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Adducts of proteins and water-soluble poly(alkylene oxides)

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Abstract. The review is devoted to methods for the modification of proteins by water-soluble diphilic block-copolymers of ethylene and propylene oxides and also to the properties of the resulting polymer–protein adducts. Two principal classes of such adducts are examined: conjugates obtained by forming covalent bonds between the protein and the polymer and noncovalent complexes. It is shown that the amphiphilic nature of the polymer chains gives rise to a series of new properties of the conjugates with the block-copolymers, such as the ability to be translocated into cells, the ability to solubilise insoluble biologically active compounds, the ability to serve as agents transporting the latter, and also the ability to interact with surfactants of different chemical nature. The bibliography includes 50 references.

I. Introduction

A vigorous development of a new aspect of protein engineering, involving the specific alteration of the properties of protein molecules as a result of their chemical modification by water-soluble polymers, began since the beginning of the 1970s. The conjugate obtained by means of such modification consists of two macromolecular fragments — the protein and polymer fragments — and combines the properties of each of them. Dextran,[1] poly-*N*-vinylpyrrolidinone,[2] and poly(ethylene oxide) (PEO)[3–5] are usually employed as the polymer fragments. Among the polymers listed above, PEO is most widely used. It is a nontoxic, nonimmunogenic synthetic polymer, which is available with a wide range of molecular masses.[6] The poly(ethylene oxide)– protein conjugates are characterised by weaker immunogenic and antigenic properties, an increased circulation period in the blood circulation system, and in many instances an increased stability.[7] These properties are to a large extent determined by the effect of the steric stabilisation of the protein globule resulting from the creation around the latter of a shell comprising attached polymer chains.

An important property of the PEO–protein conjugates is their ability to dissolve in organic solvents. This makes it possible to employ such conjugates as biocatalysts for peptide synthesis,[8] transesterification,[9] and amination of esters.[10]

The next stage in the construction of polymer–protein conjugates is the introduction of additional fragments (blocks) into the polymer chain in the stage involving the synthesis of polymeric reagents. This approach led to the formation of conjugates of proteins with block-copolymers of ethylene and propylene oxides, namely proxanols (Pro) and pluronics. These copolymers are nonionogenic surfactants in which the PEO and poly(propylene oxide) (PPO) blocks are respectively the hydrophilic and hydrophobic parts of the molecule.[11] Compared with the PEO–protein conjugates, the conjugates of proteins with the block-copolymers are characterised by a greater structural variety, manifested, firstly, by the possibility of attaching copolymers to different numbers, compositions, and arrangements of the blocks and, secondly, by the possibility of different arrangements of the polymer chains on the surface of the protein globule. The amphiphilic nature of the attached polymer chains gives rise to new properties of the conjugates, such as the ability to be transported into the cell,[12,13] the ability to solubilise effectively insoluble biologically active compounds,[14,15] the ability to serve as agents transporting the latter, as well as the ability to interact with surfactants of different chemical nature.[16] In the present review, we shall examine the methods of synthesis and characteristics of the protein–Pro conjugates as well as their physico-chemical and biological properties. Together with the traditional methods of synthesis of polymer–protein conjugates, effected via the covalent interaction of the polymer with the protein, new approaches to the synthesis of structures of this type, based on the formation of noncovalent complexes between the protein and Pro, will be considered.

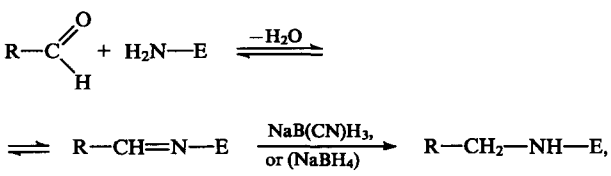
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II. Synthesis, isolation, and characteristics of the conjugates

1. Synthesis of the conjugates

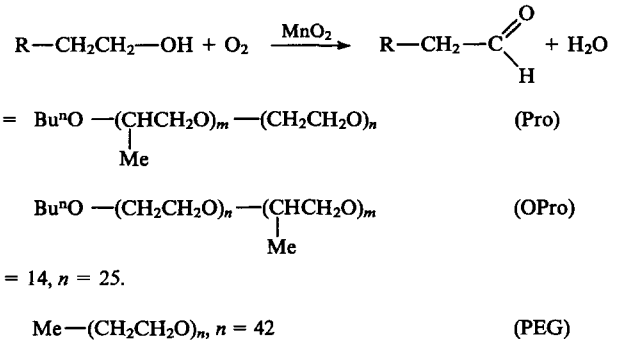
The conjugates of proteins with proxanols are synthesised by the reductive alkylation of the primary surface amino-groups of the protein by polymeric monoaldehydes,



where R is the polymer chain and E is the protein molecule.

Conjugates based on the following proteins have been obtained similarly: bovine serum albumin (BSA),[14] α-chymotrypsin (CT),[17] pyruvate kinase from the rabbit skeletal muscle, ricin from the castor bean, viscumin from the mistletoe,[18] the classical proteinase inhibitor of the Bauman–Birk type from the soya beans (BBI),[19] and cytrochrome *c*.[12] The conjugates based on CT and BBI have been obtained in the presence of reversible inhibitors of the active centres of enzymes. For example, in the synthesis of conjugates based on CT, we employed for this purpose *N*-benzoyl- or *N*-acetyl-L-tyrosine.[16] Sodium cyanohydroborate is a more convenient reducing agent for the preparation of the conjugates than sodium tetrahydroborate, which leads to the reduction of the disulfide bonds between polypeptide chains as a side reaction.

The monoaldehyde derivatives of PEO and Pro are obtained by oxidising the corresponding monofunctional polymers by atmospheric oxygen in solution in chloroform in the presence of manganese(IV) oxide as the catalyst.[20]



Evidently the only difference between the Pro and OPro (reversed proxanol) polymers consists in the position of the hydrophobic PPO block relative to the terminal aldehyde group. The use of these polymeric reagents makes it possible to synthesise conjugates with different sequences in the attachment of the polymeric blocks to the protein. The number of polymer chains in the conjugate may be regulated by varying the polymer/protein ratio in the reaction mixture.

The molecular models of the conjugates described in this review are presented in Fig. 1. Evidently the conjugates are star-shaped structures with a protein globule in the centre, the polymer chain being bound covalently to the globule. When polymeric reagents with a number-average molecular mass of 2000 are employed, the length of the polymer chains is 20.7 nm for PEO (Fig. 1, type *I*), and 21.6 nm for Pro and OPro (Fig. 1, types *II* and *III*), which is significantly greater than the diameter of the protein globule (for example, the average length of the two globule half-axes for CT is 2.5 nm). Such a structure suggests that the polymer chains provide a steric shield to the protein, diminishing its binding to macromolecular substrates, antibodies,

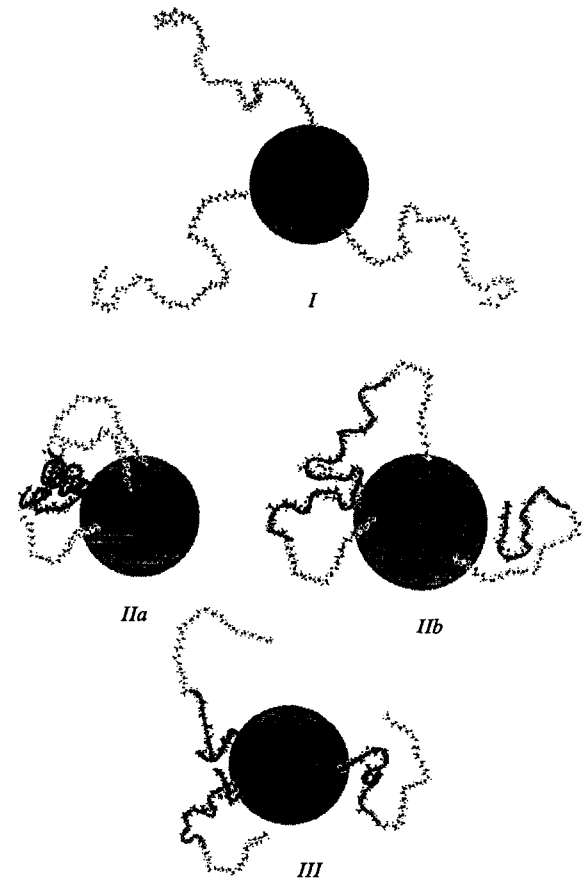


Figure 1. Molecular models of protein conjugates: (*I*) with PEO ($M = 2000$); (*IIa*) with Pro having a nonuniform distribution of polymer chains; (*IIb*) with Pro having a uniform distribution of polymer chains; (*III*) with OPro. The PPO blocks of the polymer chains of the proxanols are shown shaded; the radius of the protein globule is 25 Å (the average length of the two half-axes of the CT globule).[21]

and proteolytic enzymes. Evidently this effect of the polymer chains should depend on their number and on the nature of their distribution on the surface of the protein in the conjugate.

