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Conjugates of Proteins with Block Co-polymers of Ethylene and Propylene Oxides

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Introduction

Covalent attachment of non-ionic water-soluble polymers to proteins is a progressive way of imparting new useful properties to a protein molecule. Among the polymers so far studied for this purpose, polyvinylpyrrolidone (Von Spect and Brendel, 1977), dextrans (Marshall and Rabinowitz, 1975) and polyethylene glycol (PEG) (Abuchowski *et al.*, 1977a,b; Takoi *et al.*, 1989) are most often employed. PEG, with a history as a plasma expander, being non-immunogenic, amphiphatic and non-toxic, has been applied to various enzymes as a superior agent for modification (Kurganov and Topchieva, 1991). The products of the reaction, polymer–protein conjugates, are characterized by reduced immunogenicity, protracted retention in the circulation and, in some cases, by enhanced stability (Veronese *et al.*, 1989). These features are due to the effect of 'steric stabilization' of the protein globule caused by attachment of polymer chains.

An important property of PEG–protein conjugates is their solubility in organic solvents. That is why they are used as biocatalysts for peptide synthesis (Matsushima, Okada and Inada, 1984), transesterification reactions (Yoshimoto *et al.*, 1984) and the process of ester amination (Takahashi *et al.*, 1985).

The next stage in the construction of polymer–protein conjugates was the modification of the polymer chain by the insertion of foreign fragments (blocks) in the macromolecule by a co-polymerization process. Using this approach PEG was altered by using well-known, commercially available, co-polymers, namely co-polymers of

Abbreviations: BBI, soybean Bauman-Birk proteinase inhibitor; BF, benzaflavin (2', 3', 4', 5'-tetrabenzoyl-5-acetyl-1,5-dihydroriboflavin); BSA, bovine serum albumin; BTEE, *N*-benzoyl-*L*-tyrosine ethyl ester; CHT, α-chymotrypsin; Cyt-C, cytochrome *c*; DNP, 2,4-dinitrophenol; DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; MDA, malonic dialdehyde; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; PK, pyruvate kinase; PMR, proton magnetic resonance; POL, peroxidation of lipids; RPE, REP, pluronics, block co-polymers of ethylene oxide and propylene oxide; TNBS, 2,4,6-trinitrobenzene sulphonic acid; UV, ultraviolet.

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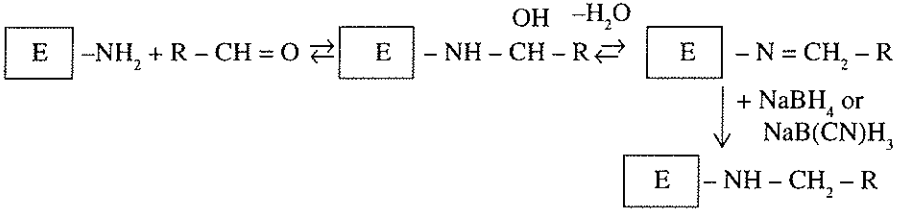
ethylene oxide and propylene oxide – ('pluronics') (Lunsted and Schmolka, 1976). These polymers are non-ionic surfactants in which polyethylene oxide and polypropylene oxide form the hydrophylic and hydrophobic parts of the compounds. In contrast to PEG–protein conjugates, conjugates based on pluronics are characterized by a diversity of polymer–protein structures. The amphiphilic character of protein–pluronic conjugates provides new functions of the modified proteins, such as a capability for translocation across biological membranes, maintenance of effective solubilization, and ultimately the transport of insoluble biologically active compounds, as well as the ability to form molecular assemblies, by interaction with various amphiphilic compounds. In this chapter we report methods of synthesis, purification and characterization of pluronic–protein conjugates, as well as their functional properties.

Synthesis, isolation and characterization of conjugates

SYNTHESIS OF CONJUGATES

Conjugates of proteins with pluronics are synthesized by the interaction of protein ε-amino groups with monoaldehyde derivatives of polyalkylene oxides leading to the formation of labile amidine groups, which are subsequently reduced by sodium borohydride ($NaBH_4$) or sodium cyanoborohydride:



where R stands for polymer chain.

The following proteins have been used for the synthesis of protein–pluronic conjugates: bovine serum albumin (BSA) (Topchieva *et al.*, 1991), bovine α-chymotrypsin (CHT) (Efremova, Mozhaev and Topchieva, 1992), pyruvate kinase (PK) from rabbit skeletal muscles, ricin from castor-oil plant seeds and viscumin from mistletoe (Teplova *et al.*, 1993), classical soybean Bauman–Birk proteinase inhibitor (BBI) (Larionova *et al.*, 1993) and cytochrome *c* (Kirillova *et al.*, 1993).

The reactions of CHT and BBI conjugation were performed in the presence of reversible inhibitors of the active site of the enzyme. Sodium cyanoborohydride is a preferable reducing agent, for its use leads to improved preservation of the protein's native structure, while $NaBH_4$ is able to reduce S–S bonds between polypeptide chains.

The monoaldehyde derivatives of PEG and pluronics of RPE and REP types (R, starting radical; P, polypropylene oxide block; E, polyethylene oxide block) were synthesized by oxidation in the presence of MnO_2 (Boccu, Largajolli and Veronese, 1983). The following monoaldehydes have been used for protein modification:

1. monomethoxy-PEG monoaldehyde (PEG-CHO),
   CH_3–$(OCH_2CH_2–)_{44}$–OCH_2CHO;
2. RPE block co-polymer monoaldehyde (RPE-CHO),
   C_4H_9–$(OCH(CH_3)CH_2)_{14}$–$(OCH_2CH_2)_{20}$–OCH_2CHO;
3. REP block co-polymer monoaldehyde (REP-CHO),
   C_4H_9–$(OCH_2CH_2)_{20}$–$(OCH(CH_3)CH_2)_{14}$–OCH_2CHO.

Evidently the only difference between block co-polymers (2) and (3) is in the position of polyethylene oxide and polypropylene oxide blocks towards the aldehyde group. By using these polymer reagents, two main types of conjugates with hydrophobic blocks at the periphery or inside conjugate structure were synthesized (*Figure 1*).

Molecular models of the conjugates studied in this work are presented in *Figure 1*. The conjugates have a star-shaped structure, where protein occupies the central place with polymer chains growing from it as branches. The length of polymer chain is the same order of, or even bigger than, the diameter of the protein molecule. That is why the protein in the conjugate should be sterically stabilized towards the action of macromolecular substrates, antibodies and proteolytic enzymes. It is evident that this property depends on the number and type of polymer chains distribution on the surface of protein.

Because of the amphiphilic properties of the polymeric reagents, two principal types of conjugate structure, of variable polymer chain distribution, may be obtained at the stage of synthesis:

1. polymer chains may be located in one part of the protein globule surface, forming a domain structure (IIa in *Figure 1*), or
2. polymer chains may be stochastically distributed on its surface (IIb in *Figure 1*).

When the conjugation reaction is carried out in aqueous buffering media, polymer reagents such as pluronics are present in the form of micellar microphases (Osipova, Topchieva and Kasaikin, 1990). It is highly probable that in such systems modification will lead to a significant heterogeneity of the conjugates (IIa-type structures). In contrast, in aqueous–organic mixtures pluronics are present in the form of single molecules (Osipova, 1990). The use of mixed solvents in the synthesis of conjugate would obviously lead to a stochastical type of polymer distribution on the protein globule surface (IIb-type structures in *Figure 1*). It should be noted that the ethanol concentration in the reaction medium was much lower than denaturing concentration, for the proteins under investigation (Khmelnitsky *et al.*, 1991).

It was necessary to find methods sensitive to different types of polymer distribution. One of the approaches to the investigation of the steric arrangement of the polymer chains involves the study of the ability of bound polymers to interact with the bulky cyclic reagent β-cyclodextrin. It has been shown that β-cyclodextrin forms inclusion-type complexes with pluronics (Topchieva *et al.*, 1993a, 1994b). It was natural to suppose that, in the case of the irregular type of distribution (structure IIa in *Figure 1*), polymer chains attached to protein will not be able to interact with a bulky reagent for steric reasons. It has been found that IIb-type conjugates (i.e. those

