicinal diols with cyanogen bromide (*XVII*) and chloroformates (*XVI*), etherification with cyanuric chloride (*XXVII*)). Cellulose, agarose (Sepharose), and cross-linked dextran (Sephadex) were the carriers most often activated by the aforementioned methods of activation of natural polysaccharides [141, 142]. Activation with cyanogen bromide still is, with regard to considerable popularity of agaroses and dextrans, probably the most often used method of activation. The reaction rate at moderate conditions and high substitution degrees are the main advantages of this simple activation procedure.

In activation of bead cellulose the procedures elaborated for activation of traditional cellulose materials, such as powdery and microcrystalline celluloses, have been applied [143, 144]. One of these procedures is oxidation of bead cellulose with periodate (*XXI*). The indisputable advantage of this method over the activation with cyanogen bromide is the nontoxic reagent, mostly sodium periodate. However, it is necessary to control the degree of oxidation in order to prevent destruction of beads. The other two universal procedures (activation with epichlorhydrin and 4-toluenesulfonyl chloride), often used in preparation of bead adsorbents [34], have not been applied so often in immobilization of enzymes. Activation with cyanuric chloride (*XXVII*) is more suitable because it provides such a product (cellulose chlorotriazine) which is, similarly as the product of periodate oxidation (cellulose 2,3-dialdehyde), sufficiently reactive for enzyme immobilization.

In the method *1a* are included also such examples where the enzyme, structurally modified, was activated by periodate oxidation [57, 137]. This example has its analogue in the procedure where the glycoenzyme was prepared synthetically and the neoglycoenzyme was then activated by periodate oxidation [78]. Covalent immobilization by the procedure *1c* is one of the ways how to achieve spatially-oriented binding of enzymes into multienzyme complexes [145]. Glycosylation of the enzyme and periodate oxidation of the (synthetic) neoglycoenzyme might be the reaction steps of a universal procedure [140], used for this purpose.

Immobilization of enzymes by ionic bond on derivatives of bead cellulose is exemplified in Table 15.

Table 12

Review of enzymes and other proteins immobilized covalently on activated bead cellulose *via* method *1a*

Derivative	Reagent used for activation	Site of activation	Activated bead cellulose	Proteins immobilized on activated cellulose	Ref.
XVIa	Ethyl chloroformate	OH	*trans*-2,3-Cyclic carbonate	Chymotrypsin A	[63, 132, 133]
XVIb	4-Nitrophenyl chloroformate	OH	*trans*-2,3-Cyclic carbonate	Trypsin	[73]
XVIc	*N*-Hydroxysuccinimidyl chloroformate	OH	*O*-Ethoxycarbonyl	Trypsin	[73]
XVII	Cyanogen bromide	OH	Imidocarbonate	Trypsin	[73]
				Glucoamylase	[134]
				Methemoglobin	[72]
				Immunoglobulin G	[115, 116]
XVIII	2,4-Diisocyanatotoluene	OH	Isocyanatophenyl	Glucoamylase	[80, 134]
				Glucose isomerase	[80]
				Invertase	[80]
XIX	Sodium nitrite/HCl	NH_2 (aromatic)	Diazonium salt	Glucoamylase	[80]
				Trypsin	[135, 136]
				Invertase	[137, 138]
XX	Glutardialdehyde	NH_2	Aldehyde	Glucoamylase	[80]
				Glucose oxidase	[57]
				Invertase	[138]
XXI	Periodate, s	OH	2,3-Dialdehyde	Trypsin	[66, 73, 135]
					[136, 139]
				Glucose oxidase	[57]
				Chymotrypsin	[64, 65]
				Lysozyme	[139]
				Ovalbumin	[139]
				Serum albumin	[139]
				Immunoglobulin G	[115, 116]

Table 12 (Continued)