Since the polymer chains in the conjugates are attached only to a proportion of the surface amino-groups of the protein, a possibility arises of their different relative dispositions. Thus the polymer chains may be uniformly distributed on the surface of the protein (Fig. 1, *IIb*) or may be concentrated in one place with formation of domains (Fig. 1, *IIa*). Naturally, the formation of structures of a particular type is specified in the stage involving the synthesis of the conjugate. During the synthesis in aqueous buffer media, where proxanols manifest a capacity for self-association due to their diphilic nature,[22] conditions arise for their nonuniform distribution on the surface of the protein globule. The conjugates thus obtained may be regarded as a kind of intramolecular micelle, stabilised by the hydrophobic interaction between the polymer chains attached to the protein. On the other hand, when the conjugates are synthesised in aqueous organic media, where the hydrophobic interactions between the proxanol molecules are suppressed,[23] their more uniform distribution on the surface of the protein globule should be promoted. Taking these considerations into account, two series of conjugates were synthesised and will be designated below as nonuniform (nonun.) and uniform (un.). It is noteworthy that the concentrations of the organic solvent in the reaction mixture in the synthesis of the uniform conjugates were much lower than the threshold concentrations inducing the denaturation of the protein.[24]

In order to make sure that the above postulates are valid, it was necessary to estimate the differences between the distributions of the polymer chains on the surface of the protein globule in conjugates synthesised under different conditions. Use was made for this purpose of the ability of the polymer chains of poly(alkylene oxides) to interact with a bulky cyclic ligand — β-cyclodextrin (β-CD). We had shown earlier[25] that β-CD forms clathrate complexes with proxanols, the number of β-CD molecules 'threaded' onto the polymer chains being determined by the number of propylene oxide units entering into the composition of the proxanol. Naturally, the interactions of the polymer chains of the conjugates with this ligand depend on the nature of the distribution of the polymer chains on the protein surface. A uniform distribution of the polymer chains should not prevent the formation of complexes with β-CD, whereas for a nonuniform distribution the interactions are hindered for steric reasons. Indeed, it has been shown that only conjugates for which a uniform distribution of the polymer chains has been postulated (Fig. 1, *IIb*), form, like the free proxanols, crystalline clathrate complexes with β-CD which are insoluble in water. On the other hand, the conjugates with a tentatively nonuniform distribution of the chains do not form complexes with β-CD.[16] Thus it has been confirmed that the conjugates synthesised in an aqueous medium are characterised by a nonuniform (domain) type of distribution of the polymer chains, in contrast to the conjugates synthesised in an aqueous organic medium and characterised by a more uniform distribution. The differences between the structures of such conjugates are responsible also for a series of differences between their physicochemical properties and biological behaviour.

2. Purification of the conjugates

The purification of the conjugates based on proxanols in order to remove the excess polymeric reagent is a more difficult problem than in the case of the PEO – protein conjugates. This is due to the amphiphilic properties of the proxanols, which are capable of forming associated species with the polymer chains entering into the composition of the conjugate. Taking this into account, two purification methods have been proposed which make it possible to suppress the hydrophobic interactions between the polymeric reagent and the polymer chains of the conjugate: precipitation of the conjugate with acetone;[17] gel-permeation chromatography involving the use of the 1:4 ethanol – water mixture as the eluent.[12] Thin-layer chromatography has been used to demonstrate the absence of a free polymer impurity in the conjugate.

3. Characteristics of the conjugates

Tables 1 and 2 present the characteristics of the conjugates obtained: composition, degree of modification (average number of polymer molecules attached to one protein molecule), and the number of primary, i.e. unmodified, amino-groups in the protein component of the conjugate.

Table 1. Characteristics of the conjugates of α-chymotrypsin with poly(ethylene oxide) and proxanols.

Polymer	Type of conjugate[a]	Designation	Degree of modification (*n*)	CT content (mass%)[b]	No. of unsubstituted NH_2 groups (*m*)
PEO	*I*	$(PEO)_3$–CT	3	81.0 (80.5)	12
PEO	*I*	$(PEO)_9$–CT	9	58.1 (58.8)	5
Pro	*IIa*	$(Pro)_2$–CT(nonun.)	2	86.2 (86.1)	13
Pro	*IIa*	$(Pro)_3$–CT(nonun.)	3	82.1 (79.8)	12
Pro	*IIa*	$(Pro)_6$–CT(nonun.)	6	67.5 (66.7)	8
Pro	*IIa*	$(Pro)_7$–CT(nonun.)	7	64.0 (64.1)	–
Pro	*IIa*	$(Pro)_{10}$–CT(nonun.)	10	55.6	4
Pro	*IIb*	$(Pro)_3$–CT(un.)	3	80.6	11
Pro	*IIb*	$(Pro)_5$–CT(un.)	5	71.5 (71.0)	9
Pro	*IIb*	$(Pro)_6$–CT(un.)	6	67.4 (67.5)	–
Pro	*IIb*	$(Pro)_{11}$–CT(un.)	11	45.5 (46.0)	–
OPro	*III*	$(OPro)_3$–CT	3	80.6 (80.7)	12
OPro	*III*	$(OPro)_7$–CT	7	64.2 (64.0)	–

[a] Here and henceforth the designations *I*, *IIa*, *IIb*, and *III* correspond to the structures presented in Fig. 1.
[b] The protein content in the conjugates was determined spectrophotometrically and also with the aid of the biuret reaction (values in brackets).

Table 2. Characteristics of the conjugates of bovine serum albumin with poly(ethylene oxide) and proxanols.

Polymer	Type of conjugate	Designation	Degree of modification	BSA content (mass%)		
				biuret reaction[a]	UV (280 nm)[a]	^{13}C NMR
PEO	*I*	$(PEO)_5$–BSA	5	87	87	–
PEO	*I*	$(PEO)_{14}$–BSA	14	70	71	–
Pro	*IIa*	$(Pro)_5$–BSA(nonun.)	5	87	87	–
Pro	*IIa*	$(Pro)_8$–BSA(nonun.)	8	81	80	78
Pro	*IIb*	$(Pro)_{15}$–BSA(un.)	15	70	69	70
OPro	*III*	$(OPro)_6$–BSA	6	85	84	–
OPro	*III*	$(OPro)_{11}$–BSA	11	75	75	–

[a] The method for the determination of the protein content in the conjugate.

The protein content in the conjugate was determined spectrophotometrically (λ = 280 nm) and also with the aid of the biuret reaction.[26] In all cases, the initial proteins were employed as the standard. It had been shown previously[3,4] that the presence of poly(alkylene oxide) does not affect the results of the assay of the protein by means of the biuret reaction. The composition was determined by the ^{13}C NMR method, which permits independent measurements of the contents of the protein and polymeric components. Such measurements have been carried out for two conjugates of BSA with Pro (Table 2). The regions where the signals of the protein and the polymer do not overlap were found from the spectra of the individual components in a preliminary experiment. For BSA, this is the 172–180 ppm region corresponding to the carbon atoms of the carboxy-groups, while for Pro this is the 70–77 ppm region corresponding to the carbon atoms of the methylene groups of the main chain. The integral intensities of selected analytical signals of the protein and the polymer were determined from the spectra of specially prepared solutions containing mixtures of the protein and Pro in specified mass proportions. The dependence of the integral intensities on the polymer/protein mass ratio for these solutions proved to be linear. The mass compositions of the polymer synthesised were determined from the integral intensities in their spectra by means of a calibration plot. It was found that the results obtained agree, within the limits of the error of the measurement, with data obtained by the spectrophotometric and chemical methods (Table 2). Subsequently we assumed that the use of only the spectrophotometric and (or) chemical methods which do not require large amounts of the test substance, was sufficient for the determination of the composition of the conjugate. In this case, the content of the polymer [m_{Po} (mass%)] in the conjugate was assumed to be

$$m_{Po} = 100 - m_{Pr}, \quad (1)$$

where m_{Pr} is the content of the protein (mol%). The degree of modification (n) was calculated from the composition of the conjugate as the average number of polymer molecules attached to one protein molecule:

$$n = \frac{m_{Po}/\bar{M}_{Po}}{m_{Pr}/M_{Pr}},$$

where $\bar{M}_{Po}$ is the number-average molecular mass of the polymer and M_{Pr} is the molecular mass of the protein.

In addition, the degree of modification was determined independently by titrating the residual unsubstituted primary amino-groups of the protein. For proteins where the total number of the primary surface amino-groups is known, the degree of modification n may be determined as the difference

$$n = r - m, \quad (2)$$

where r is the total number of surface NH_2 groups and m is the number of unsubstituted NH_2 groups.

It is noteworthy that the number of unsubstituted surface NH_2 groups in proteins is usually determined by titrating a solution of the protein with 2,4,6-trinitrobenzenesulfonic acid (TNBS).[27] However, in our case this procedure is inapplicable, since both the remaining unsubstituted NH_2 groups of the conjugates and the substituted, secondary amino-groups react with TNBS.[28] The number of surface amino-groups in the conjugates determined by the reaction with TNBS therefore remains the same as in the initial protein. In order to determine the number of residual unsubstituted amino-groups of the protein after conjugation, we resorted to Ruff's method[28] based on the interaction of the primary amino-groups with *o*-phthalic dialdehyde in the presence of mercaptosuccinic acid. The number of primary NH_2 groups was determined by this method in a series of CT conjugates. The degree of modification calculated by Eqn (2), taking into account the fact that the total number of primary amino-groups with CT is 15, proved to be close to the value calculated by Eqn (1) from the protein content in the conjugate.

A satisfactory agreement between the results obtained by the two methods confirms the fact that only the primary amino-groups of the protein are modified and that the other aminoacid residues are unaffected.

4. Catalytic properties of the conjugates

The catalytic properties of the CT-based conjugates are presented in Table 3, from which it is seen that:

1. The conjugates retain a high enzyme activity in relation to both substrates employed — the low-molecular-mass substrate [the ethyl ester of *N*-benzoyl-L-tyrosine (BTEE)] and the high-molecular-mass substrate (casein).

2. The kinetic constants of the hydrolysis of the low-molecular-mass substrate (BTEE) by the enzyme forming part of the composition of the conjugates changed only slightly compared with the native enzyme. Similar results were obtained also in the study of the hydrolysis of the high-molecular-mass substrate (casein). This is apparently associated with the fact that the active centre of α-chymotrypsin was protected in the conjugate synthesis stage by the inhibitor bound reversibly to it and so the polymer chains were unable to be bound in the immediate vicinity of the active centre of the enzyme.