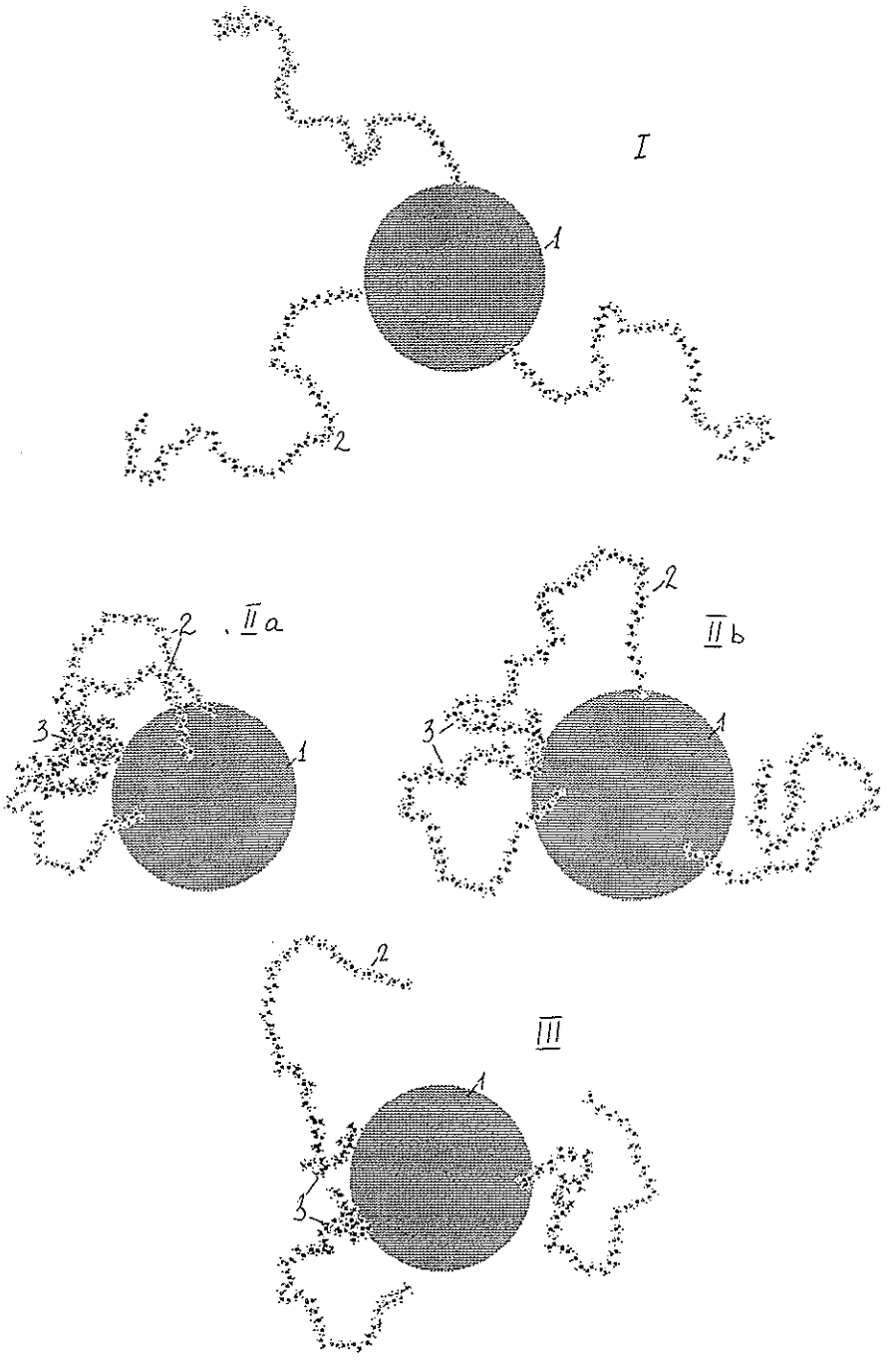


Figure 1. Molecular models of protein conjugates with PEG (I) and pluronics of various types: IIa, conjugates with irregular polymer chain distribution; IIb, with statistical distribution of polymer chains; III, with hydrophilic blocks at the periphery of the structure. 1, Protein globule; 2, polyethylene oxide block; 3, polypropylene oxide block. The radius of central globule was chosen to correspond to the average radius of a CHT molecule, 25 Å (Krigbaum and Godwin, 1968).

with a statistical distribution of polymer chains) as well as free pluronics react with β-cyclodextrin, forming water-insoluble crystalline compounds. In contrast, IIa-type conjugates (i.e. those presumed to have domain structures) cannot form complexes with β-cyclodextrin. The differences in the biological behaviour of these two structures will be reflected in the next part of this article.

PURIFICATION OF CONJUGATES

Purification of pluronic conjugates from the excess of polymer reagent offers more difficulties than purification procedures in the case of PEG–protein conjugates. This is accounted for by the amphiphilic properties of pluronics, which lead to association between unreacted reagent and polymer chains in the conjugate. This explains the low efficiency of commonly used methods of purification, such as dialysis and ultrafiltration in aqueous media. Effective purification can be achieved by using aqueous organic media, which leads to the dissociation of these complexes. Taking into account all these considerations, two methods of purification have been proposed: precipitation of conjugate by acetone and gel-permeation chromatography using 20% (v/v) ethanol as eluent. Thin-layer chromatography was used to demonstrate the absence of polymer contamination in the conjugate.

CHARACTERIZATION OF CONJUGATES

Tables 1 and *2* present characteristics of conjugates of proteins with PEG and pluronics. The protein content in the conjugates was determined either spectroscopically at 280 nm, or using a biuret reaction (Kochetov, 1980) (native proteins were used as standards). It was shown that neither PEG nor pluronics interfere with the protein assay using a spectrophotometric method (A_{280} or the biuret reaction (Efremova, Mozhaev and Topchieva, 1992). The results of both methods were in good agreement (see *Tables 1* and *2*).

Table 1. Characteristics of conjugates of CHT with PEG and pluronics

N	Polymer	Type of conjugate	Degree of modification	Wt % of CHT	Number of unmodified surface NH_2-groups (m)	Designation of conjugate
1	PEG	I[a]	3	81.0 (80.5)[b]	12	$(PEG)_3$–CHT
2	PEG	I	9	58.1 (58.8)	5	$(PEG)_9$–CHT
3	RPE	IIa	2	86.2 (86.1)	13	$(RPE)_2$–CHT-a
4	RPE	IIa	3	82.1 (79.8)	12	$(RPE)_3$–CHT-a
5	RPE	IIa	6	67.5 (66.7)	8	$(RPE)_6$–CHT-a
6	RPE	IIa	7	64.0 (64.1)	–	$(RPE)_7$ CHT-a
7	RPE	IIa	10	55.6	4	$(RPE)_{10}$–CHT-a
8	RPE	IIb	3	80.6	11	$(RPE)_3$–CHT-b
9	RPE	IIb	5	71.5 (71.0)	9	$(RPE)_5$–CHT-b
10	RPE	IIb	6	67.4 (67.5)	–	$(RPE)_6$–CHT-b
11	RPE	IIb	11	45.5 (46.0)	–	$(RPE)(_{11}$–CHT-b
12	REP	III	3	80.6 (80.7)	12	$(REP)_3$–CHT
13	REP	III	7	64.2 (64.0)	–	$(REP)_7$–CHT

[a] I, IIa, IIb and III are the conjugates having structures presented in *Figure 1*.
[b] The amount of CHT present in each conjugate was determined by UV-spectroscopy and by the biuret procedure (values in parentheses).

Table 2. Characteristics of conjugates of BSA with PEG and pluronics

N	Polymer	Type of conjugate	Degree of modification	Content of BSA, wt%			Designation of conjugate
				Biuret method	280 nm	^{13}C NMR	
1	PEG	I	5	87	87	–	$(PEG)_5$–BSA
2	PEG	I	14	70	71	–	$(PEG)_{14}$–BSA
3	RPE	IIa	5	87	87	–	$(RPE)_5$–BSA-a
4	RPE	IIa	8	81	80	78	$(RPE)_8$–BSA-a
5	RPE	IIb	15	70	69	70	$(RPE)_{15}$–BSA-b
6	REP	III	6	85	84	–	$(REP)_6$–BSA
7	REP	III	11	75	75	–	$(REP)_{11}$–BSA

^{13}C NMR spectroscopy

^{13}C NMR spectroscopy presents a direct method for determination of both protein and polymer content in conjugates, because there exist regions where signals from polymer and protein do not interfere. Two conjugates of BSA with RPE co-polymer (preparations 4 and 5 in *Table 2*) were analysed by this method; samples of BSA and RPE mixtures served as standards. The results turned out to be in good agreement with data obtained by UV-spectroscopy and a biuret method (see *Table 2*). One may see that each of the methods used is suitable for the analysis of conjugate composition.

The degree of modification, n, which denotes the average number of polymer chains coupled with a protein molecule, was calculated from data on conjugate composition using the following formula:

$$n = \frac{\text{weight \% of polymer/MW of polymer}}{100 - \text{weight \% of polymer/MW of protein}} \qquad \text{Eq. (1)}$$

Titration of surface amino groups in conjugates

Titration of NH_2-groups with 2,4,6-trinitrobenzene sulphonic acid (TNBS) is commonly used for the determination of the degree of modification of protein in conjugates. Beforehand one should determine the number of surface primary amino groups in the parent protein. Then the degree of modification, n, is given by the formula:

$$n = \alpha - m, \qquad \text{Eq. (2)}$$

where α stands for the total number of surface NH_2-groups in the protein, and m for the number of unmodified NH_2-groups in the conjugate. In our case, however, not only unmodified NH_2-groups but also the resulting secondary amino groups of conjugate could interact with TNBS (Fields, 1971). Hence, the number of amino groups in conjugates as determined by this reaction remained the same as in the parent protein. Thus, it was necessary to use a reagent for primary amino groups only. We have chosen *o*-phthalic aldehyde and titrated NH_2-groups by the method of Roth (1971). This method was applied to a number of conjugates on the basis of CHT (column 4 in *Table 1*). The degree of modification calculated by equation (2),

assuming $\alpha = 15$ for CHT (Birktoft and Blow, 1972), was in good agreement with the results obtained on the basis of protein content determination by UV-spectroscopy and the biuret method, and equation (1).

