THIOL DERIVATIVES OF CELLULOSE AS SUPPORTS
FOR THE IMMOBILIZATION OF NON-THIOL ENZYMES

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Dedicated to honour the memory of Professor Dr Ľ. Drobnica.

Thiosulfate derivatives, which can be reduced with mercaptoacetic acid, are suitable intermediates for the preparation of thiol derivatives of polymers. Thiosulfate derivatives of cellulose were prepared *via* chlorodeoxy- or *via* 3-chloro-2-hydroxy-propylcellulose, while mercaptodeoxycellulose prepared *via* chlorodeoxy derivative had more convenient properties for the immobilization of non-thiol enzymes (acetylcholine esterase, butyrylcholine esterase and trypsin). Before immobilization SH groups were introduced into choline esterases by *i*) reduction of the cystine residues, *ii*) reaction with methyl 4-mercaptobutyrimidate, and the isothiocyanate groups were introduced into trypsin on reaction with 3-isothiocyanatopropyl 1-isocyanate. The immobilization of the enzymes treated in this way was carried out under the conditions of the oxidation of thiol groups (*i*), thiol-disulfide exchange reaction (*ii*), or an addition nucleophilic reaction of isothiocyanates with thiols. In contrast to the proteolytic activity of the immobilized trypsin the esterolytic activity of immobilized choline esterases attained satisfactory values.
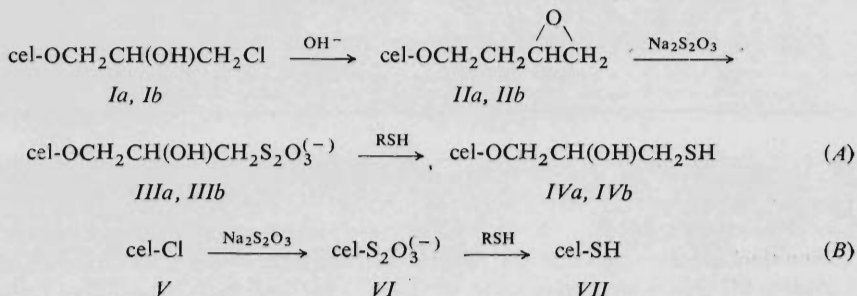
Water-insoluble supports containing thiol groups have wide application possibilities in various fields of solid-phase biochemistry. They are most frequently used in affinity chromatography of thiol enzymes[1] and in the immobilization of enzymes[2]. With the exception of polyacrylamide matrix (Enzacryl Polythiol) the polythiols prepared exclusively on the basis of polysaccharides, mainly agarose[3], are today commercially available.

In the preparation of polysaccharides containing thiol groups the starting reaction is the activation of the polysaccharide with cyanogen bromide[3] or the O-alkylation of agarose with bis-epoxides or α-halohydrins[4,5], but in the case of cellulose these procedures do not give satisfactory results[6]. On the other hand chlorodeoxycellulose seems a suitable starting material for the preparation of thiol derivatives of polysaccharides. This cellulose derivative can be relatively easily prepared under mild reaction conditions and with a relatively high degree of substitution[7] ($D_{Cl} = 0.3$). Chlorine in this chlorodeoxy derivative can be substituted by common nucleophilic substituents, containing, for example, —NH$_2$, —SH or —OH groups. Thus, in the case of chlorodeoxydextran, the substitution with the —S$_2$O$_3^-$ groups has been de-

scribed giving rise to thiosulfatodeoxydextran[8]. Such thiosulfatodeoxydextran is then converted to mercaptodeoxydextran by alkaline cleavage under argon.

As a further starting derivative of cellulose 3-chloro-2-hydroxypropyl may serve, which can be easily prepared under the conditions of acid catalysis[9-11] with sufficiently high degrees of substitution. The advantage of this intermediate consists in the high reactivity of the α-halohydrin grouping.