3. A significant loss of enzyme activity was observed only after the attainment of high degrees of modification (11 and more attached polymer chains). A similar trend has been described by Chiu et al.[30] for conjugates based on CT and PEO. Evidently the decrease in activity in this case is not a consequence of the decrease in k_{cat} but occurs as a result of the decrease in the number of active centres in the conjugates [Table 3, $(Pro)_{11}$–CT(un.)].

5. Thermal inactivation of the conjugates

An important practical consequence of the modification of proteins by polymers is a change in the resistance of the protein globule to the effect of elevated temperatures.[31] The thermal stabilities of the conjugates between proteins and proxanols have

Table 3. The enzyme properties of the conjugates of α-chymotrypsin with poly(ethylene oxide) and proxanols.

Protein, conjugate	Activity in enzymic hydrolysis of casein (%)	Enzymic hydrolysis of BTEE			Content of active centres (%)
		activity (%)	k_{cat}/s^{-1}	$10^5\ K_m$/mol litre^{-1}	
CT	100	100	31.2 ± 0.5	6.27 ± 0.94	80
$(Pro)_5$–CT(nonun.)	102	97	42.4 ± 2.8	13.0 ± 2.9	61
$(Pro)_3$–CT(un.)	103	115	–	–	80
$(Pro)_6$–CT(un.)	96	98	32.5 ± 1.2	6.18 ± 0.82	67
$(Pro)_{11}$–CT(un.)	50	57	37.0 ± 0.4	21.7 ± 3.5	40
$(PEO)_6$–CT	97	98	40.4 ± 0.8	9.3 ± 1.5	80
$(OPro)_3$–CT	98	100	–	–	80

been investigated by a method involving the kinetic study of the enzyme properties of the conjugates at the elevated temperature.[17] The corresponding kinetic curves (the time variations of the activity) are presented in Fig. 2. Evidently the rate of the thermal inactivation of CT diminishes significantly even after the addition of only three polymer chains (curves *2* and *3* in Fig. 2). With increase in the degree of modification of the conjugate, this effect becomes more pronounced (curves *4*–*6* in Fig. 2), i.e. the thermal stability of the conjugates increases in parallel with the increase in the degree of modification. The modification of the enzyme by PEO has the greatest stabilising effect (curve *6* in Fig. 2; the conjugate contains nine polymer chains), the specimen retains 80% of the initial activity after heating for 3 h at 45 °C.

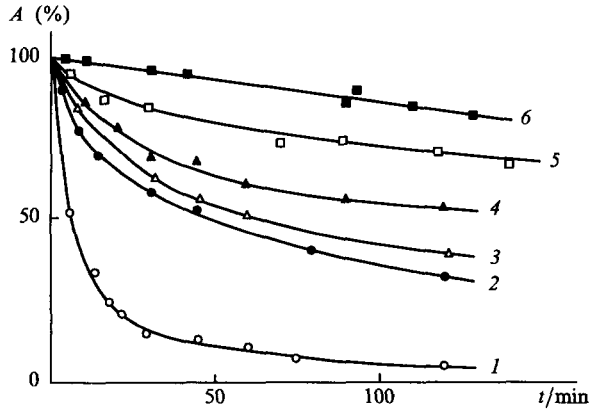


Figure 2. Kinetic curves for the thermal inactivation of CT and its conjugates with poly(alkylene oxides): (*1*) CT; (*2*) $(OPro)_3$–CT; (*3*) $(Pro)_3$–CT(nonun.); (*4*) $(Pro)_6$–CT(nonun.); (*5*) $(Pro)_5$–CT(un.); (*6*) $(PEO)_9$–CT. The thermal inactivation conditions here and in Figs 3 and 4 were as follows: 0.2 M tris.HCl, pH 8.0, 45 °C; the enzyme concentration in each specimen was 0.1 μM.

It was shown in a specially formulated experiment that simple addition of poly(alkylene oxides) to a solution of CT does not affect its thermal stability.

The difference between the shapes of the kinetic thermal inactivation curves for the CT–PEO conjugate (curve *6* in Fig. 2) and three conjugates with block-copolymers (curves *2*–*4*) is striking. In all cases, the curves were treated mathematically in terms of the variables of a first-order reaction. The kinetic data for the inactivation of the $(PEO)_9$–CT conjugate in terms of such variables can be described by a linear relation. The rate constant calculated from these data is $k = (2.3 \pm 0.1) \times 10^{-5}$ s^{-1}.

These relations for the remaining conjugates are represented by curves consisting of two or more regions with different shapes. The molecular causes of such heterogeneity in the case of native CT were discussed previously.[32] It is most likely that in the case of the conjugates this is also associated with their inhomogeneity as regards both structure and composition, which may be manifested both by a distribution with respect to the degrees of modification of different protein globules and by a nonuniform arrangement of the chains on the surface of an individual protein globule. Such inhomogeneity has a particularly notable effect on the properties of the $(OPro)_3$–CT(nonun.), $(Pro)_3$–CT(nonun.), and $(Pro)_6$–CT(nonun.) conjugates synthesised under conditions such that the polymeric reagents are in the micellar form, which promotes a nonuniform filling of the surface of the protein globule by the polymer (Fig. 1, *IIa*). On the other hand, the $(PEO)_9$–CT conjugate, containing polymer chains incapable of forming micellar structures,[33] should be characterised by a much more uniform distribution of the polymer chains. It may be that this is in fact one of the causes of the high thermal stability of the $(PEO)_9$–CT conjugate.

The thermal stability of the $(Pro)_5$–CT(un.) conjugate, synthesised under conditions which rule out the possibility of a micelle formation reaction between the molecules of the block-copolymers, approaches (curve *5* in Fig. 2) the thermal stability of the $(PEO)_9$–CT conjugate. This effect is particularly pronounced, since the degree of modification in the $(Pro)_5$–CT conjugate is smaller almost by a factor of two than in $(PEO)_9$–CT.

Thus the thermal stability of the conjugates increases in parallel with the increase in their content of polymer chains. For the same number of polymer chains, the thermal stability of conjugates with a uniform distribution of the chains is higher than that of the conjugates with a nonuniform distribution (curves *5* and *4* in Fig. 2). On the other hand, the stability of the conjugates in which the hydrophobic block is on the periphery is higher than that of the conjugates with the reverse order in the arrangement of the blocks. The results can be explained in terms of the ideas, being developed in the literature, according to which the modification of the protein surface by hydrophilic reagents should stabilise the protein molecule, since it makes it possible to shield the surface hydrophobic groups of the protein from unfavourable contact with water.[34,35] Indeed, as can be seen from Fig. 1 in the case of a nonuniform distribution of the polymer chains in the conjugate, a considerable proportion of the protein surface remains unprotected, whereas for a uniform distribution there is a greater possibility of the shielding of the protein globule. This in fact leads to an increase in the thermal stability of the conjugates with a uniform distribution of the chains compared with those having a nonuniform distribution.

6. Interaction of the conjugates with diphilic compounds

One of the remarkable properties of the conjugates of proteins with proxanols is their ability to interact with both synthetic and natural diphilic compounds.[17,36] The polymer chains then play the role of a reinforcing construction, organising around themselves assemblies of other diphilic compounds. In supramolecular structures of this kind, diphilic substances may have an indirect effect on the properties of the protein globule forming part of the composition of the conjugate. For example, we may quote the increase in the thermal stability of the conjugate following the introduction of the free proxanol (Fig. 3). This is apparently due to the binding of the polymer chains of the conjugate to the additionally introduced polymer with formation of a more compact polymer shell around the protein, which leads to the shielding of the hydrophobic domains on its surface.

Another picture is observed when β-CD, capable of forming clatrate complexes with proxanols, is introduced as a diphilic additive into the system. As shown previously, in the interaction with β-CD the polymer chains of the proxanols assume a more

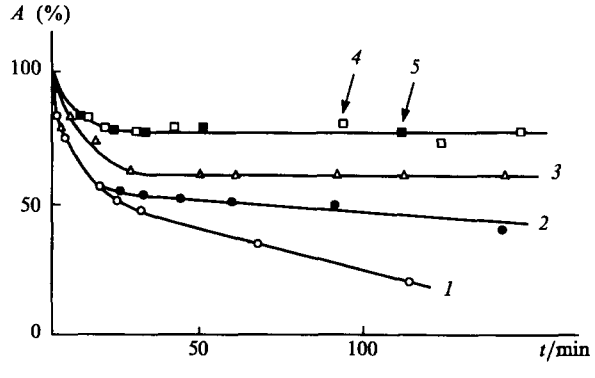


Figure 3. Kinetic curves for the thermal inactivation of the pure $(Pro)_4$–CT(un.) conjugate (curve *1*) and the conjugate with added Pro (curves *2*–*5*). Molar excess of Pro relative to the conjugate: (*2*) 50; (*3*) 100; (*4*) 500; (*5*) 750. For thermal inactivation conditions, see Fig. 2.

extended conformation.[37] Such chains are apparently no longer able to shield the hydrophobic sections of the protein and hence the thermal stability of the protein in the conjugate is somewhat reduced (Fig. 4), although it still remains higher than that of the native protein. It is noteworthy that β-CD affects only the thermal stability of the conjugates with a uniform distribution of the polymer chains on the protein surface (Fig. 1, *IIb*), but does not influence at all the thermal stability of the conjugates with a nonuniform distribution of the chains, with which it does not interact for the reasons stated previously.[16]

Thus the study of the thermal stability of the conjugates may constitute in many instances an extremely informative method, whereby complex formation reactions between the conjugates and diphilic substances may be discovered.