Enzymatic properties of conjugates

ENZYMATIC PROPERTIES OF CHT–PLURONIC CONJUGATES

Kinetic characteristics of the reactions of enzymatic hydrolysis of conjugates on the basis of CHT are presented in *Table 3*. These data show that:

1. Conjugates retain high enzymatic activity towards both substrates investigated, i.e. a low molecular weight substrate BTEE and casein. This presents a strong argument in favour of the retention of the protein native structure in conjugates.
2. There are insignificant changes in the values of K_m and catalytic constant for hydrolysis of low molecular weight substrate BTEE by conjugates, compared to the parent enzyme. Analogous results were obtained for hydrolysis of high molecular weight substrate, such as casein. The preservation of enzymatic properties may be explained by the fact that the modification of CHT is carried out in the presence of a reversible inhibitor (*N*-acetyl-L-tyrosine or *N*-benzoyl-L-tyrosine), whose binding to enzyme sterically screens the active site.
3. A noticeable decrease in enzymatic activity was observed for conjugates with high degrees of modification (11 and more, with maximal = 15). It is seen that the decrease in activity is due to the destruction of active sites in conjugates, but not to the lowering of the value of k_{cat} (*Table 3*, $N = 5$).

Table 3. Enzymatic properties of CHT conjugates with PEG and pluronics

N	Enzyme, conjugate	Enzymatic hydrolysis of casein	Enzymatic hydrolysis of BTEE			Content of active sites (%)
		Activity (%)	Activity (%)	k_{cat}, s^{-1}	K_m, $M \times 10^5$	
1	CHT	100	100	31.2±0.5	6.27±0.94	80
2	$(RPE)_5$–CHT-a	102	97	42.4±2.8	13.0±2.9	61
3.	$(RPE)_3$–CHT-b	103	115	–	–	80
4.	$(RPE)_6$–CHT-b	96	98	32.5±1.2	6.18 0.82	67
5.	$(RPE)_{11}$–CHT-b	50	57	37.0±0.4	21.7 3.5	40
6.	$(PEG)_6$–CHT	97	98	40.4±0.8	9.3 1.5	80
7.	$(REP)_3$–CHT	98	100	–	–	80

THERMAL STABILITY OF CONJUGATES

It is well known that chemical modification of proteins is an effective method to change their thermal stability. In the case of PEG–protein conjugates it has been shown that attachment of polymer chains either increases or does not change thermal stability of proteins (Abuchowski *et al.*, 1977b; Veronese *et al.*, 1989). The peculiarity of conjugates with pluronics consists of the possibility of regulating thermal stability of modified protein by variation of a number of parameters, such as the

degree of modification, the number and type of block arrangement, hydrophilic–lipophilic balance, and the type of polymer distribution on the surface of protein globule. For these reasons we studied the thermal inactivation of all types of conjugates of CHT. The thermal stabilities of PEG–CHT conjugate and of native CHT were studied for comparison. Kinetic curves of thermoinactivation of CHT and its conjugates with polymers are presented in *Figure 2*. The thermostability of conjugates of CHT decreases in the order: $(PEG)_9$–CHT > $(RPE)_5$–CHT-b > $(RPE)_6$–CHT-a > $(RPE)_3$–CHT-a > $(REP)_3$–CHT-a > CHT. It should be noted that all CHT–pluronic conjugates with the exception of $(RPE)_5$–CHT-b are of the IIa type.

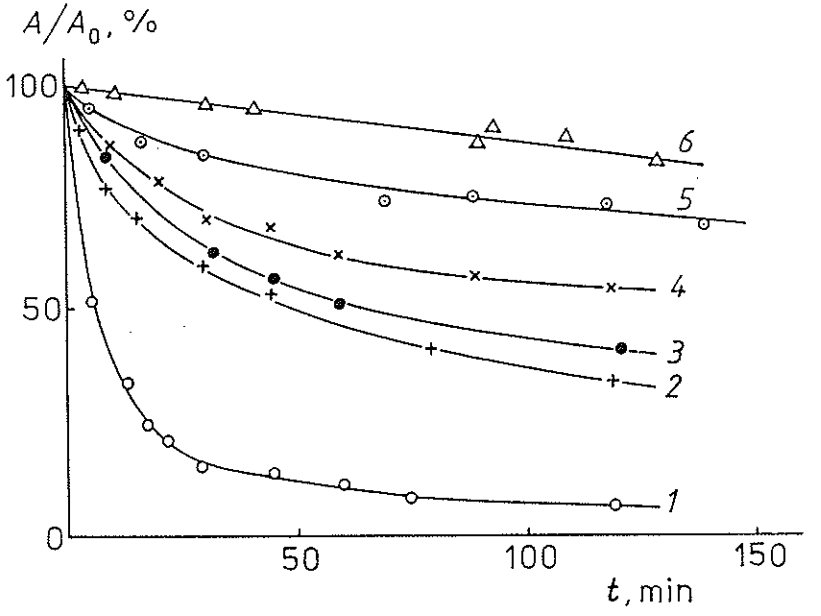


Figure 2. Kinetics of thermal inactivation of CHT and its conjugates with polyalkylene oxides: 1, CHT; 2, $(REP)_3$–CHT; 3, $(RPE)_3$–CHT-a; 4, $(RPE)_6$–CHT-a; 5, $(RPE)_5$–CHT-b; 6, $(PEG)_9$–CHT. Conditions of thermal inactivation: 0.2 M Tris–HCl, pH 8.05; 45°C. Concentration of protein in probe is 0.1 μM. The solution of CHT or its conjugate was incubated in a temperature-controlled cell at 45.0±0.05°C. After selected time intervals, the residual activities of aliquots were assayed by determining the initial rate of hydrolysis of BTEE. The enzymatic activity is expressed as a percentage of the initial activity of corresponding preparations.

It is seen that the rate of thermal inactivation of the enzyme decreases significantly as a result of the attachement of three polymer chains. The stabilizing effect becomes even more pronounced with an increase in the degree of modification of CHT. The maximal stabilizing effect is achieved for CHT–PEG conjugates with nine polymer chains. It is remarkable that the conjugate $(RPE)_5$–CHT-b, with a statistical mode of distribution, possesses higher thermostability than conjugate $(RPE)_6$–CHT-a, with a domain type of distribution. At the same time, the difference in the placement of hydrophobic blocks in conjugates with an equal number of co-polymer chains leads to change in their thermostability: a conjugate of type IIa is more stable than a conjugate of reversed type (III).

One of the unique properties of protein conjugates with pluronics is their ability to interact with both synthetic (surfactants) and natural (lipids) amphiphilic compounds

(Topchieva *et al.*, 1989; Efremova, Mozhaev and Topchieva, 1992). The functional aspect of these interactions was demonstrated by study of thermoinactivation. Thermal stability of conjugates may be enhanced by the addition of free pluronics into the incubation system. This phenomena is illustrated in *Figure 3*, which demonstrates thermal inactivation of conjugate $(RPE)_4$–CHT-a (type IIa) in the presence of added free pluronic in molar excess over the conjugate, ranging from 50 to 750. The kinetic curves in *Figure 3* show that addition of free pluronics to the solution up to a 500-fold molar excess increases the stability of enzyme in the conjugate. Further increase in the pluronic concentration does not significantly influence the thermal inactivation kinetics, perhaps due to saturation of the conjugate by associated co-polymer.

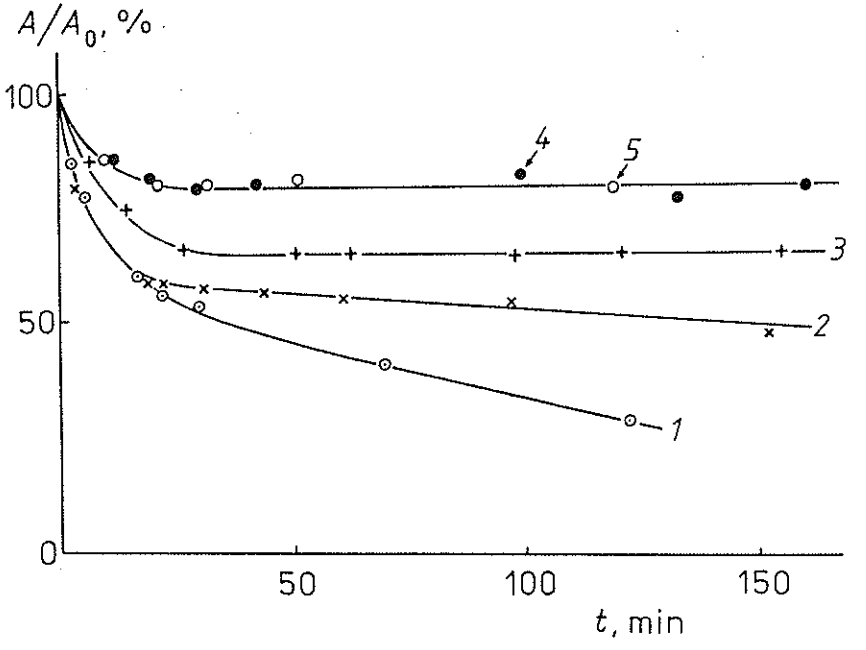


Figure 3. Kinetics of thermal inactivation of conjugate $(RPE)_4$–CHT-a alone (1) and in the presence of added free RPE co-polymer in molar excess (with respect to CHT) of 50 (2), 100 (3), 500 (4) and 750 (5). Thermal inactivation conditions were as in *Figure 2*.

One can assume that an increase in the stability of CHT–pluronic conjugates compared to the parent protein is due to the ability of polymer chains to screen hydrophobic domains at the surface of protein (Mozhaev and Martinek, 1984; Mozhaev, Martinek and Berezin, 1988). An additional increase in the stability of CHT–pluronic conjugates in the presence of free pluronics can be accounted for by two explanations. The first is complex formation between pluronics and the protein of the conjugate during incubation with heating. For steric reasons this type of interaction is mostly improbable. The second is interaction between polymer chains of conjugate and added pluronics. Probably the interaction between free and bound pluronics, leading to formation of a polymer coat, are mainly due to hydrogen bonds bridging polyether chains, involving water molecules. As a result, the contacts stabilizing the structure of the protein globule are not destroyed and encagement of the protein is realized.