In the present paper the two mentioned routes for the preparation of thiol derivatives of cellulose (equations (A), (B)) are compared and the suitability of these deriva-

$$\text{cel-OCH}_2\text{CH(OH)CH}_2\text{Cl} \xrightarrow{\text{OH}^-} \text{cel-OCH}_2\text{CH}_2\overset{\displaystyle O}{\overset{\displaystyle /\backslash}{\text{CHCH}}}_2 \xrightarrow{\text{Na}_2\text{S}_2\text{O}_3}$$
$$\textit{Ia, Ib} \qquad\qquad\qquad \textit{IIa, IIb}$$

$$\text{cel-OCH}_2\text{CH(OH)CH}_2\text{S}_2\text{O}_3^{(-)} \xrightarrow{\text{RSH}} \text{cel-OCH}_2\text{CH(OH)CH}_2\text{SH} \qquad (A)$$
$$\textit{IIIa, IIIb} \qquad\qquad\qquad \textit{IVa, IVb}$$

$$\text{cel-Cl} \xrightarrow{\text{Na}_2\text{S}_2\text{O}_3} \text{cel-S}_2\text{O}_3^{(-)} \xrightarrow{\text{RSH}} \text{cel-SH} \qquad (B)$$
$$V \qquad\qquad VI \qquad\qquad VII$$

tives for the immobilization of non-thiol enzymes is discussed. In the majority of thiol enzymes the SH groups represent simultaneously the groups essential for catalysis. Also in view of the fact that mercaptodeoxycellulose does not contain the necessary spacer it would be suitable to introduce it into the enzymes (choline esterase and trypsin) before immobilization. Therefore we used the mentioned non-thiol enzymes for immobilization after introduction of new groups (SH, NCS) which react with the thiol groups of modified cellulose.

EXPERIMENTAL

Chemicals

Cellulose, powdered, was supplied by Whatman, Maidstone; from it cross-linked cellulose[12] was prepared with 1-chloro-2,3-epoxypropane (epichlorohydrin). 3-Isothiocyanatopropyl 1-isocyanate was obtained using the procedure given in the preceding paper[13].

Acetylcholine esterase from bovine erythrocytes (28·9 U/mg) (acetylcholine hydrolase, EC 3.1.1.7) was a product of Koch-Light, Colnbrook; acetylcholine esterase from electric eel (25·4 U/mg) was a product of Sigma, St. Louis. Butyrylcholine esterase (9·3 U/mg) (acylcholine acylhydrolase, EC 3.1.1.8) was obtained from horse plasma[14]. Crystalline trypsin from bovine pancreas (EC 3.4.21.4) 1·0 TU Cas/mg was supplied by Léčiva, Prague; casein according to Hammarsten was from Lachema, Brno. The choline esterase monotest for the determination of the activity of butyrylcholine esterase by means of butyrylthiocholine chloride as substrate[15] was a product of Boehringer Mannheim. [1-^{14}C]Acetylcholine chloride (155 MBq/mmol) was supplied by The Radiochemical Centre, Amersham; [^{125}I]-labelled rat prolactin (277·5 kBq/ml) was prepared by Dr O. Földeš.

3-Chloro-2-Hydroxy-propyl Cellulose (*Ia, Ib*)

Cellulose *Ia*, prepared by acid catalyzed reaction (with $BF_3.[C_2H_5]_2O$) of powdered cellulose with epichlorohydrin in dichloromethane[10,11], contained 2·8% of chlorine. In contrast to this cellulose *Ib* was prepared by acid catalyzed reaction (with $Zn[BF_4]_2$) of crosslinked cellulose ($q_n = 16$) with epichlorohydrin in aqueous medium[9]; and it contained 12·7% chlorine.

2-Hydroxy-3-mercaptopropyl Cellulose (*IVa, IVb*)

A suspension of 1 g of cellulose *Ia* or *Ib* in 10 ml of NaOH of a concentration which would provide for a 1·5-fold excess of OH^- ions with respect to the chlorine present in cellulose *I* (ref.[9]) was stirred at room temperature for 1 h. It was then washed with water and acetone. After drying cellulose *IIa* (with traces of oxirane groups) or *IIb* (1·2 mmol of oxirane groups/g) was suspended in such a volume of 1M sodium thiosulfate in 0·5M phosphate buffer of pH 6·3 as to provide for optimum concentration conditions[4]. Cellulose *IIIa* (traces of S, Cl) or *IIIb* (5·94% S, 4·37% Cl) was washed with water and acetone. After drying it was resuspended in 8 ml of 0·1M--NaHCO$_3$ containing 5 mM EDTA. One ml of a 80% mercaptoacetic acid (10 mmol) was added and the mixture stirred at room temperature for 30 min. The pH value was kept within the 7·7 to 7·9 limits. The product, *IVa* or *IVb*, was washed with water containing 1 mM-EDTA, then pure water and finally with acetone. After drying the content of the thiol groups was determined.