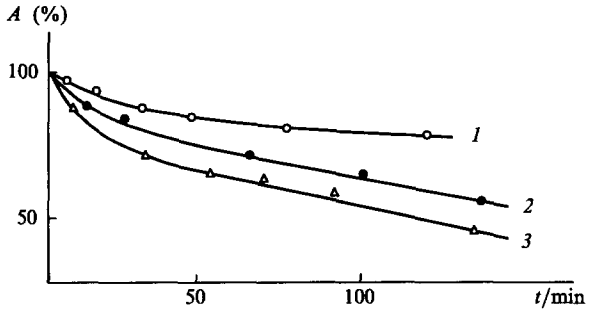


Figure 4. Kinetic curves for the thermal inactivation of the $(Pro)_3$–CT(un.) conjugate (curve *1*) and the same conjugate in the presence of β-CD (curve *2*) and egg lecithin (curve *3*) (Molar ratio conjugate : additive = 1 : 50). For thermal inactivation conditions, see Fig. 2.

III. Physicochemical properties of the conjugates

1. Differential scanning calorimetry

The data on the thermal stability of the conjugates obtained by the kinetic method have been supplemented by the results of the study of the thermal denaturation of the conjugates by differential scanning calorimetry (DSC). Fig. 5 presents heat absorption curves for aqueous solutions of CT and its conjugates. Evidently a significant alteration of the shape of the heat absorption peak is observed for the conjugates. Apart from the first maximum, located in the temperature range 47.6 – 49.6 °C as in the initial CT, a second maximum appears on the thermograms for the conjugates in the region of higher temperatures (54.5 – 55.5 °C), while for the $(OPro)_3$–CT and $(Pro)_7$–CT(un.) conjugates there is a shoulder in the same temperature range. Thus the appearance of an additional thermal transition is characteristic of all the conjugates. The most trivial explanation of this phenomenon is that the appearance of additional transitions is associated with the heterogeneity of the conjugates: the first maximum on the heat absorption curve corresponds to the fraction of the conjugate with a low degree of modification, while the second maximum corresponds to the fraction with a high degree of modification. However, this hypothesis conflicts with the DSC data for the conjugates with a higher degree of modification — $(Pro)_7$–CT(un.) (curve *6* in Fig. 5). As can be seen from the figure, there is only one maximum on the heat absorption curve for this conjugate (T_{max} = 51.2 °C) and there is a shoulder in the range 54.5 – 55.5 °C.

During the denaturation of the protein globule, the hydrophobic sections of the polypeptide chains are exposed to the solvent, becoming accessible to the hydrophobic units of the polymer chains entering into the composition of the conjugate. The appearance of a second maximum on the heat absorption curves for the conjugates is apparently due to the fusion of such a polymer – protein complex.

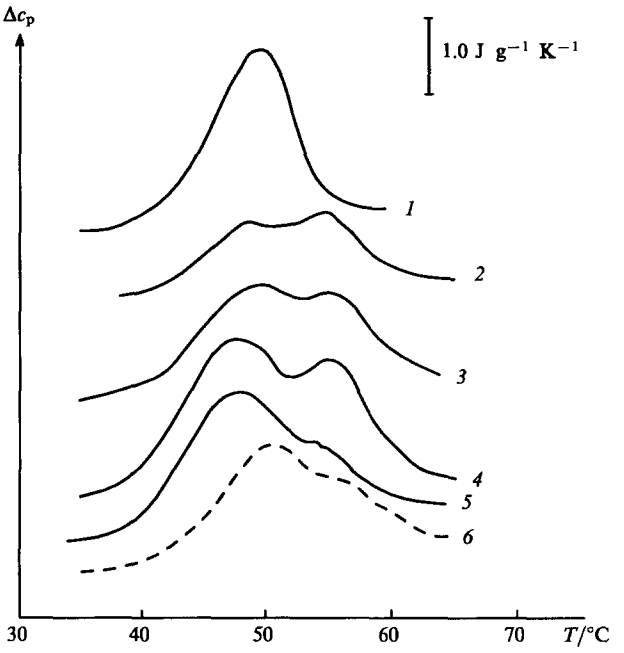


Figure 5. Heat absorption curves for CT (curve *1*) and its conjugates $(PEO)_3$–CT (curve *2*), $(Pro)_3$–CT(un.) (curve *3*), $(Pro)_3$–CT(nonun.) (curve *4*), $(OPro)_3$–CT (curve *5*), and $(Pro)_7$–CT(un.) (curve *6*). Buffer — 0.02 M sodium phosphate, pH 7.0; concentration of preparations 1 mg ml^{-1}; heating rate 1 K min^{-1} (c_p = heat capacity at constant pressure).

In order to demonstrate the validity of this hypothesis, additional studies were made. These confirmed the ability of proxanol to interact with CT at an elevated temperature. It was shown that:

1. During the scan heating from 20 to 60 °C (1 K per minute) of the solution of CT containing Pro (molar ratio 1 : 20), this protein does not aggregate.

2. Similar heating of a solution containing a mixture of CT and PEG leads to the aggregation of the enzyme similar to that occurring in the solution of CT under the same conditions.

3. The product of the interaction, obtained as a result of the heating of a mixture of CT with Pro to 60 °C and separated from the excess of the free proxanols, contains only 50% of the protein. According to ^1H NMR data, the remainder comprises the bound proxanol.

4. A peak whose maximum is close to the second maximum on the thermograms of the conjugate (T_{max} = 54.5 °C) is observed on the heat absorption curve for the solution containing a CT – Pro mixture.

The facts listed above show that the polymer – protein complex is formed at elevated temperatures. The complex has a fusion peak located in the same temperature range as the second maximum on the heat absorption curves for the conjugates.

Thus the DSC study of the conjugates showed that (a) the conjugates are thermally more stable than the initial protein, (b) that the thermal stability of the conjugates based on proxanol is comparable to that of the PEO – protein conjugates, and (c) that the appearance of the second maximum on the heat absorption curves for the conjugates is probably associated with the formation of intramolecular polymer – protein complexes.

2. Sedimentation of the conjugates

The hydrodynamic properties of the conjugates have been investigated by the high-speed sedimentation method. The sedimentation coefficients of the conjugates and the initial proteins are listed in Table 4, which shows that the modification of the proteins by polymers does not alter appreciably the sedimentation coefficient. This permits the conclusion that

Table 4. Sedimentation coefficients of proteins (s_{20}) and their conjugates with poly(ethylene oxide) and proxanols (T = 20 °C, 48 000 rev min^{-1}, 0.02 M phosphate buffer, pH 7.0).

Protein, conjugate	Concentration/mg ml^{-1}	s_{20}/Sv
BSA	1	4.3 ± 0.2
$(PEO)_{15}$ – BSA	1	4.2 ± 0.2
$(Pro)_8$ – BSA(nonun.)	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
$(OPro)_6$ – BSA	1	4.1 ± 0.2
CT	1	2.0 ± 0.15
	4	2.02 ± 0.15
	8	2.2 ± 0.17
	10	2.45 ± 0.12
$(Pro)_3$ – CT(nonun.)	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
$(Pro)_3$ – CT(un.)	1	1.91 ± 0.13
	3	1.99 ± 0.15
	5	2.11 ± 0.08
	10	2.25 ± 0.13

solutions of the conjugates do not contain aggregates or products of intermolecular interaction in the experimental concentration range.

It might have been expected that, even in the absence of intermolecular interactions, the covalent attachment of polymer chains to the protein should have affected its sedimentation coefficient. The fact that significant changes in the sedimentation coefficients of the conjugates compared with those of the initial proteins were not observed apparently means that the increase in molecular mass in the modified protein is compensated by the flotation effect due to the polymer chain. It is noteworthy that the poly(alkylene oxides) used to modify the protein have a density close to that of water and they do not therefore undergo sedimentation in the ultracentrifuge.[38]

3. Hydrophobic chromatography

The hydrophobicity of the conjugates has been estimated with the aid of reversed-phase hydrophobic high-performance liquid chromatography (HPLC). Fig. 6 illustrates chromatograms of the native BBI and the $(Pro)_5$ – BBI conjugate.[18] Evidently the retention volume of the conjugate is smaller than that of the native protein. This indicates its lower affinity for the hydrophobic carrier. The same trend has been observed also for the conjugates of CT with proxanol. At first sight, these results may appear surprising, since the modification by diphilic proxanols should impart an additional hydrophobicity to the protein. However, they can be accounted for by assuming that the hydrophobic blocks of the proxanol participate in intramolecular interaction with the hydrophobic region on the protein surface, whereas the hydrophilic blocks of the polymer occur mainly on the periphery of the conjugate.

4. Conformational models of the proxanol – protein conjugates

The entire set of data obtained with the aid of hydrophobic chromatography, sedimentation, and DSC indicate the existence of common features in the formation of the structures of the conjugates synthesised by the attachment of Pro to the protein.

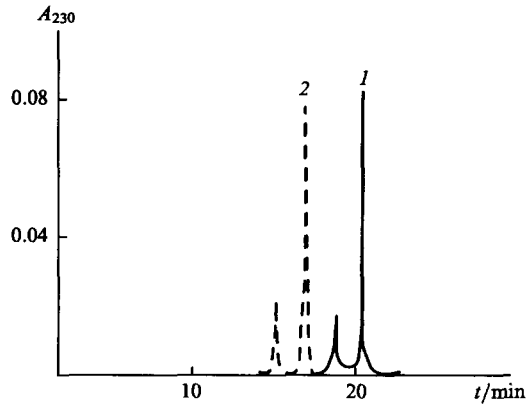


Figure 6. High performance liquid chromatography of native BBI (curve *1*) and of its conjugate $(Pro)_5$ – BBI(nonun.) (curve *2*). The chromatography was carried out on a Zorbax C-8 column with 70% acetonitrile as the eluent; A_{230} = optical density at 230 nm.