Addition of egg-yolk lecithin and β-cyclodextrin produces an opposite effect on the stability of protein in conjugates (*Figure 4*). All additives themselves do not affect the behaviour of the native enzyme. Probably in the latter case these additives are stronger competitors for hydrophobic bond formation with pluronics than the corresponding domains of the protein globule. As a result, the steric arrangement of polymer chains is changed in such a way that the hydrophobic domains of protein become exposed again and destabilization of the enzyme structure as compared with conjugate takes place.

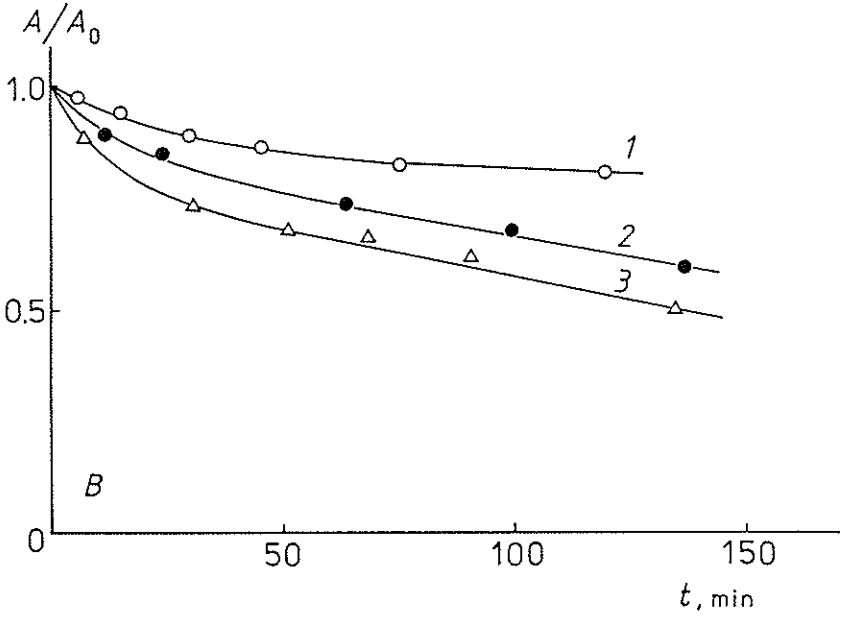


Figure 4. Kinetics of thermal inactivation of a CHT–proxanol conjugate obtained by method B $(RPE)_3$–CHT-b (1); the same conjugate in the presence of β-cyclodextrin (2) and egg-yolk lecitin (3). Molar ratio of conjugate:additive, 1:50. Thermal inactivation conditions were as in *Figure 2*.

The data presented above give examples of changes in thermal stability of conjugates due to their interaction with amphiphilic molecules. As a matter of fact, study of thermal stability may be successfully used for detection of interactions between conjugates and different partners. On the other hand, non-covalent supramolecular structures based on conjugates and additives initiate new approaches to the elucidation of the mechanisms of transport and the functioning of proteins as drugs.

Physico-chemical properties of conjugates

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Taking into consideration the results on thermal stability of conjugates, gained through investigation of their enzymatic properties (see previous section), it was interesting to obtain proof of the increase in conjugate thermal stability by the direct method of DSC.

Thus, thermally induced transitions in proteins modified with pluronics and PEG have been studied by the method of DSC. *Figure 5* shows DSC data for CHT and its conjugates with polymers in aqueous buffering media. The heat sorption peak for CHT is slightly asymmetric and possesses one maximum with T_{max}=49.6°C, which is in good agreement with the results presented by Tischenko, Tiktopulo and Privalov (1974).

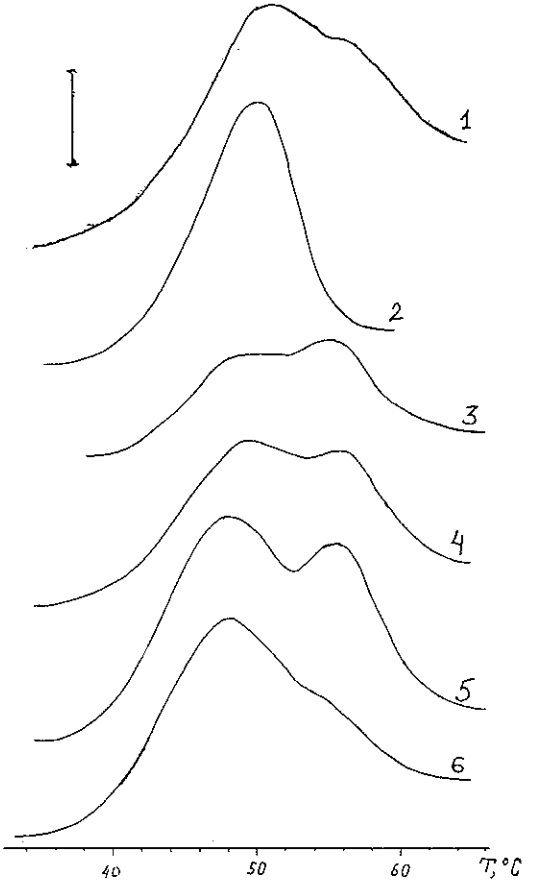


Figure 5. Temperature dependences of excess heat capacity (Δc_p) for CHT (2), and its conjugates $(PEG)_3$–CHT (3), $(RPE)_3$–CHT-b (4), $(RPE)_3$–CHT-a (5), $(REP)_3$–CHT (6), $(RPE)_7$–CHT-b (1). The concentration was 1.0 mg ml^{-1}; buffer, 20 mM phosphate, pH 7.0. The vertical bar corresponds to 1.0 kJ $K^{-1}kg^{-1}$.

Considerable changes in the form of heat sorption peaks are observed for CHT conjugates with polymers (curves 3–6 on *Figure 5*). Besides the first maximum, between the temperature limits of 47.6 and 49.6°C, there arises a second maximum on heat sorption curves of conjugates $(RPE)_3$–CHT-a, $(RPE)_3$–CHT-b and $(PEG)_3$–CHT, which is located at 54.5–55.5°C. On the heat sorption curve of $(REP)_3$–CHT there arises a shoulder in the same temperature region. A trivial explanation of this phenomenon would be that the existence of two maxima on heat sorption curves is due to the heterogeneity of conjugates, namely that the conjugate fraction with a low

degree of modification gives rise to the first maximum and the fraction with higher degree of modification to the second. This suggestion is not confirmed by DSC data on conjugates with a high degree of modification (curve 1) which revealed only one maximum (T_{max} = 51.2) and a shoulder in the region 54.5–55.5°C.

One may suppose that in the course of protein globule denaturation hydrophobic parts of the protein molecule become accessible to interaction with hydrophobic blocks of co-polymer, coupled to protein. The emergence of the second maximum on heat sorption curves of conjugates may be due to the melting of such polymer–protein complexes.

In order to investigate this suggestion we conducted some additional experiments, using mixtures of CHT with PEG and pluronics. It was shown that:

1. Heating of a solution of CHT, containing pluronic (1:20), from 20°C to 60°C (1 k min^{-1}), does not lead to aggregation and precipitation of protein.
2. Analogous heating of solution, containing pure CHT or a mixture of CHT and PEG (1:20), leads to aggregation of protein followed by precipitation.
3. Protein-containing product, gained from a CHT–pluronic mixture after heating and purification from excess pluronic, contained only 50% of protein. The rest, according to PMR data, is a bound pluronic.
4. The heat sorption curve of a solution containing a mixture of CHT and pluronics has only one peak with T_{max} = 54.5°C, which coincides with the position of the second maximum on DSC curves of conjugates.

These facts demonstrate the ability of pluronics to interact with CHT in aqueous solutions under heating.

We should focus our attention on the fact that on the curve of heat sorption of $(PEG)_5$–CHT conjugate two peaks of melting were observed. An explanation of this effect relies on the concept of polyalkylene oxide solubility in water. According to the theory of Kjellander and Florin (1981), solubility of PEG is due to its ability to fit into the crystal structure of water. The coupling of hydrophobic groups or complex fragments containing both hydrophobic and polar groups to the end groups of PEG leads to a sufficient change of the conformational state of polymer chains in conjugates. One may suppose that fragments of PEG chains, located in the vicinity of the protein globule, lose their ability to fit into the structure of water and hence become hydrophobic.

Thus, DSC study of CHT conjugates with PEG and pluronics showed that:

1. Conjugates are more stable than parent protein.
2. Thermal stability of conjugates on the basis of pluronics is comparable with thermal stability of PEG–protein conjugates.
3. The emergence of the second maximum on DSC curves of conjugates is very likely due to the formation of intramolecular polymer–protein complexes.

SEDIMENTATION OF CONJUGATES

Hydrodynamic properties of conjugates were investigated using sedimentation analysis. Sedimentation coefficients obtained for the conjugates and for the initial proteins are summarized in *Table 4*. One may see that alteration of the sedimentation

coefficients of proteins caused by the modification is negligible. Thus sedimentation data show that solutions of conjugates do not contain products of intermolecular interaction under the investigated conditions.