Derivative	Reagent used for activation	Site of activation	Activated bead cellulose	Proteins immobilized on activated cellulose	Ref.
XXII	Benzoquinone	OH	Benzoquinone	β-Galactosidase	[67, 68, 140]
				Chymotrypsin	[70]
				Subtilisin	[70]
				Protease, neutral	[70]
XXIIIa	Epichlorhydrin + α,ω-diaminoalkane	OH	Alkylamine	Glucose oxidase	[57]
XXIIIb	Epichlorhydrin + benzidine	OH	Arylamine	Glucose oxidase	[57]
XXIV	Carbodiimide	COOH	Activated ester	Glucose oxidase	[57]
XXV	4-Isothiocyanato-benzaldehyde	NH_2	Formylphenyl	Trypsin	[66]
XXVI	Bromoacetaldehyde Diethyl acetal/HCl	OH	Formylmethyl	Trypsin	[135, 136]
				Invertase	[138]
XXVII	Cyanuric chloride	OH	Chlorotriazine	Invertase	[137, 138]
				Immunoglobulin G	[114]
XXVIII	Tosyl chloride	OH	Tosyl	Immunoglobulin G	[114]
XXIX	Dimethyl sulfoxide—carbodiimide	OH	Aldehyde	Immunoglobulin G	[114]
XXX	Chromium trioxide	OH	Carbonyl	Immunoglobulin G	[113, 114]

Table 13

Review of enzymes immobilized on bead cellulose derivatives covalently *via* the method *1b* using homobifunctional reagents

Derivative	Homobifunctional reagent	Site of activation	Activated bead cellulose	Enzymes immobilized	Ref.
XXXI	Glutardialdehyde	Amino, aromatic	Aldehyde	Glucoamylase	[80]
XXXII	2,4-Diisocyanatotoluene/water	Hydroxy	Amino	Glucoamylase	[80]
XXXIII	3-Aminopropylethoxysilane/glutardialdehyde	Hydroxy	Aldehyde	Glucoamylase	[80]
XXXIV	Polyethylenimine/glutardialdehyde	Unknown	Aldehyde	Invertase	[137]

Table 14

Review of enzymes and other proteins immobilized on bead cellulose derivatives covalently *via* the method *1c* using activated enzymes

Derivative	Functional group of cellulose	Functional group of activated protein	Immobilized proteins	Ref.
XXXV	Hydrazide	2,3-Dialdehyde	Invertase	[137]
XXXVI	Hydrazide, hydrazinodeoxy	2,3-Dialdehydoglucose, -galactose, -cellobiose, -lactose	Serum albumin	[78]
XXXVII	Alkylamine	2,3-Dialdehyde imine	Glucose oxidase	[57]
XXXVIII	Alkylamine/cyclohexyl isocyanide	2,3-Dialdehyde imine	Invertase	[77]

Table 15

Review of enzymes immobilized on bead cellulose derivatives with ionic bonds

Derivative	Method of preparation and reagents used	Derivative	Immobilized enzyme	Ref.
XXXIXa	Cross-linking followed by 2-chlorotriethylamine	DEAE	Glucoamylase	[134]
			Glucose isomerase	[134]
XXXIXb	2-Chlorotriethylamine followed by cross-linking	DEAE	Glucoamylase	[80]
			Glucose isomerase	[146]
XXXIXc	Cross-linking followed by 2-chlorotriethylamine and repeated cross-linking	DEAE	Glucoamylase	[147]
XL	Diisocyanato, water, *O*-methylisourea	Guanidino	Glucoamylase	[80]
			Glucose isomerase	[80]
			Invertase	[147]
XLI	Epichlorhydrine, diethylamine	DEAHP	Invertase	[148, 149]

Table 16

Intrinsic kinetic constants of immobilized invertase measured in packed bed reactors

Bead cellulose prepared from	Bead cellulose derivative	Immobilization	Constants		Ref.
			K_m/(mmol dm^{-3})	K_i/(mmol dm^{-3})	
Cellulose acetate	Guanidino	Ionic (*2*)	46.9	4.884	[147]
Cellulose xanthate	Chlorotriazine	Covalent (*1a*)	33.0	—	[137]
Cellulose xanthate	Chlorotriazine	Covalent (*1a*)	48.5	1752.0	[138]
Cellulose xanthate	Chlorotriazine	Covalent (*1a*)	47.6	1748.7	[138]

K_i — constant of substrate inhibition.