These properties are manifested particularly strikingly for conjugates with a hydrophobic block on the periphery of the molecule (Fig. 1, structure *II*). The excess hydrophobicity introduced by the proxanols in such conjugates may be compensated either by the formation of intermolecular associated species or as a result of intramolecular interaction. Since associated species were not detected in dilute solutions of the conjugates, it is natural to postulate the formation of structures stabilised by intramolecular hydrophobic interactions. This hypothesis is confirmed by hydrophobic chromatographic data, indicating that the conjugates behave as substances which are more hydrophilic than the initial protein. Such structures are apparently constructed in the form of 'intramolecular micelles', in which the PPO blocks enter into the composition of the hydrophobic core, while the PEO blocks form part of the hydrophilic environment.

One can also postulate that the formation of such structures facilitates the complex formation reaction between the polymer chains of the proxanols and the protein occurring at elevated temperatures. It should be noted especially that the proposed structures have a definite conformational lability. This is manifested in the presence of hydrophobic or diphilic 'partners' such as lipids,[36] proxanol,[17] and cyclodextrins,[16] which are capable of interacting with the hydrophobic block of the polymer chains entering into the composition of the conjugates. There is probably in this case competition between the hydrophobic sections on the surface of the protein and the hydrophobic 'partners' for a chance to interact with the PPO block of the conjugate, as a result of which the 'intramolecular micelle' decomposes and mixed associated species or complexes are formed.

IV. Membranotropic properties of the conjugates

A fundamentally new property of the conjugates of proteins with proxanols is their ability to interact with biological membranes. Since the hydrophilic proteins forming part of their composition are not membranoactive, one can postulate that this property is due to the presence of the diphilic polymer chains of the proxanols. As shown previously, proxanols have the capacity to become incorporated in a biological membrane and to be translocated through it.[36]

In order to investigate the translocation of the conjugates through a biological membrane, use was made of the approach involving the study of the influence of these compounds on the rate of consumption of oxygen by liver mitochondria and the thymus lymphocytes.[12] It is known that the diphilic substances capable of being translocated through a cell membrane can

reach the NADH-dehydrogenase complex, located in the mitochondrion and responsible for cell respiration, and inhibit the NADH-dehydrogenase activity, which is manifested by a decrease in the rate of consumption of oxygen. When such experiments were carried out with the conjugates, it was found that some of them are capable of suppressing cell respiration of T-lymphocytes. This indicates their translocation to the cell. We investigated the dependence of the translocation of the conjugates on the nature of the attached chains and of their distribution relative to the protein globule.[13]

In order to elucidate the question of the influence of the nature of the arrangement of the hydrophilic and hydrophobic blocks in the proxanol chains in relation to the protein in the conjugate on its ability to suppress the respiration of T-lymphocytes, the effects of the type *II* and *III* conjugates based on CT and BSA were compared (Fig. 1): Fig. 7 shows that only type *II* conjugates, in which the hydrophobic block is on the periphery of the molecule, are able to suppress respiration in the lymphocytes. On the other hand, neither the PEO–protein conjugate, nor the conjugate with the hydrophilic block on the periphery of the molecule (type *III*), nor the initial CT affect respiration in the lymphocytes. The effectiveness of the influence of the type *II* conjugate on respiration is comparable to that of the pure proxanol. The concentrations which induce 50% suppression of the respiration were 9.8 ± 2.4 μg mol^{-1} for the proxanol and 30.0 ± 6.2 μg mol^{-1} for the conjugates (Table 5, A). Comparison of the corresponding concentrations of the free proxanol and the proxanol incorporated in the conjugates showed that they are similar (Table 5, B). Similar results were also obtained for the conjugates based on BSA and cytochrome *c*. This means that the capacity for translocation is determined by the polymer chains and depends little on the nature of the protein.

The conjugates of the three proteins investigated showed a similar effect also on respiration in isolated mitochondria (Table 5).

The dependence of the suppression of respiration on the number of polymer chains attached to the protein and needed to effect its translocation has been investigated in relation to the inhibiting effect of a number of CT conjugates with different contents of polymer chains. Fig. 8 shows that the suppression of cell respiration by the conjugates begins to be manifested to an appreciable extent in the presence of not less than three polymer chains per protein molecule.

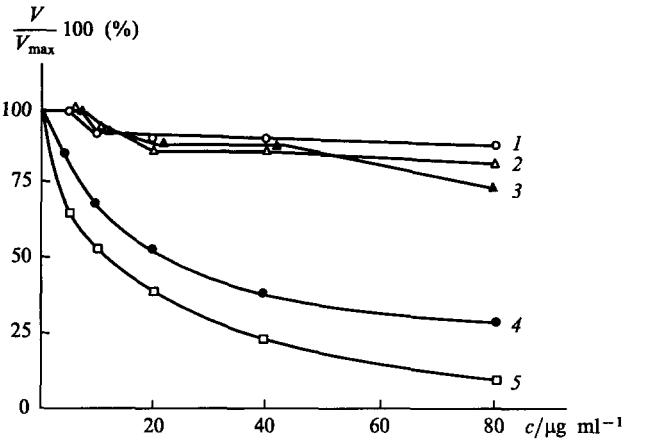


Figure 7. The influence of conjugates of proteins with various polymers on the rate of consumption of oxygen (V) by thymus lymphocytes: (*1*) initial CT; (*2*) $(PEO)_9$–CT; (*3*) $(OPro)_7$–CT; (*4*) $(Pro)_8$–BSA(nonun.); (*5*) $(Pro)_{10}$–CT(nonun.). The incubation medium for the lymphocytes contained (the concentrations are expressed as mM) NaCl (145), KCl (3.6), KH_2PO_4 (2), morpholinopropanesulfonic acid (8), and pyruvate (10); pH 7.4; T = 37 °C; the conjugates were added after oligomycin (0.2 μg ml^{-1}) and dinitrophenol (60 μM); in the control sample, the incubation medium was added instead of the conjugates to the lymphocytes; the concentration of lymphocytes in the cell was 10^8 cells in 1 ml.

Fig. 9 presents the results of a comparative study of the inhibiting effect of BSA-based conjugates, differing in the type of distribution of polymer chains on the surface of the protein globule, on the consumption of oxygen by T-lymphocytes. Evidently only the conjugates with a nonuniform distribution of chains cause a significant inhibition of respiration. CT conjugates with the corresponding structure exhibited the same effect.

As regards the mechanism of the action of conjugates on cell respiration, it is possible to examine several alternative versions: (1) the conjugates interact with the receptors located on the outer surface of the cell membrane and these receptors then transmit the signal to the cell; (2) the conjugates disrupt the cells; (3) the polymer chains are split off from the protein during the incubation of the conjugates with the lymphocytes and then

Table 5. The effectiveness of the suppression of the uncoupled respiration of lymphocytes and mitochondria by the Pro polymer and its conjugates with CT, cytochrome *c* (CYT), and BSA: $(Pro)_6$–CT(nonun.), $(Pro)_8$–CYT(nonun.), and $(Pro)_7$–BSA(nonun.).

Object of study	Pro	$(Pro)_6$–CT(nonun.)	$(Pro)_8$–CYT(nonun.)	$(Pro)_7$–BSA(nonun.)
	A. Concentration of Pro and the conjugates/μM (μg ml^{-1})			
Lymphocytes	4.9 ± 1.2 (9.8 ± 2.4)	0.66 ± 0.1 (30.6 ± 6.2)	1.1 ± 0.1 (33.0 ± 3.4)	0.75 ± 0.1 (61.0 ± 6.2)
Mitochondria	4.7 ± 1.2 (9.3 ± 2.4)	0.48 ± 0.06 (21.6 ± 4.7)	– –	– –
	B. Concentrations of free Pro and the Pro in the conjugates/μM			
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	–	–

Remarks. The absorption of oxygen was recorded in the presence of 50–60 μM DNP, 4 mM glutamate, and 1 mM malate. The result was calculated as the arithmetical mean from 5–6 experiments. The table lists the concentrations of Pro and the conjugates inducing a 50% inhibition of the consumption of oxygen. The efficiencies of free Pro and the Pro in the conjugate are compared in Part B of the table.

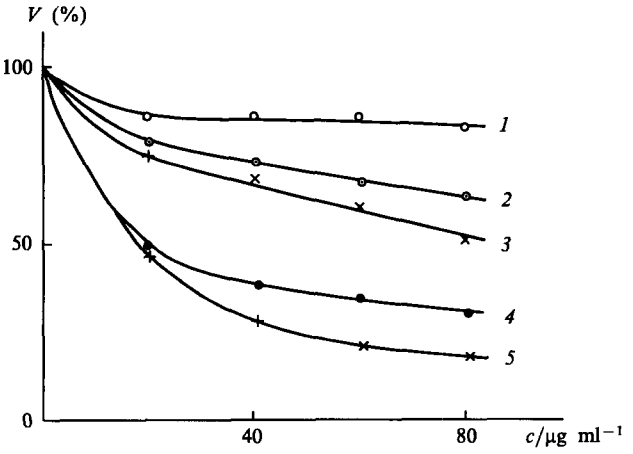


Figure 8. The influence of CT conjugates containing different numbers of Pro chains on the rate of consumption of oxygen by thymus lymphocytes: (*1*) $(Pro)_2$–CT(nonun.); (*2*) $(Pro)_3$–CT(nonun.); (*3*) $(Pro)_6$–CT(nonun.); (*4*) $(Pro)_{10}$–CT(nonun.); (*5*) $(Pro)_{15}$–CT(nonun.). For the experimental conditions, see Fig. 7.