Table 4. Sedimentation coefficients for proteins and their conjugates with PEG and pluronics

N	Protein conjugate	Concentration (mg ml^{-1})	S_{20}, S
1	BSA	1	4.3±0.2
2	$(PEG)_{15}$–BSA	1	4.2±0.2
3	$(RPE)_8$–BSA-a	1	4.5±0.3
		2	4.0±0.2
		4	4.0±0.2
		6	4.2±0.2
		10	4.6±0.2
4	$(REP)_6$–BSA	1	4.1±0.2
5	CHT	1	2.0±0.15
		4	2.02±0.15
		8	2.2±0.17
		10	2.45±0.12
6	$(RPE)_3$–CHT-a	1	2.3±0.1
		3	2.42±0.15
		5	2.51±0.16
		6	2.61±0.21
		7	2.60±0.18
7	(RPE_3)-CHT-b	1	1.91±0.13
		3	1.99±0.15
		5	2.11±0.08
		10	2.25±0.13

T=20°C, 48 000 min^{-1}, 0.02 M phosphate buffer, pH 7.0.

Even in the absence of intermolecular interactions, the attachment of polymer chains might have influenced the value of sedimentation coefficients. The fact that no considerable alterations of sedimentation coefficients of proteins after modification was observed should mean that the effect of growth of molecular mass is compensated for by the flotation action of polymer chains. It should be noted that the polyalkylene oxides used in this work are characterized by a flotation factor value near 1, so they do not sediment in the ultracentrifuge, but have a tendency to flotation (Bailey and Koleske, 1966).

HYDROPHOBIC CHROMATOGRAPHY

Reverse-phase hydrophobic high-performance liquid chromatography (HPLC) was used to evaluate the relative hydrophobicity of conjugates. *Figure 6* shows the results of hydrophobic HPLC for native BBI and $(RPE)_5$–BBI conjugate (Larionova, 1993). The retention volume of the conjugate is less than that of the native protein, thus indicating its lesser affinity to the hydrophobic sorbent. The same tendency was observed for CHT and its conjugates with pluronic. At first glance, this result would seem surprising since the polymer chains entering the conjugate contain hydrophobic blocks. However, this may be accounted for by the fact that the surface of the protein globules of the conjugate is covered mainly with the hydrophilic blocks of the polymer, due to the intramolecular interactions of the hydrophobic blocks of pluronic and the hydrophobic regions of the inhibitor molecule.

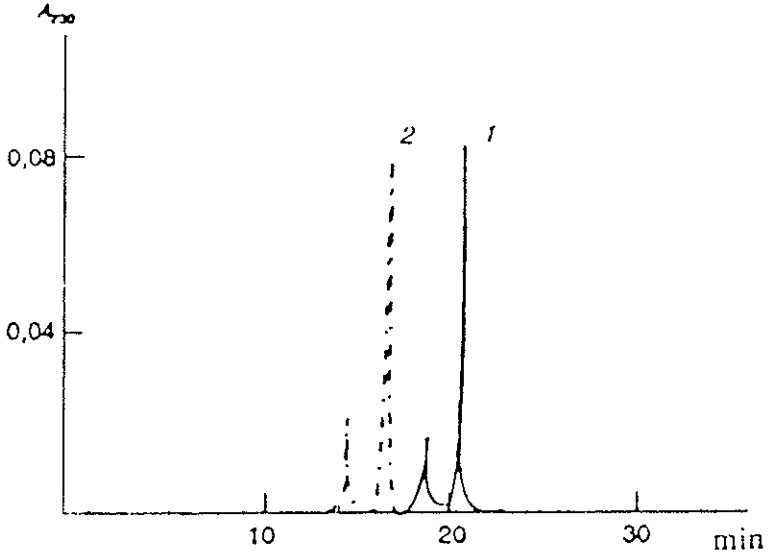


Figure 6. High-performance liquid chromatography on a Zorbax C-8 column. The eluent was 70% acetonitrile. (1) Native BBI; (2) $(RPE)_5$–BBI-a conjugate.

Conformational models of conjugates on the basis of proteins and pluronics

The whole complex of data obtained by applying hydrophobic chromatography, sedimentation and DSC, indicate the presence of general regularities in structure formation in conjugates synthesized by coupling of pluronics to proteins. These properties have been demonstrated most clearly in the case of conjugates with hydrophobic blocks at the periphery of the conjugate structure (structure II). For these conjugates excess of hydrophobicity may be compensated either by formation of intermolecular associates, or intramolecular structures. As association products of these conjugates in diluted aqueous buffers were not detected, it is natural to propose the formation of structures stabilized by intramolecular interactions. The increase in hydrophilicity of conjugates as compared to the native protein, shown by the method of hydrophobic chromatography, leads to suggestion of the formation of 'intramolecular micelles' in which polypropylene oxide blocks 'adhere' to the surface of protein.

It may be suggested that formation of compact structures of similar type presents prerequisites for complexation between polymer chains of pluronics and protein at elevated temperatures.

It should be particularly emphasized that these structures possess a certain conformational lability. This property is revealed in the presence of hydrophobic or amphiphilic 'partners', such as lipids (Topchieva, 1989), pluronics (Efremova, Mozhaev and Topchieva, 1992) and cyclodextrins (Polyakov, 1993; Topchieva, 1993a; Topchieva, 1994b), and leads to the formation ofmixed association products or complexes. Presumably, these processes may proceed by unwrapping of the intramolecular micelle of the conjugate and the interaction with hydrophobic parts of the 'partner'.

Membranotropic properties of conjugates

The ability of pluronics to cross the biological membrane (Topchieva, 1989) leads one to expect that this property could be transmitted to their conjugates with hydrophilic proteins. Translocation of conjugates through the biological membrane was investigated by using the following approach: the influence of these compounds upon the rate of oxygen consumption by liver mitochondria and thymus lymphocytes was measured. If the hydrophobic substances cross the lymphocyte plasma membrane and reach the NADH dehydrogenase site of the mitochrondrial respiratory chain, capable of binding hydrophobic substances, one should observe inhibition of oxygen consumption.

We investigated conjugates of pluronics with BSA, CHT and cytochrome *c*, possessing a variety of molecular masses, with the idea of establishing a structure–function (membranotropic properties) correlation. Detailed investigations were performed using conjugates on the basis of CHT (Topchieva, 1994b). To elucidate the influence of the position of the hydrophilic and hydrophobic blocks of pluronics in protein conjugates on their ability to suppress the respiration of lymphocytes, the effects of conjugates II and III (see *Figure 1*) were compared. As *Figure 7* demonstrates, only conjugate II, with the hydrophobic block located at the periphery of the molecule, is able to suppress the respiration of lymphocytes. At the same time, PEG–protein conjugates, conjugates with hydrophilic blocks at the periphery of the molecule (type III) and CHT itself had no influence on lymphocyte respiration. The efficiency of conjugate II can be compared with the influence of free pluronic.

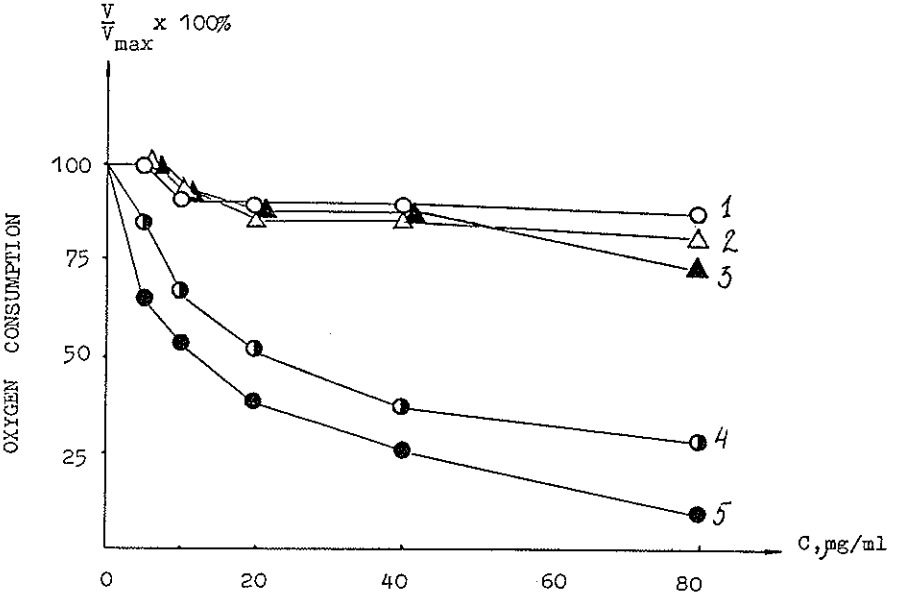


Figure 7. Influence of protein conjugates with different polymers on the rate of oxygen consumption by T lymphocytes: 1, native CHT; 2, $(PEG)_9$–CHT; 3, $(REP)_7$–CHT; 4, $(RPE)_8$–CHT-a; 5, $(RPE)_{10}$–CHT-a. Lymphocytes (10^8 cells ml^{-1}) were incubated in 8 mM MOPS buffer containing 145 mM NaCl, 3.6 mM KCl, 2 mM KH_2PO_4, and 10 mM pyruvate (pH 7.4, 37°C). Conjugates were added after oligomycin (0.2 μg ml^{-1}) and 60 μM DNP. In the control sample incubation medium was added instead of solutions of conjugates.