Bead cellulose is a porous carrier. The size and geometry of pores and the size and conformation of the enzyme determine the distribution of the enzyme on the carrier, on its surface and along the pores. In the study of kinetics of "porous" immobilized enzymes, except mass transport limitations, the size--exclusion principle is a dominant factor. It is confirmed by empirical quantitative relations, respecting the relative catalytic activity of trypsin immobilized on various derivatives of bead cellulose (Perloza) *vs.* molecular mass [66], available distribution coefficient [135], and hydrodynamic diameter of the substrate [136]. The constants of these equations were tested as parameters of quantitative relationships between the activity of the immobilized enzyme and structure of the bead cellulose—trypsin conjugates [66, 135, 136]. In preparation of conjugates attention was paid to reductive alkylation, *i.e.* immobilization procedure utilizing aldehyde derivatives of bead cellulose, 2,3-dialdehyde inclusive [66, 135, 136].

For characterization of suitability of the enzyme carrier numerous criteria are used: availability (price), easy activation, resistance to chemical and microbial attack, hydrodynamic properties (*e.g.* drop pressure), *etc.* It is customary to compare bead cellulose as enzyme carrier with other spherical biopolymers, mainly with agaroses (Sepharoses) and cross-linked dextrans (Sephadexes). As it follows from this review, bead cellulose is equal to traditional biopolymers in all criteria mentioned above, moreover, in price, hydrodynamic properties, resistance to chemical attack, and easy activation even surpasses them.

Another criterion for suitability of the carrier are the properties of the enzyme after immobilization. In this respect the first source of information are the kinetic constants of immobilized and free enzyme and the effect of various factors on their values. Such an information was obtained from packed bed reactors (PBR), packed with immobilized invertase [137, 138, 147, 149] and glucoamylase [140] and from stirred tank reactors (STR), packed with immobilized invertase [137, 148]. Serious confrontation of kinetic data is impeded by diverse methods of immobilization (Tables 12, 13, 15), different procedures of preparation of bead cellulose [3, 4] as well as by the origin and quality of free enzyme. It follows also from the differences in values of kinetic constants of invertase from *Candida utilis* [147] and *S. cerevisiae* [137, 138], immobilized in two different ways on two different bead celluloses (Table 16). Availability of the Czechoslovak cellulose both at inland and foreign workplaces at present offers a possibility to unify the properties of the carrier. The enzymes (proteins) and the methods used for their immobilization on the Czechoslovak bead cellulose are reviewed in Table 17.

Most recently developed mathematical models predicting kinetics of STR and PBR were extended by considering the role of cellulose bead geometry [137].

Table 17

Proteins covalently immobilized on bead cellulose (Perloza®), produced in Czechoslovakia

Protein	Method of immobilization	Activated bead cellulose	Ref.
Chymotrypsin	*1a*	2,3-Dialdehyde	[64, 65]
		Benzoquinone	[70]
Trypsin	*1a*	2,3-Dialdehyde	[66, 73, 135, 136, 139]
		Formylphenyl	[66]
		Formylmethyl	[135, 136]
		2-(4-Aminophenylsulfonyl)ethyl[a]	[135, 136]
Subtilisin	*1a*	Benzoquinone	[70]
Protease, neutral	*1a*	Benzoquinone	[70]
Invertase	*1a*	Chlorotriazine	[137, 138]
		2-(4-Aminophenylsulfonyl)ethyl[a]	[137, 138]
		Formylmethyl	[138]
	1b	PEI-cellulose/aldehyde	[137]
	1c	Hydrazide	[137]
	2	DEAHP	[148, 149]
β-Galactosidase	*1a*	Benzoquinone	[67, 68]
Glucose oxidase	*1a*	Aldehyde	[57]
	1c	2,3-Dialdehyde	[57]
Hydroxysteroid Dehydrogenase + diaphorase	*1a*	Imidocarbonate	[71]
Polynucleotide Phosphorylase	*1a*	2-(4-Aminophenylsulfonyl)ethyl[a]	[150]
Serum albumin	*1a*	2,3-Dialdehyde	[139]
	1c	Hydrazides, hydrazinodeoxy	[78]

a) Commercially available as Ostsorb AV (United Chemical and Metallurgical Works, Ústí n/L).