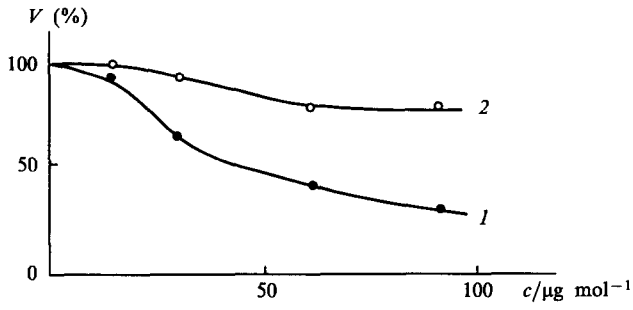


Figure 9. The influence of conjugates of BSA with a nonuniform (curve *1*) and uniform (curve *2*) arrangement of the Pro chains on the rate of consumption of oxygen by thymus lymphocytes: (*1*) $(Pro)_8$–BSA(nonun.); (*2*) $(Pro)_{15}$–BSA(un.). For the experimental conditions, see Fig. 7.

permeate through the cell membrane; (4) the conjugates permeate through the plasma membrane into the cell and reach the mitochondrial membranes.

The first hypothesis conflicts with the fact that the conjugates at virtually identical concentrations inhibit the separate respiration of both the cell as a whole and of an isolated mitochondrion. The substances capable of affecting the respiration of lymphocytes by interacting with the receptors on the outer surface of the plasma membranes, in particular with concanavalin A, increase the rate of their respiration only by 20% and do not affect the respiration of isolated mitochondria.[39]

It has been shown in special experiments that inhibition of the respiration of lymphocytes by proxanols and their conjugates is a reversible process and that it is fully restored after the cells have been washed. Consequently the inhibiting effects are not associated with the breakdown of the integrity of the plasma membranes of T-lymphocytes.

The separation of the polymer chains from the protein is also hardly likely to be the cause of the membrane activity of the conjugates. Indeed, on the basis of the idea that the $NH-CH_2$ bonds formed during the synthesis of the conjugate are hydrolytically stable and that their susceptibility to the action of hydrolytic enzymes is low, such separation appears fairly improbable. Nevertheless, in order to be able to reach the final conclusion that the polymer chains are not split off during the incubation of the conjugate with T-lymphocytes, an additional biological test of the ability of cells to cleave the conjugates has been carried out.[13] It confirmed with a high degree of reliability the absence of free proxanols in the supernatant after the separation of the cells. Thus one may conclude that the influence of the conjugates on the respiration of T-lymphocytes is the result of their permeation through the plasma membranes into the interior of the cells. Hydrophilic substances such as the proteins used to synthesise the conjugates are known to be incapable of permeating into the cells directly through the cell membranes. However, the same proteins acquire the capacity for translocation as a result of their being rendered hydrophobic by aliphatic acids.[40] The results permit the hypothesis that the modification of proteins by synthetic polymers (proxanols), capable of permeating through the cell membrane,[12] acts similarly to the addition of aliphatic acids. It must be emphasised that the necessary conditions for the translocation of such conjugates are the location of the hydrophobic blocks on the periphery of the conjugate molecule and also a nonuniform distribution of the polymer chains (Fig. 1, structure *IIa*). The type *IIb* and *III* conjugates of proteins with proxanols and the conjugates with PEO do not affect the respiration of cells. In certain cases, the PEO–protein conjugates may permeate into the cells via the endocytosis mechanism,[41] but the rate of this process is several orders of magnitude lower than that of the diffusion of the conjugate with membranotropic properties. Furthermore, endocytosis cannot occur in mitochondria.

Thus it has been shown that the covalent attachment of proxanols to hydrophilic proteins can serve as a method of imparting membranotropic properties to the latter.

V. Solubilising properties of the conjugates

1. The solubilisation of benzaflavin by conjugates of BSA with poly(alkylene oxides)

One of the important functions of serum albumins is their ability to transport nonpolar compounds of different chemical nature—aliphatic acids, hormones, and vitamins.[42] Bearing in mind the diphilic properties of proxanols described above, it might have been expected that their attachment to serum albumins would permit the creation of systems with an enhanced solubilising capacity in relation to water-insoluble biologically active substances. Such compounds include a dihydroriboflavin ester—5-acetyl-2′,3′,4′,5′-tetrabenzoyl-1,5-dihydroriboflavin [benzaflavin (BF)]. Dihydroriboflavin esters have a broad spectrum of biological activity and are of interest not only as long-acting vitamin B_2 preparations but also as medicinal drugs for metabolic therapy.[43]

The solubilisation of BF in aqueous solutions of BSA and its conjugates with poly(alkylene oxides) has been observed by UV spectroscopy at a wavelength of 306 nm, which corresponds to the BF absorption maximum. The extinction coefficients of the solubilised BF in a solution of pure BSA and of the $(PEO)_{14}$–BSA and $(Pro)_5$–BSA(nonun.) conjugates were determined from the dependence of the optical density on the concentration of the solubilised BF.

Protein, conjugate	BSA	$(PEO)_{14}$–BSA	$(Pro)_5$–BSA(nonun.)
10^{-3} ε/litre mol^{-1} cm^{-1}	4.9	4.7	3.5

The solubilisation limit (p), equal to the number of molecules of the solubilised substance per molecule of the solubilising agent, was determined by high-speed sedimentation in an analytical ultracentrifuge with an optical recording system. The use of the sedimentation method was based on the fact that the bound BF undergoes sedimentation together with the protein or the

conjugate.[13] The fact that the absorption by BF corresponds to a longer-wavelength part of the spectrum compared with BSA and the conjugates makes it possible to select a wavelength where only the bound ligand absorbs and its concentration can be determined.

In order to compare the solubilising capacities of BSA and the conjugates, a series of experiments were carried out in which the BF concentration was varied for a constant content of the protein or the conjugate. It is seen from the results that BSA has an appreciable solubilising capacity in relation to BF (Table 6). It was demonstrated by special experiments that proxanols do not solubilise BF. On the other hand, in all the systems containing the conjugates, the BF solubilisation limit proved to be higher than for BSA, the highest value corresponding to the $(Pro)_5$–BSA(nonun.) conjugate, in which the hydrophobic blocks are located on the periphery of the conjugate.

An increase in the number of polymer chains per protein molecule in the conjugate leads to a decrease in the solubilisation limit. This apparently occurs owing to the greater shielding of the regions responsible for the solubilisation of BF. Two procedures for the solubilisation of BF were used in the above investigation — by suspending BF and by introducing it into the above systems from an alcohol solution. The second procedure makes it possible to include a greater amount of BF, probably for kinetic reasons. The study by the nonelastic light scattering method of the system obtained by both procedures showed that, together with the particles whose size corresponded to the conjugate present in the system (less than 30 nm), larger particles with radii from 200 to 3000 nm (possibly BF aggregates) were observed when BF was introduced from an alcoholic solution. On solubilisation by the suspension method, only particles with radii less than 30 nm are formed.

In order to obtain systems with the optimum BF content, a study was made of the dependence of the solubilising capacities of BSA and the $(Pro)_5$–BSA(nonun.) conjugate on the pH of the medium (Fig. 10). The figure shows that the dependence is bell-shaped. The maximum solubilisation is observed in the pH range 7.6–9.0. Study of the dependence of the solubilisation limits on the phosphate buffer concentration (pH 6.2 and 7.2) showed that an increase in the buffer concentration impairs the solubilising

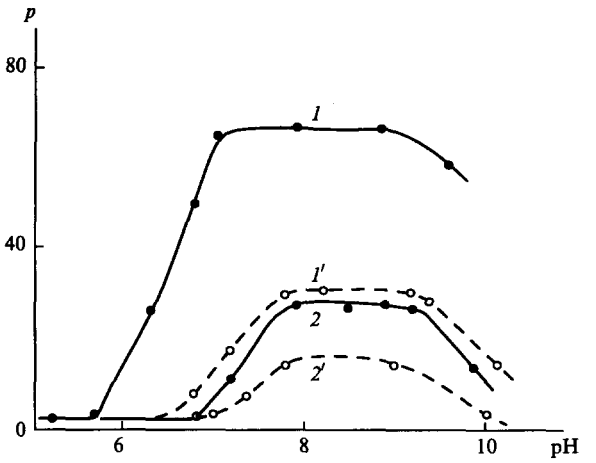


Figure 10. Dependence of the BF solubilisation limit on the pH of the medium: (*1*) visual method; (*2*) sedimentation method; BSA — dashed line; $(Pro)_5$–BSA(nonun.) conjugate — continuous lines.

capacities of both the protein and the conjugate. Thus, the attainment of the maximum solubilising effect requires the use of solutions in the pH range 7.6–9.0 at the lowest ionic strength.

Experiments in which the solubilisation of BF in solutions of BSA and the conjugates was investigated at an elevated temperature (40 °C) made it possible to observe an appreciable heterogeneity of such systems in contrast to the systems containing the pure solubilising agent (Table 6). A definite fraction of the substance undergoing sedimentation in such systems is characterised by a sedimentation coefficient exceeding by a factor of approximately two the corresponding sedimentation coefficients of the protein and the conjugates. It may be that the BF in such systems promotes the formation of 'bridges' between the protein molecules.

The results suggest that the BF molecules in the conjugates investigated are localised in the hydrophobic regions of the BSA

Table 6. The solubilising properties of BSA and its conjugates with poly(alkylene oxides) in relation to BF (0.02 M phosphate buffer, pH 7.2; the BF was introduced from alcoholic solution).