Table 5. The efficiency of suppression of lymphocyte and mitochondrial uncoupled respiration by block co-polymer RPE and its conjugates with α-chymotrypsin $(RPE)_6$–CHT-a), cytochrome *c* ($(RPE)_8$–Cyt-C-a) and BSA ($(RPE)_7$–BSA-a)

		Concentration of RPE and its conjugates (μM)			
		RPE	$(RPE)_6$–CHT-a	$(RPE)_8$–Cyt-C-a	$(RPE)_7$–BSA-a
a	Lymphocytes	4.9±1.2	0.66±0.1	1.1±0.1	0.75±0.1
	Mitochondria	4.7±1.2	0.48±0.06	–	–
		Concentration of RPE and its conjugates (mg ml^{-1})			
		RPE	$(RPE)_6$–CHT-a	$(RPE)_8$–Cyt-C-a	$(RPE)_7$–BSA-a
b	Lymphocytes	9.8±2.4	30.6±6.2	33.0±3.4	61.0±6.2
	Mitochondria	9.3±2.4	21.6±4.7	–	–
		Concentration of free RPE and of RPE in the conjugates (μM)			
		RPE	$(RPE)_6$–CHT-a	$(RPE)_8$–Cyt-C-a	$(RPE)_7$–BSA-a
c	Lymphocytes	4.9±1.2	6.6±1.9	3.3±0.3	5.3±0.5
	Mitochondria	4.7±1.2	4.8±1.5	–	–

Oxygen consumption was recorded in the presence of 50–60 μM DNP + 4 mM glutamate + 1 mM malate (mitochondria). Results are expressed as means±SEM for five or six experiments. The concentrations of pluronic in μM (a) and mg ml^{-1} (b) are shown which inhibit the oxygen consumption by 50%. The concentrations of free polymer and of polymer bound in a conjugate are compared for their efficiencies (c).

Concentrations of the compounds inhibiting respiration by 50% were 9.8±2.4 μg ml^{-1} (pluronic) and 30.0±6.2 μg ml^{-1} (conjugate; *Table 5*).

Conjugates produced similar effects on the respiration of isolated mitochondria. Both substances, pluronic and conjugate II, suppressed the rate of uncoupled mitochondrial respiration and lymphocyte respiration at the same concentrations. Average concentration of the same compounds reducing the respiration of mitochondria to 50% were 9.3±2.4 μg ml^{-1} and 21.6±4.7 μg ml^{-1}, respectively (*Table 5*).

The properties of conjugates with other proteins were similar (*Table 5*). Proteins *per se* had no influence on lymphocyte and mitochondrial respiration.

The dependence of the inhibitory effect of the conjugate on the number of polymer chains coupled to the protein and required for translocation is illustrated by the inhibitory effects of a series of CHT conjugates with variable content of polymer chains. As seen from *Figure 8*, the suppression of cell respiration becomes detectable by the action of conjugates with not less than three polymer chains per protein molecule.

Data on the inhibitory effect of BSA conjugates differing in the type of polymer-chain distribution on the protein surface on the rate of oxygen consumption by lymphocytes are presented in *Figure 9*. It is seen that more pronounced inhibition of respiration occurs with the conjugate having the irregular distribution of polymer chains. The same effect was observed with conjugates of CHT with similar types of structure (data not shown).

Concerning the mechanism of conjugate action on cellular membrane, some alternative explanations may be considered:

1. the conjugates interact with receptors on the external surface of the plasma

membrane, which transfer a signal into the cell;
2. the conjugates damage cells;
3. polymer chains split from proteins during lymphocyte incubation with the conjugates; or
4. the conjugates pass through the plasma membrane and reach the mitochondrial membrane.

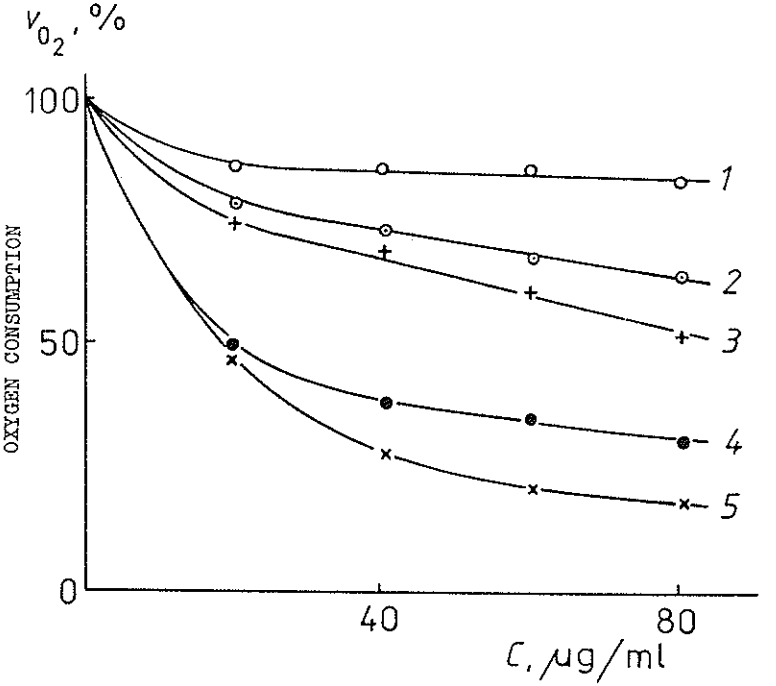


Figure 8. Influence of CHT conjugates containing different numbers of pluronic chains on the rate of oxygen consumption by T lymphocytes: 1, $(RPE)_2$–CHT-a; 2, $(RPE)_3$–CHT-a; 3, $(RPE)_6$–CHT-a; 4, $(RPE)_8$–CHT-a; 5, $(RPE)_{10}$–CHT-a. Experimental conditions were as in *Figure 7*.

The first assumption gives no explanation as to why the conjugates at virtually the same concentrations inhibit the uncoupled respiration both in whole cells and isolated mitochondria. In addition, the mitogenic lectin, concanavalin A (which is known to stimulate lymphocyte respiration by interacting with the receptors on the external surface of plasma membrane) increases their respiration rate by 20% only and has no effect on the respiration of isolated mitochondria (Lakin-Thomas and Brand, 1988). We have obtained a threefold stimulation of lymphocyte respiration by pluronic conjugates with CHT in the presence of oligomycin (Kirillova, 1993).

The experiments showing that the suppression of respiration of lymphocytes by pluronics and protein–pluronic conjugates is completely reversed by subsequent washing of the cells provide evidence that inhibitory effects were not associated with plasma-membrane injury.

The splitting of block-co-polymeric chains from the protein can hardly account for the membranotropic activity of the conjugates. The -NH-CH_2 bond, formed during conjugate formation, is resistant to hydrolysis and sterically inaccessible to enzymic

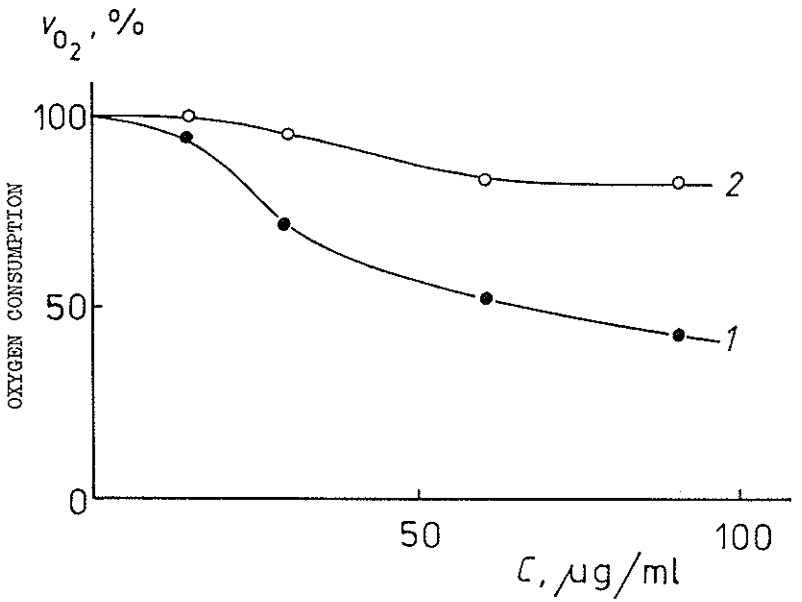


Figure 9. Influence of BSA conjugates with irregular (1) and statistical (2) distribution of pluronic chains on the rate of oxygen consumption by T lymphocytes: 1, $(RPE)_8$–BSA-a; 2, $(RPE)_{14}$–BSA-b. Experimental conditions as in *Figure 7*. The designations are given in *Table 2*.

cleavage. If this bond is split, then the inhibitory effect of the conjugates would be present independent of the sequence of hydrophilic and hydrophobic blocks of the polymer attached to protein and would occur in the presence of both conjugates II and III. However, this effect was observed in the presence of conjugate II only. We conclude that the effect of the conjugates on thymus-derived lymphocytes is a result of their transport through the plasma membrane. Hydrophilic substances, in particular proteins, are unable to enter a cell unless the plasma membrane contains corresponding carriers. However, hydrophilic substances may be translocated into a cell by means of fatty acid hydrophobization (Hu, Olson and Olson, 1986). The evidence obtained allows us to suggest that binding of synthetic polymers (pluronics) capable of crossing cellular membranes (Topchieva, 1989; Kirillova, 1993) to proteins has a similar effect to the attachement of fatty acids. It should be emphasized that hydrophobic blocks in such conjugates should be located at the periphery of the conjugate molecule and polymer chains should be distributed irregularly (structure IIa in *Figure 1*). Protein–pluronic conjugates of IIb and III types and conjugates with PEG had no influence on the respiration of these cells. In some cases PEG–protein conjugates may enter cells by endocytosis (Beckman, 1988). This process is slower by several orders of magnitude than diffusion of conjugates with membranotropic properties, and cannot occur in mitochondria.