In the PBR, owing to the Sherwood number kept below 2 (flow rates 0.5—4 $cm^3 min^{-1}$), deformation of the ideal spherical shape of beads was observed. The spherical shape of cellulose beads was partly destroyed in STR, particularly when the number of revolutions exceeded 200 min^{-1} When preservation of the original shape of beads is required, other types of reactors or reinforcement of beads is recommended [137]. Invertase was used as the model enzyme.

The same model (invertase immobilized on derivatives of bead cellulose) was used when the intrinsic kinetic constants of hydrolysis of saccharose were estimated using both direct (enzyme thermistor) and indirect (differential reactor system) methods [138]. The effect of heat losses and flow nonideality on the values of intrinsic kinetic constants (K_m, K_i, V_m) was eliminated, however, the effect of flow rate on the thermistor sensor signal was evident. The obtained kinetic parameters were superverified by means of an independent differential reactor system. Of several Perloza derivatives, the chlorotriazine derivative was used successfully in all studies [137, 138].

Although a number of bead cellulose derivatives have been employed for immobilization of various enzymes, it soon became clear that the traditional methods for cellulose solubilization [3, 4] resulted in rapid enzyme inactivation. *Linko et al.* succeeded in developing such solvent systems for α-cellulose that allowed the regeneration of the biocatalyst of high enzyme loading as beads from water [151—154]. They entrapped inside cellulose beads numerous bacteria and yeasts instead of enzymes; the solvent system consisted of *N*-ethylpyridinium chloride, dimethylformamide, and dimethyl sulfoxide. Catalytic activity recoveries of glucose isomerase, invertase, β-galactosidase, and urease ranged from 22 % to 85 % [152]. Similar techniques were also developed to prepare bead-shaped biocatalysts using cellulose di- and triacetates as carriers [153, 154]. Activity yields of entrapped cells, *e.g.* invertase (*S. cerevisiae*), up to 85 % were obtained [154].

Marconi et al. found that cellulose diacetate having a degree of polymerization around 300 can be advantageously used as a matrix for cell entrapment. Acetone was used as the solvent, and the necessary aggregation of cells was achieved by addition of a polycation (polyethylenimine) [155, 156]. In such a way bacteria and yeasts were entrapped; catalytic activity of their glucose isomerase [155, 156], penicillin acylase, and β-galactosidase [156] was used for industrial applications.

When the cells are to be cultured *in vitro*, it is advantageous to utilize microcarriers. As such, also bead DEAE-, DEAHP-, and TMAHP-celluloses with the exchange capacity of 0.3 to 1.5 mmol g^{-1} and particle size 70 to 300 μm may be used. The authors in [157] employed this method and succeeded in

preparing cells L, Hela, BHK, and others, suitable for diagnostic purposes or for industrial production of various biologically active compounds, *e.g.* human and animal virus vaccines, interferons, hormones, enzymes, *etc.*

Standard methods of determination of the amount of biocatalyst bound to a water-insoluble carrier, such as a material balance in solution, analysis of amino acids, determination according to a modified Lowry method, determination by ninhydrin after hydrolysis, and radiometric determination, are correlable also in the case of bead cellulose [139]. Moreover, a direct spectrophotometric method based on dissolution of protein-cellulose conjugate in cadmium tris(ethylenediamine) hydroxide (trivial name: cadoxene) has been developed. Cadoxene is a solvent for celluloses, bead cellulose included, that is optically transparent in the region of the UV spectrum in which proteins absorb. Authenticity of this method is based on the requirement to exclude: (i) absorbance contribution caused by chemical bonds between protein and cellulose, (ii) change in the UV spectrum of the protein, caused by chemical modification. Reductive alkylation performed by binding the protein on a periodate-oxidized bead cellulose followed by reduction with borohydride fulfils both requirements [139]. Reductive alkylation is a simple, cheap, and rapid procedure that can be applied to many enzymes since it is known that ε-amino groups of lysine residues utilized for binding are not essential for catalytic activity of numerous enzymes. This was proved in experiments with immobilization of chymotrypsin [64, 65, 158], glucose oxidase [57], and trypsin [73, 135, 136] on different aldehyde derivatives, 2,3-dialdehyde inclusive, where highly active preparations were obtained. The storage stability of dry chymotrypsin—bead cellulose conjugates prepared *via* reductive alkylation with 2,3-dialdehyde was observed to be excellent [158]. Drying was performed by lyophilization and in air at 25 °C to 75 °C.