Solubilising agent	T/°C	p	s_{20}/Sv (solubilising agent)	s_{20}/Sv (complex with BF)	Fractions of different forms (%)
BSA	20	17	4.2	6.3	100
	40	17	6.7	11.0	47
				6.2	53
$(Pro)_5$–BSA(nonun.)	20	40	4.4	10.1	100
	40	40	6.8	14.2	21
				7.3	79
$(OPro)_{11}$–BSA	20	19	4.2	6.8	100
	40	19	6.6	13.2	47
				5.9	53
$(PEG)_{14}$–BSA	20	21	4.2	6.6	100
	40	21	6.5	10.5	49
				6.8	51
$(Pro)_{100}$–BSA[a]	20	18	2.9	6.2	100
	40	18	5.8	12.0	80
				6.6	20

[a] The solubilising agent was obtained from the conjugate $(Pro)_5$–BSA(nonun.) by adding free proxanol.

protein globule. This is also confirmed by the finding that the attachment of proxanol to other proteins, for example to class G immunoglobulins or to CT, which are unable to bind this ligand, has no effect on their solubilising properties.

2. The transfer of benzaflavin into mitochondria with the aid of BSA conjugates

The data presented in this Section presuppose the possibility of using the conjugates of proteins with poly(alkylene oxides) as agents transporting water-insoluble biologically active substances into the target cells. In order to test this hypothesis, a study was made of the transfer of benzaflavin into rat heart mitochondria.[15] This phenomenon was observed by studying the relation between the amount of BF bound by the mitochondria and the amount of BF present in solution in the solubilised form. The amount of BF bound by the mitochondria was determined from the difference between the total content of BF in solution and its content in the supernatant after incubation with the mitochondria. This quantity reaches a maximum after 1 min and remains constant on further incubation with the mitochondria.

It was observed that the BF solubilised by BSA and its conjugates with PEO and Pro is capable of being transported into the mitochondrial membranes. Quantitative data on the transfer of BF are presented by the binding isotherms in Fig. 11. In the BF concentration range investigated (up to 8×10^{-4} M), the dependences of the amount of bound BF on the concentration of free BF are linear:

$$\frac{[BF]_{bound}}{[MC]} = K[BF]_{free}, \quad (3)$$

where K is the proportionality coefficient, which depends on the nature of the solubilising agent, and [MC] is the concentration of the mitochondrial protein. The linearity of these relations showed that the experimental data correspond to the initial sections of the binding isotherms. It is important to emphasise that the experimental points obtained for different concentrations of the solubilising agent lie on the same isotherm characteristic of the given solubilising agent. Thus the proportionality coefficient in Eqn (3) is independent of the concentration of the solubilising agent.

It is of interest that BSA and its conjugate with PEO proved to have identical capacities to transport BF. Both solubilising agents may be characterised by the same proportionality coefficient $K = 0.31 \pm 0.01$ litre (g of mitochondrial protein)$^{-1}$. For the conjugate of BSA with proxanol, $K = 0.96 \pm 0.01$ litre (g of mito-chondrial protein)$^{-1}$. The higher capacity of the BSA–proxanol conjugate to transport BF can apparently be explained by the formation of a weaker complex between BF and this conjugate than the complex between BSA and its conjugate with PEO.

Thus the BSA–proxanol conjugates have definite advantages over other systems investigated as regards both their solubilising and transport capacities, which creates preconditions for their employment in the development of new medicinal preparations.

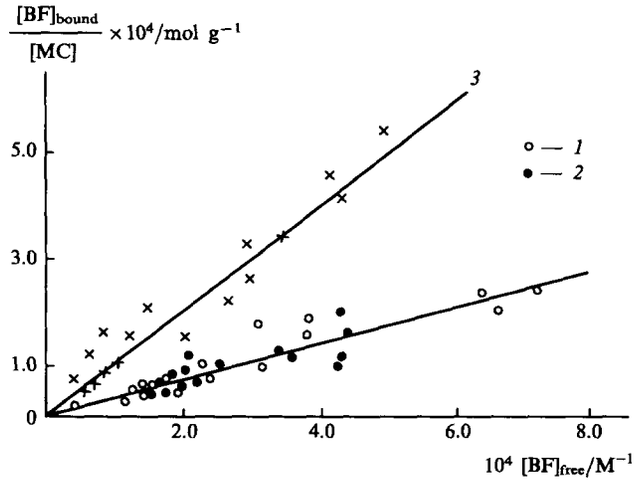


Figure 11. Dependence of the amount of bound BF [in moles per gram of the mitochondrial protein (MC)] on the concentration of free BF. Solubilising agent: (*1*) BSA; (*2*) $(PEO)_{14}$–BSA; (*3*) $(Pro)_5$–BSA(nonun.).

VI. Noncovalent complexes between α-chymotrypsin and the block-copolymer of ethylene and propylene oxides

The complex formation reactions between proteins and water-soluble proteins constitute one of the effective methods in protein engineering. Thus many synthetic linear polyelectrolytes form complexes with proteins.[44,45] Protein molecules are bound to polyions by electrostatic interaction, which may be accompanied by the formation of hydrogen bonds and hydrophobic contacts. In contrast to this, water-soluble polymers which contain no ionogenic group, for example PEO, do not form complexes with proteins.[46] When solutions of BSA or CT are mixed with solutions of proxanols at room temperature, complexes are not produced either.[21]

As mentioned above, we demonstrated in relation to CT that stable protein–proxanol complexes can nevertheless be obtained when their solutions are heated to temperatures corresponding to the onset of the thermal denaturation of the protein (this problem has been examined in greater detail elsewhere[46]).

The first results indicating the formation of mixed CT–Pro particles were obtained with the aid of a visual method for the observation of the turbidity of solutions of a protein and poly(alkylene oxide). The formation of such particles is also indicated by data obtained by the DSC method for aqueous solutions of CT, CT–PEG, and CT–Pro. Fig. 12 presents thermograms for the fusion of CT (curve *1*), CT in the presence of PEO (curve *2*), and CT in the presence of Pro (curve *3*). Evidently PEO does not affect the fusion of the protein globules: the fusion curves *1* and *2* virtually coincide (the heat of fusion is 1090 kJ mol^{-1} in the former case and 1080 kJ mol^{-1} in the latter). In contrast to this, in the presence of Pro, a shift of the fusion peak towards higher temperatures and an increase of its height (the heat of fusion is

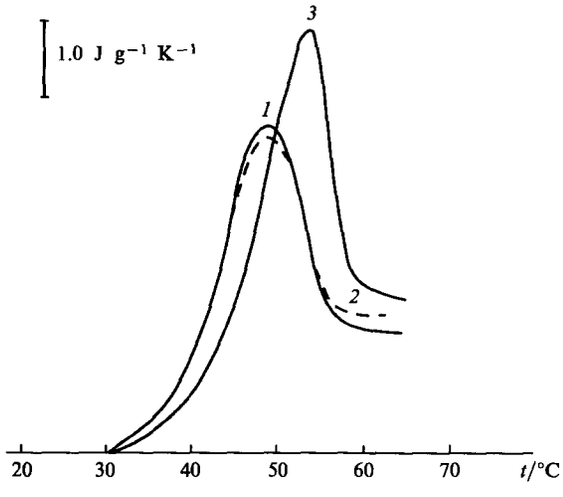


Figure 12. Heat absorption curves for CT (concentration 2 mg ml^{-1}, curve *1*), for a mixture of CT and PEO (2 mg mol^{-1} and 0.48 mg ml^{-1}, curve *2*), and for a mixture of CT and Pro (2 mg ml^{-1} and 0.48 mg ml^{-1}, curve *3*). Buffer—0.02 M sodium phosphate, pH 7.0; heating rate 1 K min^{-1}.

Table 7. Characteristics of the CT–Pro complexes (heating rate 1 K min^{-1}, initial temperature of the solution 20 °C, incubation time in the thermostat before the attainment of the upper limit in the heating process 5 min).

Complex	Preparation conditions		Content of polymer in complex/mol (mol of CT)$^{-1}$	Activity (% of initial protein)		Content of active centres (%)	10^5 K_m/M[a]	k_{cat}/s^{-1}
	upper limit in heating process/°C	CT:Pro molar ratio		before isolation	after isolation			
CT–$(Pro)_{3.5}$	44	1:2.5	3.5	–	50	–	–	–
CT–$(Pro)_5$	44	1:10	5	82	120	–	–	–
CT–$(Pro)_5$	44	1:20	5	87	125	82	7.1+0.8	25+3
CT–$(Pro)_5$[b]	44	1:20	5	76	118	83	9.5+0.7	27+1
CT–$(Pro)_6$	48	1:20	6	50	97	74	13.2+0.8	30+2
CT–$(Pro)_7$	48	1:20	7	45	92	57	16.7+0.8	32+2
CT–$(Pro)_{10}$[b]	48	1:20	10	34	79	–	–	–
CT–$(Pro)_{10}$	52	1:20	10	22	74	32	13.9+0.6	48+4
CT–$(Pro)_{13}$	52	1:20	13	26	51	14	18+2	166+19
CT–$(Pro)_{11}$	60	1:20	11	0	0	–	–	–
Native CT		0	–	100		92	5.6+0.4	24+2

[a]The kinetic constants were obtained by studying the enzymic hydrolysis of BTEE (pH 8.0; 25 °C). The errors were determined with a fiducial probability of 0.95.

[b]The complexes were cooled and isolated immediately after the attainment of the upper temperature limit of the heating process without subsequent incubation in a thermostat.