Thus, it has been shown that covalent coupling of pluronics to hydrophilic proteins which are not transported through the cell membrane renders them membranotropic.

Solubilization efficiency of conjugates

SOLUBILIZATION OF BENZAFLAVIN BY BSA–POLYALKYLENE OXIDE CONJUGATES

On the basis of the above-described properties of pluronics, it might be expected that coupling of amphiphilic molecules to carrier proteins, e.g. BSA, would result in an enhanced ability of solubilization of water-insoluble substances, for example 2', 3', 4', 5'-tetrabenzoyl-5-acetyl-1,5-dihydroriboflavin (benzaflavin, BF) (Topchieva, 1993b).

Dihydroriboflavin esters exhibiting a wide spectrum of biological activities are important both as long-acting vitamin B preparations and as metabolic drugs (Avakumov, Kovler and Kruglikova-L'vova, 1992).

BF solubilization in aqueous solutions of BSA and its conjugates with polyalkylene oxides was estimated using a UV method at 306 nm, a wavelength corresponding to maximum absorption of BF. Absorption coefficients were measured on the basis of a plot of absorbance versus concentration of BF solubilized by BSA ($\varepsilon = 4.7 \times 10^3$ M^{-1} cm^{-1}), conjugate of BSA with PEG ($(PEG)_{14}$–BSA) ($\varepsilon = 4.7 \times 10^3$ M^{-1} cm^{-1}).

The limit of solubilization (p), being equivalent to the number of molecules bound to one molecule of the solubilizing agent, was estimated using high-speed sedimentation analysis at $\lambda = 306$ nm. The use of the sedimentation technique is based on co-sedimentation of BF bound to protein or conjugate (Topchieva, 1991).

Two methods of BF solubilization were employed, namely suspending dry material in aqueous solutions containing a solubilizing agent (method 1) and introduction of BF dissolved in ethanol into these systems (method 2). Examination of both systems using the quasi-elastic light-scattering technique showed that introduction of BF from ethanolic solutions resulted in the occurrence of small particles of the size of conjugates (less than 30 nm) along with larger ones with a radius of 200–3000 nm, presumably corresponding to BF aggregates. Solubilization using the former technique (method 1) yielded only small particles (<30 nm). To compare the two approaches, the systems containing BF and solubilizing agents of different types were subjected to centrifugation. Solubilization limit values of BF by BSA and its conjugates are presented in *Table 6*. BSA showed a marked capacity for solubilization of BF. However, direct experiments revealed an inability of pluronics to solubilize BF.

Table 6 demonstrates that all conjugate-containing systems were characterized by higher p values as compared with those containing BSA. Conjugate $(RPE)_5$–BSA-a, with hydrophobic regions located on the periphery of macromolecules, showed the highest limit of solubilization. However, method 2 allowed more BF to be introduced into a solubilization system, supposedly owing to a specific kinetic behaviour of the process. It should be emphasized that an increased number of polymer chains in the conjugate, due to a higher degree of modification (samples 4 and 5), did not appear to have any appreciable effect on the efficiency of solubilization. At the same time, a markedly enhanced density of polymer chains in the vicinity of the protein, which resulted from the interaction of the introduced free pluronics with pluronic chains covalently bound to protein, led to a decrease in the limit of solubilization, probably owing to enhanced shielding of the sites responsible for BF solubilization. Optimum incorporation of BF into the conjugate occurred at pH 7.6–9.0 (*Figure 10*). The limit of solubilization decreased with increasing ionic strength of the buffer solution.

Experiments conducted to study temperature-dependent solubilization of BF and

Table 6. Temperature-dependent solubilization of BF by BSA and its conjugates (0.02 M phosphate buffer, pH 7.2)

No.	Solubilizing agent	T(°C)	p	s_{20} (S) (solubilizing agent)	s_{20} (S) (BF complex)	Proportion of different forms (%)
1	BSA	20	17	4.2	6.3	100
		40	17	6.7	11.0	47
					6.2	53
2	$(PEG)_{14}$–BSA	20	21	4.2	6.6	100
		40	21	6.5	10.5	49
					6.8	51
3	$(REP)_{11}$–BSA	20	19	4.2	6.8	100
		40	19	6.6	13.2	47
					5.9	53
4	$(RPE)_{24}$–BSA-a	20	40	4.4	10.1	100
		40	40	6.8	14.2	21
					7.3	79
5	$(RPE)_{100}$–BSA[a]	20	18	2.9	6.2	100
		40	18	5.8	12.0	80
					6.6	20

[a] The solubilizing agent was prepared from compound 4 by addition of free pluronic. method 2 was used for introduction of BF into the system.

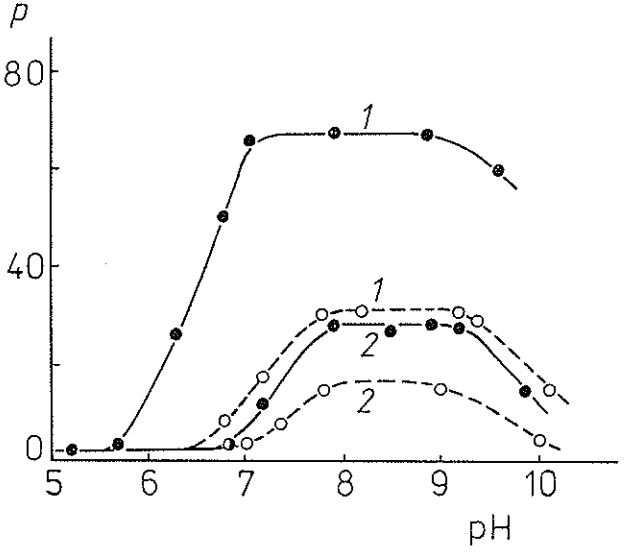


Figure 10. Relationship between BF solubilization limits (p) and pH (at 20°C). 1, Visual method; 2, sedimentation method; - - - -, BSA; ——, conjugate $(RPE)_5$–BSA-a.

BSA and its conjugates revealed marked heterogeneity of the solubilization systems at 40°C which was not normally observed in solutions containing pure protein components. Certain portions of sedimentograms indicated mean sedimentation coefficients which were approximately twice as high as those intrinsic to protein components (*Table 6*). In such systems, BF could probably facilitate the 'bridging' of protein molecules to give rise to aggregates. Comparison of the relative amounts of different particles in the experimental systems demonstrated that the proportion of

aggregates in solutions containing BSA–pluronic conjugates was significantly lower than in all other BSA-containing systems. Polymer chains of these conjugates appear to be responsible for steric hindrance inherent in BF-mediated intermolecular interactions between proteins.

It is appropriate to surmise, on the basis of these findings, that BF molecules are located in hydrophobic parts of the conjugated BSA. This is confirmed by the fact that attachment of pluronics to other proteins, e.g. immunoglobulin G (IgG) or CHT, with lack of ability to bind this ligand, had no effect on their solubilizing potency.

BF TRANSFER FROM SOLUBILIZED FORMS IN RAT HEART MITOCHONDRIA

The above data suggested the possibility of using conjugates of BSA with polyalkylene oxides for the delivery of BF to target cells. Therefore, we investigated the transport of solubilized BF in biological systems, specifically BF transfer to rat iver mitochondria. This phenomenon is demonstrated by the relationship between amount of bound BF and concentration of free BF ($[BF]_{free}$) in the presence of either BSA or its conjugates. Quantitative data on the BF transfer are presented as binding isotherms in *Figure 11*. At the BF concentration range studied the plot of $[BF]_{bound}$ versus $[BF]_{free}$ is linear:

$$\frac{[BF]_{bound}}{[MT]} = K[BF]_{free}$$

where K denotes the coefficient of proportionality related to the chemical nature of the solubilizing agent, and [MT] is the concentration of mitochondrial protein. The linear correlation between the two variables indicates that for experimental results characterizing the initial portions of the binding isotherms the values of K were independent of the amount of the solubilizing agent. Also, *Figure 11* shows an identity of the ability of BSA and BSA–PEG conjugates to transfer BF to membranes. Both solubilizing agents may be characterized by the same K value: $K = 0.31 + 0.01$ (gram of mitochondrial protein per litre)$^{-1}$. Higher transfer efficiency of BSA conjugates with pluronics for BF transport, as compared to BSA and its conjugate with PEG ($K = 0.96 + 0.01$) (gram of mitochondrial protein per litre)$^{-1}$, is probably due to weaker association between the BSA–pluronic conjugate and solubilized BF. To summarize, BSA–pluronic conjugates have certain advantages over the remaining solubilization systems, both at the degree of solubilization and transport of BF. This makes them useful tools for the development of new drug formulations.