4. Conclusion

In the present review numerous examples of use of bead cellulose in biochemistry and biotechnology have been analyzed in order to verify the assumption that the new form of the regenerated cellulose will surpass the traditional form and will be capable to substitute other current carriers. Bead celluloses have found application mainly in methods and processes utilizing solid-phase techniques, mostly liquid chromatographies and immobilized biosystems (heterogeneous biocatalysis). While the solid-phase methods are well elaborated and represent the dominant part of the material, the shortage of data on solid-phase bioprocesses utilizing bead celluloses is obvious. At the same time, many data on typical properties of bead celluloses (spherical shape, size of

particles and pores as well as their distribution, porosity, hydrophilicity, chemical reactivity, adsorptivity, chemical stability, thermal resistance, mechanical strength, and also availability and price), necessary for bioprocess engineering, are available in the literature.

The aim of this review was, beside actualization of the knowledge about bead cellulose, to draw attention to less-elaborated fields, *e.g.* engineering of solid-phase bioprocesses utilizing bead cellulose. This may then help in evaluation of bead cellulose as a material with manifold applicability by methods corresponding to the up-to-date level of knowledge in biochemistry and biotechnology.

List of abbreviations and symbols

BSA	bovine serum albumin
CB	Cibacron Blue
CBD	Cibacron Blue-dextran
CM	carboxymethyl
DEAE	diethylaminoethyl
DEAHP	diethylaminohydroxypropyl
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
FPC	*O*-[*N*-(4-formylphenyl)thioureidoethyl]cellulose
IMA	immobilized metal affinity
LDH	lactate dehydrogenase
MEC	2-mercaptoethylcellulose
MHPC	3-mercapto-2-hydroxypropylcellulose
MPC	[2-(4-mercaptophenylsulfonyl)ethyl]cellulose
NADH	nicotinamide adenine dinucleotide, reduced
P	phosphate
PBR	packed-bed reactor
PHP	3-phenoxy-2-hydroxypropyl
PHPC	3-phenoxy-2-hydroxypropylcellulose
RB	Remazol Blue
RBD	Remazol Blue-dextran
SE	sulfoethyl
SEC	size-exclusion chromatography
SHP	sulfohydroxypropyl
STR	stirred tank reactor
THPC	3-thiosulfato-2-hydroxypropylcellulose
TMAHP	3-trimethylammonio-2-hydroxypropyl
TSGT	thermal sol-gel transition
B_e	amount of a substance adsorbed from bulk concentration c at equilibrium
$\bar{B}_e$	amount of a substance adsorbed from infinite bulk concentration at infinite time
c_B	concentration of an adsorptive at $B_e = 0.5\bar{B}_e$

c_v	concentration of an adsorptive at $v_0 = 0.5\,\bar{v}_0$
I—L	immobilized ligand
K_i	inhibition constant
K_{I-L}	dissociation constant of the immobilized ligand—enzyme complex
K_m	Michaelis constant
K_{M-L}	dissociation constant of the mobile ligand—enzyme complex
p	solid—liquid partition coefficient
P	liquid—liquid partition coefficient
V, V_0	elution volumes for LDH from dye-cellulose and cellulose columns of equivalent dimensions
V_m	maximum reaction rate
δ	Hildebrand solubility parameter
ε	dielectric constant

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