1250 kJ mol^{-1}) are observed. Hence it follows that during heating the CT and Pro are bound to one another in some way, giving rise to a new product, in which the protein component acquires a somewhat higher thermal stability. The thermally induced binding of CT and Pro is irreversible under the experimental conditions. After such solutions have been cooled, the mixed reaction product is preserved in them, which is indicated by the determinations of the protein content in the products isolated (Table 7) (the method of isolation of these products from the reaction mixture ensured the complete removal of the excess free polymer, which was checked by thin-layer chromatography.)

The question of the nature of the interactions between Pro and the protein is of fundamental importance. The hypothesis of the formation of a noncovalent complex is most natural. In order to determine the nature of the bonds between Pro and CT, we studied the dissociation of the complexes in the presence of urea by thin-layer chromatography (TLC). The method is based on the difference between the chromatographic mobilities of the dissociation products and the initial complex. The rate of dissociation of the complexes in aqueous solutions is low and the dissociation becomes appreciable only after 24 h. The process accelerates significantly in the presence of 8 M urea. These data show that the reaction products are noncovalent complexes between CT and Pro.

On the basis of ideas concerning the complex formation process, one may postulate that the composition of the complexes may be regulated in two ways: by varying the polymer/CT ratio at a fixed heating temperature and by varying the heating temperature for a fixed reactant ratio. Indeed, Table 7 shows that, for a fixed excess of Pro in the system, the number of polymer chains entering into the composition of the complex increases in parallel with the increase in the maximum heating temperature. For example, in the system with a 20-fold molar excess of Pro, the number of polymer chains entering into the composition of the complex increases from 5 to 11 when the upper temperature limit of the heating process rises from 44 to 60 °C. The same tendency towards an increase in the number of polymer chains in the complex is observed also with increase in the molar excess of the polymeric component for a fixed upper temperature limit of the heating process.

We may note that the protein fraction isolated from a mixture of CT and PEG after heating to 60 °C represents the pure protein, which indicates the absence of interaction between the components. This conclusion agrees well with the results of differential scanning calorimetry.

Fig. 13 presents the ^{1}H NMR spectra of pure CT and two of its complexes with Pro obtained by heating to 44 and 48 °C respectively. Evidently, with increase in the temperature of the formation of the complexes, the denaturation changes in the protein become more appreciable, although a complete breakdown of the native structure is not observed. On the other hand, the positions and width of the signals due to the polymeric component of the complex indicate a fairly high molecular mobility of the majority of the units of the polymer chains in the complex.

A notable feature of the complexes is that they retain enzyme properties. It was of interest to elucidate how the specific enzyme activity of the complexes depends on the conditions of their preparation and isolation. Table 7 presents data on the enzyme activity of the complexes directly after the completion of the heating process but before their isolation from the reaction mixture. They reflect the properties of the complexes closest to those at the instant of their formation. The enzyme activity of the complex was measured also after their isolation from the reaction mixture. Comparison of the activities of the complexes before and after their isolation shows that the activity of the isolated complexes was higher for all the systems investigated. It may be that this is associated with the fact that the molecules of the free Pro, present in excess, form micellar structures incorporating CT. As a result of this, the active centre of the enzyme becomes less accessible to the substrate. During the isolation of the complex from the solution, the micellar structures are disrupted, which leads to a greater accessibility of the enzyme active centre to the substrate. It is important to note that in certain cases an increase in the enzyme activity of the isolated complexes compared with the initial CT was actually observed. Similar superactivity phenomena had been observed earlier in a study of the enzymic hydrolysis of the ethyl ester of *N*-acetyl-L-tyrosine catalysed by CT in inverted micelles.[48–50]

It was therefore of interest to determine the kinetic constants for the hydrolysis of BTEE catalysed by the complexes and to compare them with the corresponding constants for the initial CT. The contents of the active centres in the complexes were determined simultaneously. The corresponding data are presented in Table 7. Evidently, with increase in the upper

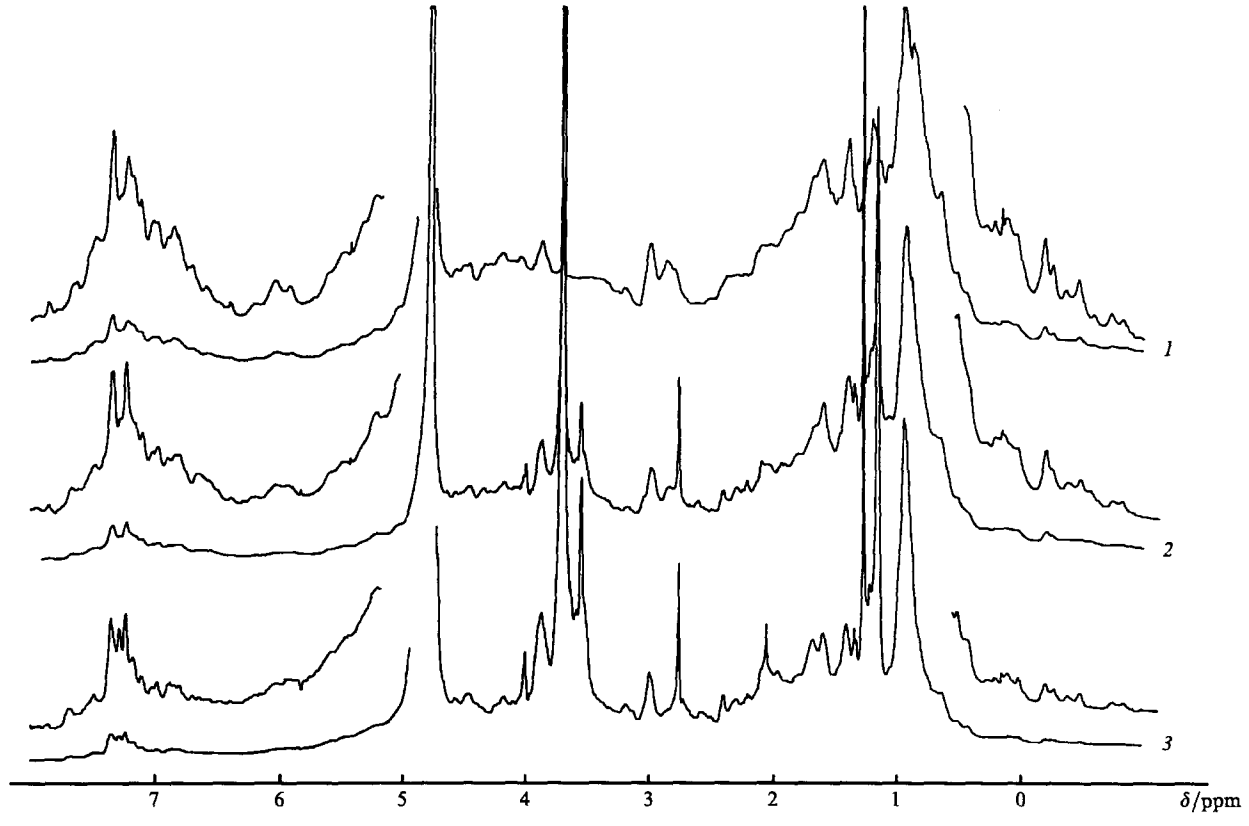


Figure 13. ^{1}H NMR spectra of solutions of CT (curve *1*), the CT–$(Pro)_5$ complex obtained by heating to 44 °C (curve *2*), and the CT–$(Pro)_7$ complex obtained by heating to 48 °C (curve *3*) in D_2O; concentration of protein in the specimen 2 mg ml^{-1}.

temperature limit in the heating of a mixture of CT and Pro and also in the incubation time for a fixed temperature limit in the heating process, the content of the active centres in the complex diminishes. The values of K_m increase steadily in the same sequence, which indicates a decrease in the affinity between the complex and the substrate. On the other hand, the increase in k_{cat} for the same series of catalysts indicates an increase in the catalytic effectiveness of the CT active centres entering into the composition of the complex.

On the basis of the overall results obtained, the mechanism of the formation of complexes between Pro and CT can be represented as follows. On heating, the protein globule uncoils, as a result of which the hydrophobic aminoacid residues appear on the surface of the protein. This is accompanied by the simultaneous dehydration of the hydrophobic Pro blocks, which promote the formation of compatible polymer–protein structures. During cooling and during the isolation of the complex from solution, sections of the hydrophobic Pro block are fixed near the protein globule via a multipoint interaction with the hydrophobic and polar groups located on the surface of the protein. The fact that the dissociation of the complex intensifies in the presence of a denaturing agent (urea) does not rule out the possibility that, together with the hydrophobic interactions, hydrogen bonds between Pro and the protein globule make a significant contribution to the stabilisation of the complex.

Thus the thermally induced interaction of Pro with CT leads to the formation of a new type of supermolecular structure—stable catalytically active complexes. The simplicity of the method of preparation of such structures and the retention of functional properties by the latter makes it possible to regard this procedure for the modification of proteins as one of promising methods in protein engineering.

VII. Conclusion

The methods developed for the preparation of conjugates of proteins and proxanols make it possible not only to vary within wide limits the structures and physicochemical properties of the products but also to simulate with their aid the biological functions of membranotropic proteins. The properties of the conjugates described above permit their use in enzyme therapy, in the creation of new therapeutic agents, and in the manufacture of biocatalysts for biotechnology soluble in organic media. Furthermore, these compounds may find applications in biochemical studies on water-insoluble biologically active compounds. The proposed alternative method for the modification of proteins by proxanols, based on the formation of nonvalence complexes between them, constitutes a new approach to the preparation of proteins modified by polymers. The set of covalent and noncovalent methods for the modification of proteins leads to extensive possibilities for the preparation of polymer–protein adducts with specified properties.

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