Mercaptodeoxycellulose (*VII*)

Cellulose *V* (6·4% Cl) was prepared on reaction of powdered cellulose with thionyl chloride at room temperature[7]. 1 g of cellulose *V* was suspended in 10 ml of 2·1M sodium thiosulfate and kept at 100°C for 40 h. Cellulose *VI* (5·63% S, 3·17% Cl) was washed with water and acetone and dried. Reduction of mercaptoacetic acid was carried out similarly as in the preparation of cellulose *IV*.

Introduction of Thiol and Isothiocyanate Groups into Enzymes and their Immobilization

Procedure 1. Choline esterase (5 mg) in 0·1M carbonate buffer of pH 8·5 containing dithiothreitol (1 mM) was stirred under nitrogen for 1 h. The mixture was introduced onto a Sephadex G-25 column (2 × 50 cm) equilibrated with 0·1M carbonate buffer of pH 8·5 and the enzyme was eluted with the equilibrium buffer. 5 ml of a solution containing mercaptocholine esterase (5 mg, activity given in Table I) was further stirred with mercaptodeoxycellulose (*VII*, 30 mg) resuspended in 0·1M carbonate buffer (pH 10·6). After addition of 0·1 ml of a solution of potassium ferricyanide prepared from 1·82 g of potassium ferricyanide, 5 g of ammonium chloride, 7·1 ml of ammonium hydroxide and 100 ml of water was kept at 4°C for 20 h. The immobilized choline esterase was washed with a 10% sodium chloride solution in 0·1M carbonate buffer of pH 8·5, 2% solution of bovine serum albumin and 10% of sodium chloride in 0·1M carbonate buffer of pH 8·5 until the reaction of the filtrate for proteins was negative.

Procedure 2. Choline esterase (5 mg) in 0·1M carbonate buffer of pH 8·5 (1 ml) was bubbled through with nitrogen for 30 min. Methyl 4-mercaptobutyrimidate (1 mg) dissolved in 0·1M carbonate buffer (0·2 ml, pH 8·5) was added and the mixture was further bubbled through with nitrogen for another 60 min at room temperature. The unreacted methyl 4-mercaptobutyrimidate was eliminated by gel filtration on Sephadex G-25 (2 × 50 cm) equilibrated with 0·1M sodium hydrogen carbonate containing 1 mM of dithiothreitol, using the equilibration solution for elu-

tion. In the meanwhile mercaptodeoxycellulose (150 mg) was added to a 1·5 mM solution of 2,2'--dipyridyl disulfide (50 ml) in 0·1M carbonate buffer of pH 8·5, similarly as in the case of Thiopropyl-Sepharose[16] 6B. The suspension was stirred at room temperatue for 1 h, the product was washed with 0·1M sodium hydrogen carbonate (50 ml), 5% sodium chloride (20 ml) and 1 mM-EDTA (100 ml). Finally, mercaptobutylamidinecholine esterase (5 mg, activity given in Table I) was dissolved in 0·1M sodium hydrogen carbonate solution (5 ml) and "activated" mercaptodeoxycellulose (30 mg) was added under stirring to the reaction mixture. The suspension was kept at 25°C for 24 h. The immobilized choline esterase was washed with 0·1M sodium hydrogen carbonate (50 ml), 0·1M sodium hydrogen carbonate in 5% sodium chloride, then with 0·1M sodium acetate of pH 5·4 in 5% sodium chloride (50 ml each) and 5% sodium chloride (100 ml).

Procedure 3. 3-Isothiocyanatopropyl 1-isocyanate (2·1 µmol/ml) was added to a mixture containing trypsin (5 mg/ml) and [125I]prolactin (5·2 kBq/ml) in 0·2M borate buffer of pH 9 and the mixture was stirred for 15 min. After acidification to pH 5·5 it was dialyzed against water and then lyophilized. A solution (1 ml) of isothiocyanatoprolytrypsin (5 mg, activity given in Table I) in 0·2M borate buffer of pH 8, containing EDTA (1 mM), was added to the thiol derivative of cellulose *IV* or *VII* (25 mg) and the suspension was shaken for 24 h. For the determination of non-specific sorptions a solution of trypsin (5 mg/ml) in the same buffer and with the addition of [125I]prolactin was used.