INFLUENCE OF BF ON PEROXIDATION OF LIPIDS IN MICROSOMES OF RAT LIVER

Water-soluble forms of BF may be employed to investigate the molecular mechanisms of its action in model biochemical systems. It was shown (Avakumov, Kovler and Kruglikova-L'vova, 1992) that the tetrabutyrate of riboflavin, a compound similar in structure to BF, inhibits peroxidation of lipids (POL) in rat liver mitochondria, initiated by the addition of adriamycin, an antitumour agent. Based on these observations it might be expected that BF would also be able to inhibit POL. Quantitatively the process of NADPH-dependent POL and the influence of solubi-

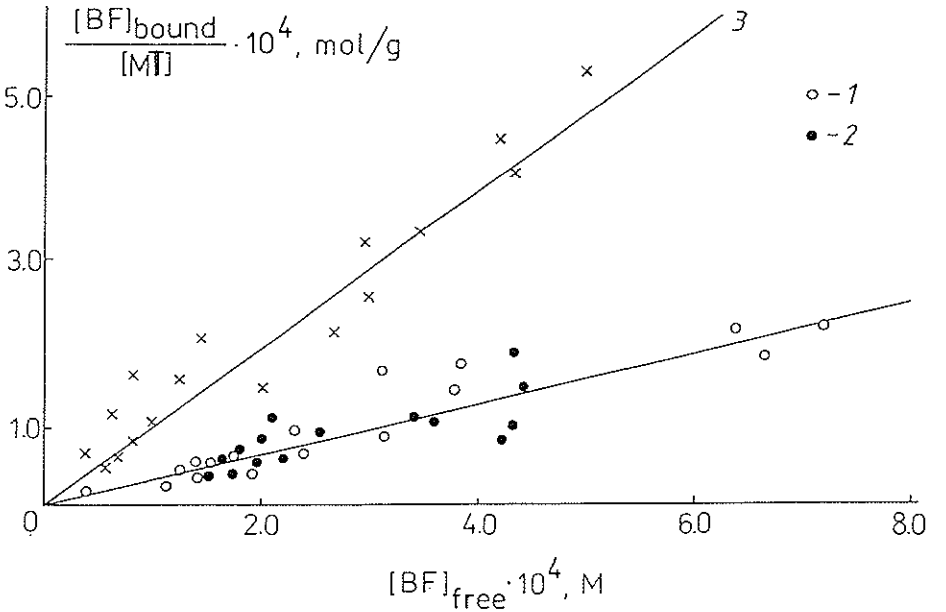


Figure 11. Relationship between concentration of bound BF (mol g^{-1} of mitochondrial protein) and concentration of free BF. Solubilizing agent: 1, BSA; 2, $(PEG)_{14}$–BSA; 3, $(RPE)_{24}$–BSA-a (structure IIa). [MT], concentration of mitochondrial protein.

lized forms of BF on its rate was studied by measuring the concentration of malonic dialdehyde (MDA), the final product of POL, in liver microsomes. In preliminary experiments the influence of BSA, PEG and pluronics on the rate of NADPH-dependent POL was studied. BSA at a concentration up to 0.7×10^{-4} M and PEG at a concentration up to 4.1×10^{-4} M had no effect on the NADPH-dependent POL. On the basis of decreasing MDA production in the presence of solubilized BF forms, we may conclude that BF, solubilized by BSA and its conjugates with polyalkylene oxides, inhibits the process of NADPH-dependent POL in rat liver microsomes. It should be noted that the inhibitory action of BF does not depend on the nature of solubilizing agent. This is depicted in *Figure 12*, which shows the relationship between the extent of POL inhibition and the concentration of BF solubilized by BSA and its conjugates with polyalkylene oxides. The concentration of BF corresponding to 50% inhibition under the conditions studied is 0.5×10^{-4} M. These investigations demonstrate the ability of BF to inhibit the POL process in biological membranes.

INFLUENCE OF BF ON THE ENZYME ACTIVITY OF D-AMINO-ACID OXIDASE FROM PIG LIVER

It has been shown in other experiments that the addition of BF dissolved in ethanol to the reaction mixture does not influence the enzymatic activity of D-amino-acid oxidase. However, solubilized forms of BF do produce an inhibitory effect. *Figure 13* shows the experimental values of the relative activity of D-amino-acid oxidase, V/V_0 (V_0 and V are the rates of enzymic reaction in the absence and in the presence of BF, respectively), plotted against the concentration of BF. The inhibitory effect of BF

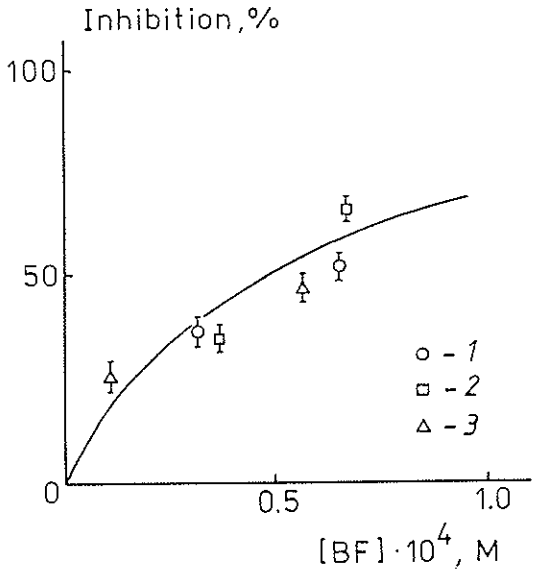


Figure 12. Relationship between degree of inhibition of MDA production in the process of NADPH-dependent POL in rat liver microsomes and concentration of BF, solubilized by BSA (1), $(PEG)_{14}$–BSA (2) and $(PRE)_{24}$–BSA-a (3). Each experimental point is an average of six measurements.

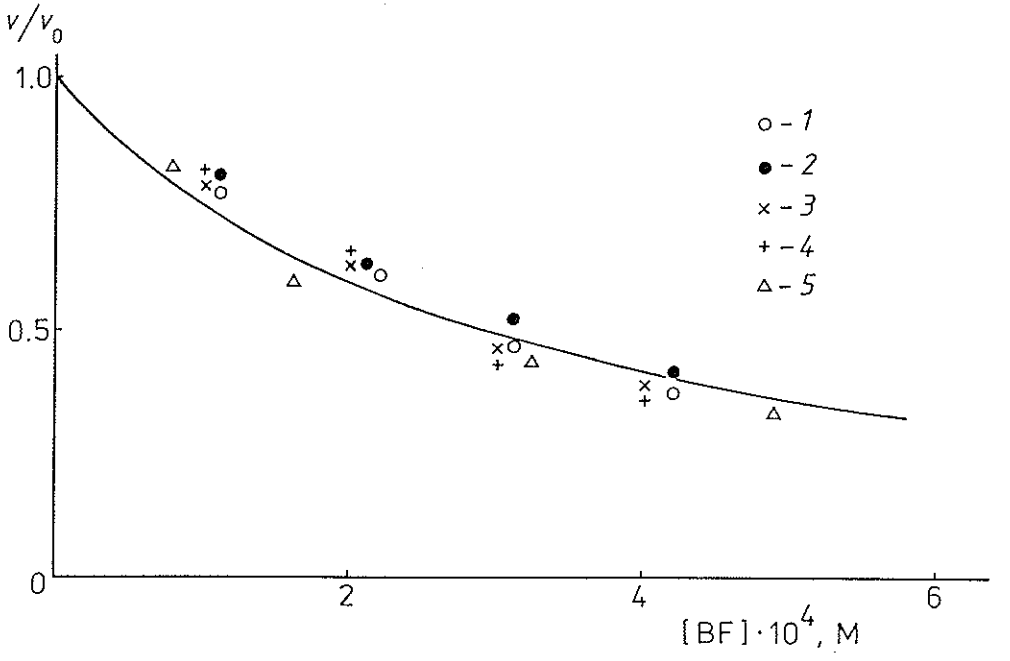


Figure 13. Inhibition of D-amino-acid oxidase by BF, solubilized by BSA (1 and 3), conjugate (PEG)14–BSA (2 and 4) and conjugate (RPE)24T–BSA (5). The enzymatic activity of D-amino-acid oxidase was registered by spectrophotometric (1,2) and polarographic (3,4,5) methods. Conditions: 0.1 M Tris–HCl, pH 8.3, 25°C; concentration of BSA in conjugates and free BSA in solution was 1 mg ml^{-1}.

does not depend on the nature of the solubilizing agent. It should be noted that the course of the curve V/V_0 versus [BF] remains unchanged by alterations in the solubilizing agent concentration. In order to estimate the inhibitory ability of BF, the inhibition constant, K_i, was calculated as follows:

$$\frac{V}{V_0} = \frac{1}{1 + [I]/K_i}$$

where [I] is the concentration of BF. The value of K_i appeared to be 0.29 + 0.02 mM. It should be noted that the sensitivity of D-amino-acid oxidase entrapped in hydrated reversed micelles to the inhibition of BF is essentially higher: $K_i = 4.5 \times 10{-}5$ M at ($[H_2O]$/[aerosol-OT] = 23). This is because, in reversed micelles, localized concentration of BF in the vicinity of enzyme takes place as a result of entrapment of BF on the surface of the micelle.

Thus, the solubilization efficiency of BSA conjugates with polyalkylene oxides (PEG and pluronics) for BF was studied. Conjugates of BSA with pluronics were shown to have greater solubilizing activity for this substance than the free protein. Solubilized forms of BF are able to inhibit NADPH-dependent POL in rat liver microsomes. The inhibitory action of BF does not depend on the type of solubilizing agent. The inhibitory properties were demonstrated by a study of the influence of solubilized forms of BF on the enzyme activity of D-amino-acid oxidase from pig kidney. Thus conjugates of BSA with pluronics are a new type of carrier for the delivery of biologically active water-insoluble compounds and their screening of biological systems.

Conclusion

The design of conjugates on the basis of proteins and block co-polymers of ethylene oxide and propylene oxide allows one to mimic biological functions of membranotropic proteins and is of great importance for progress in enzymotherapy, for creation of new formulations in the pharmaceutical industry, and for production of organosoluble biocatalysts in biotechnology. Also, these compounds are promising tools in biochemical studies of water-insoluble biologically active agents and mechanisms of action of membrane-bound enzymes.

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