Analytical Methods

The amount of the α-halohydrin, chlorodeoxy, thiosulfate and thiol groups in celluloses *I—VII* was determined by microdetermination of chlorine and sulfur, the concentration of the thiol groups in celluloses *IV* and *VII* by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB

TABLE I

Immobilization of choline esterases, trypsin and their derivatives on mercaptodeoxycellulose. Symbols: AChE I acetylcholine esterase from bovine erythrocytes; AChE II acetylcholine esterase from electric eel, BChE butyrylcholine esterase

Enzyme	Amount of bound protein mg/g support	Catalytic activity before after immobilization		Relative activity %	Activity of the preparation U/g of the support
		U/mg	U/mg		
Mercapto-AChE I	18·2	28·2	3·7	13·1	67·3
Mercapto-AChE II	17·4	24·4	3·3	13·5	57·4
Mercapto-BChE	24·5	8·3	1·2	14·5	29·4
Trypsin[a]	20·9[b]	1·0	0·04	4·1	0·8
Mercaptobutylamidine-AChE I	63·5	25·3	3·2	12·6	203·2
Mercaptobutylamidine-AChE II	71·4	23·7	2·7	11·4	192·8
Mercaptobutylamidine-BChE	7·1	1·9	1·9	26·8	153·0
Isothiocyanatopropyltrypsin[a]	79·1[b]	0·3	0·004	1·4	0·3

[a] Catalytic activity is given in Kunitz units (TU^{Cas}/mg). [b] Cellulose *IVb* (1 g) bound 36·3 mg of trypsin or 49·4 mg of isothiocyanatopropyltrypsin.

(ref.[17]). In these determinations a $0.1M$ phosphate buffer of pH 7·6 (5 ml) was used, containing 1 mM DTNB and 1 mM-EDTA. After elimination of polythiol (1—25 mg) absorbance at 412 nm was measured and for the calculation of the concentration of the thiol groups the value $\varepsilon_{412} = = 13\,600M^{-1}\,cm^{-1}$ was used. The content of the oxirane groups in cellulose *II* was determined acidimetrically after reaction with sodium thiosulfate[4]. The presence of SH groups in choline esterase introduced by procedures 1 and 2 was checked by the reaction with DTNB (ref.[18]).

The content of the proteins on the support after immobilization of choline esterases was determined from the differences in *i*) catalytic activity, *ii*) the concentration of the proteins in the solution before immobilization and after it. The activity of acetylcholine esterases was determined using [1-^{14}C]acetylcholine chloride[18,19], the activity of butyrylcholine esterase using butyrylthiocholine chloride and DTNB (ref.[15]). The concentration of the proteins was determined using bovine serum albumin as a standard[20]. The binding properties of the thiol derivatives of cellulose *IVb* and *VII* with respect to trypsin and its isothiocyanatopropyl derivatives were followed radiometrically[13] and in some cases by means of amino acid analysis[21]. The proteolytic activity of trypsin, its isothiocyanatopropyl derivatives as well as the immobilized preparations was determined using casein as substrate[13,21].

RESULTS

The lower content of chlorine (2.8%) in cellulose *Ia*, reached by alkylation in anhydrous medium, follows from the unsufficient swelling of cellulose in this medium. Higher degrees of substitution were achieved when 2-hydroxypropyl derivatives of cross-linked cellulose or dextran were used[10,11]. During the conversion of cellulose *Ib* to *IIb* under formation of oxiranes we obtained a 34.0% conversion of α-halohydrin groups, while in the case of viscose staple fiber[9] a $70-90\%$ conversion was obtained under the same conditions. We were unable to reproduce the experiments with non-cross-linked cellulose[9], because the course of the preparation of derivative *Ib* is not easily controlled. Striking differences were also observed in the reduction of 2-hydroxy-3-propanethiosulfate derivative. If the latter is bound to agarose the conversion[4] is between 55 and 98% while in the reduction of cellulose *IIIb* it is only 15%. Good conversions (about 40%) were performed, however, in the reduction of thiosulfatodeoxycellulose *(VI)*.

A higher content of chlorine (Table II) in cellulose *VII* (5.02%) could be considered as undesirable from the point of view of competitive reactions with proteins. However, under the conditions of the immobilization of enzymes this chlorine is stable. This is not so in the case of the "excess" chlorine (6.60%) in cellulose *IVb*. This "excess" chlorine can be considered as the originator of the competitive reactions in the immobilization of trypsin. This is indicated by the high sorptions of trypsin to cellulose *IVb* $(36.3\ mg/g,\ Table\ I)$ which considerably exceed the current non-specific sorptions of proteins to the derivatives of cellulose[12,13,17]. These nonspecific sorptions of trypsin on cellulose *IVb* are close, by their bonding properties, to those of cellulose *IVb* with respect to isothiocyanatopropyltrypsin $(49.4\ mg/g)$.

For the evaluation of the suitability of mercaptodeoxycellulose for the immobilization of non-thiol enzymes acetylcholine esterase from bovine erythrocytes and electric

eel, as well as butyrylcholine esterase and trypsin were used. Before their binding, however, it was necessary to introduce such groups into those enzymes which are able to react with the SH groups of cellulose. In the case of choline esterases they were the SH groups, in the case of trypsin they were the isothiocyanate groups. In the case of choline esterases this was carried out in two ways: i) by reduction of the disulfide bonds of cystine under formation of mercaptocholine esterases (procedure 1), ii) by introduction of new SH groups under formation of mercaptobutylamidine derivatives of choline esterases (procedure 2). In the first cases the immobilization was carried out by mild oxidation of the SH groups of the enzyme and mercaptodeoxycellulose, while in the second case mercaptodeoxycellulose was "activated" by reaction with 2,2'-dipyridyl disulfide and the immobilization was made via the thiol--disulfide exchange. The isothiocyanate groups were introduced into trypsin on reaction with 3-isothiocyanatopropyl 1-isocyanate (procedure 3) under formation of isothiocyanatopropyltrypsin which was then immobilized under the conditions of nucleophilic addition reaction[17,18].

The presence of the thiol groups in modified choline esterases was tested by the reaction with DTNB. All these preparations contained SH groups. The bonding properties of mercaptodeoxycellulose to mercaptocholine esterases are at the level of non-specific sorptions of trypsin (Table I). In contrast to this the bonding properties of mercaptobutylamidinecholine esterases are substantially higher and they are at the same level as the bonding properties of isothiocyanatopropyltrypsin.

The relative catalytic activities of the immobilized choline esterases are higher than in non-specifically sorbed trypsin and covalently bound isothiocyanatopropyltrypsin (Table I). The highest relative activity was displayed by the immobilized mercaptobutylamidinebutyrylcholine esterase.

TABLE II

Characterization of thiol derivatives of cellulose. In the preparation of cellulose IVa, cellulose Ia as starting material which was prepared in anhydrous medium (dichloromethane) under catalysis with $BF_3.(C_2H_5)_2O$, and in the preparation of cellulose IVb cellulose Ib prepared in aqueous medium under catalysis with $Zn(BF_4)_2$ as starting material

Cellulose	% S	% Cl	mmol S^a	mmol SH^b
			g	g
IVa	traces	traces	—	0·005
IVb	3·59	6·60	1·12	0·14
VII	2·38	5·02	0·74	0·35

^{a} Calculated from microdetermination of sulfur; ^{b} by tritration with DTNB.

DISCUSSION

In addition to the mentioned methods of preparation of thiol derivatives other procedures were tested as well. Attention was also paid to the direct substitution of chlorine in celluloses *Ia*, *Ib* and *V* by the SH groups[8-11], using the reaction with sodium sulfide. However, these experiments did not lead to the desired result.

The bonding properties of mercaptocholine esterases are — in contrast to mercapto-butylamidinecholine esterases — at the level of non-specific sorptions of trypsin (Table I). The distinct participation of chemisorption during the immobilization of mercaptobutylamidinecholine esterases as well as isothiocyanatopropyltrypsin is ascribed to the contribution of the spacer (butyl or propyl. respectively) introduced into the enzyme. When a longer spacer was introduced into the matrix (*p*-amino-benzyl cellulose "activated" with succinic acid anhydride) and the enzyme (acetyl-choline esterase from bovine erythrocytes) was immobilized directly (with 1-cyclo-hexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate), still better re-sults were achieved[12] (475·2 U/g of the support as against 203·2 U/g of the support in this paper) (Table I, mercaptobutylamidineacetylcholine esterase I).

As demonstrated earlier[12,13], unless a sufficiently porous matrix is used, a con-siderable difference may appear between the activity of the enzyme towards a high-or low-molecular substrate. This is also true in this case, *i.e.* immobilized cholineester-ases (esterolytic activities) and trypsin (proteolytic activity) differ distinctly in their relative activities. The results indicate that the enzymes with a low-molecular sub-strate are preferred (Table I). In our preceding paper[14] we also compared the amido-lytic and the proteolytic relative activities of isothiocyanatopropyltrypsin immo-bilized to cross-linked polyethyleneimine. While the amidolytic relative activities (N-α-benzoyl arginine *p*-nitroanilide) ranged from 35 to 60%, the relative proteolytic activities were about 6%.

From the point of view of the correct choice of the physical structure of the support the physico-chemical properties of the enzyme play an important role. In the case of choline esterases it is problematic so far to discuss this effect, due also to the fact that the views on their molecular masses and thus also on their amino acid com-position[23-25] are not unified. A certain preference of butyrylcholine esterase both with respect to its bonding properties and its relative catalytic activities in comparison with acetylcholine esterase (Table I) was observed[25,26] however, even during the immobilization with the cyanogen bromide method on Sepharose SB.

In the preceding paper[13] the advantages of the two-step covalent immobilization of enzymes as a method enabling the study of the effect affecting catalytic activity were mentioned. In this paper too the procedures of two-step covalent immobiliza-tion (procedures 1 — 3) were used, and the favourable results in choline esterases indi-cate the possibility of using them during a fully controlled process. Reserves exist in the direction of the improvement of the physical structure of the matrix, enabling

the achievement of satisfactory results even during the catalytic conversion of high-molecular substrates.

REFERENCES

1. Jakoby W. B., Wilchek M. (Eds): Methods Enzymol. *34, 1974.*
2. Zaborsky O. R.: *Immobilized Enzymes.* CRC, Cleveland 1974.
3. Leaflet of Pharmacia Fine Chemicals, Uppsala: *Affinity Chromatography, Principles and Methods.* Ljungföretagen AB, Orebro 1979.
4. Axén R., Drevin H., Carlsson J.: Acta Chem. Scand. B *29,* 471 (1975).
5. Nishikawa H., Bailon R.: J. Solid-Phase Biochem. *1,* 33 (1976).
6. Zemek J., Kuniak Ľ., Gemeiner P.: Czech. 201 363 (1980).
7. Vigo T. L., Welch C. W.: Text. Res. J. *40,* 109 (1970).
8. Kolova A. F., Komar V. P., Skornyakov I. V., Virnik A. D., Zhbankov R. G., Rogovin T. A.: Cell. Chem. Technol. *12,* 553 (1978).
9. Sharkova E. F., Virnik A. D., Rogovin Z. A.: Vysokomol. Soedin. *8,* 1450 (1966).
10. Ellingboe J., Allmé B., Sjövall J.: Acta Chem. Scand. *24,* 463 (1970).
11. Allmé B., Nyström E.: J. Chromatogr. *59,* 45 (1971).
12. Gemeiner P., Kuniak Ľ., Zemek J.: This Journal *45,* 2847 (1980).
13. Gemeiner P., Halák P., Poláková K.: J. Solid-Phase Biochem. *5,* 197 (1980).
14. Jansz H. S., Cohen J. A.: Biochim. Biophys. Acta *56,* 531 (1962).
15. Ellman G. L., Courtney D. K., Anders V., Featherstone R. M.: Biochem. Pharmacol. *7,* 88 (1961).
16. Brocklehurst K., Carlsson J., Kierstan M. P. J., Crook E. M.: Biochem. J. *133,* 573 (1973); *cf.* Leaflet of Pharmacia Fine Chemicals, Uppsala: Thiopropyl-Sepharose 6B, 1977.
17. Gemeiner P., Augustín J., Drobnica Ľ.: Chem. Zvesti *30,* 254 (1976).
18. Gemeiner P., Drobnica Ľ., Poláková K.: J. Solid-Phase Biochem. *2,* 289 (1977).
19. Siakotos A. B., Filbert M., Hester R.: Biochem. Med. *3,* 1 (1969).
20. Bradford M. M.: Anal. Biochem. *72,* 248 (1975).
21. Gabel D., Axén R.: Methods Enzymol. *44,* 383 (1976).
22. Bergemeyer H. U. (Ed): *Methoden der enzymatischen Analyse,* Bd. I., S. 962. Verlag Chemie, Weinheim 1970.
23. Froede H. C., Wilson I. B. in the book: *The Enzymes* (P. D. Boyer, Ed.), 3rd Edn, Vol. V, p. 87. Academic Press, New York 1971.
24. Silver A.: *The Biology of Cholinesterases.* North-Holland, Amsterdam 1974. •
25. Vigny M., Bon S., Mausoullié J., Letterrier F.: Eur. J. Biochem. *85,* 317 (1978).
26. Axén R., Heilbronn E., Winter A.: Biochim. Biophys. Acta *191,* 478 (1969).

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