and horse blood serum butyrylcholinesterase have been investigated.

It has been established that a change in the structure of the organophosphorus compounds has a definite effect on the hydrophobic interaction of the enzymes with the inhibitor.

The effect of such interaction is connected with the presence of hydrophobic regions in the neighborhood of the anionic points of the cholinesterases, the structures of which differ in ACE and BuCE.

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COVALENT IMMOBILIZATION OF HEPARIN ON A COLLAGEN FILM

T. I. Velichko, A. N. Shtopenko, N. V. Fedoseeva, and G. S. Katrukha

UDC 547.962.9:615.33

Four procedures for the covalent immobilization of heparin (Hp) on a collagen film (CF) have been investigated. In three of them (methods (I-A, B, C), the CF was first treated with epichlorohydrin and ammonia and the Hp was added with the aid of CMBC (method I-A), by reductive amination in the presence of NaCH CN (method I-B), and with the aid of CMEC after succinylation (method I-C). In the fourth procedure (method II), the CF was activated by treatment with alkali and the Hp was added with the aid of CMEC. It was shown that the maximum amount of Hp was immobilized by method II.

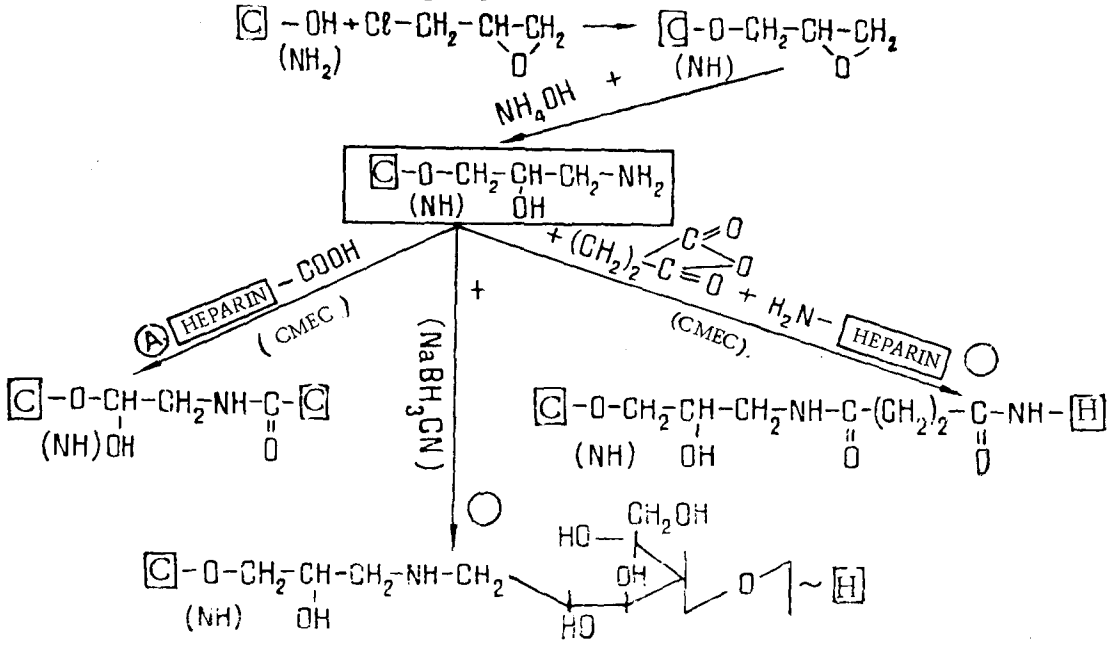
At the present time, heparin-containing materials are being investigated intensly [1]. The ionic and covalent immobilization of heparin on various supports is being studied most widely. Synthetic or natural polymers are used as supports for the covalent immobilization of heparin [2]. Over a number of years, we have been investigating the covalent immobilization of heparin on collagen — a natural fibrillar protein which is free from a number of the unfavorable properties of synthetic polymers.

It is known that heparin — a natural sulfated acidic polysaccharide — is an antigoagulant with a broad action spectrum and a regulator of many biochemical and physiological processes taking place in the organism. Heparin fixed to a polymer retains its anticoagulant properties.

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 700-704, September-October, 1987. Original article submitted February 5, 1987.

0009-3130/87/2305-0582$12.50

For the covalent immobilization of heparin on collagen it is possible to make use of its free carboxy, hydroxy, or amine groups.



II. $\boxed{C}\xrightarrow{NaOH}\boxed{C}-COOH\xrightarrow[(CMEC)]{H_2N-\boxed{HEPARIN}}\boxed{C}-CO-NH-\boxed{H}$

Methods for the Covalent Immobilization of Heparin on Collagen

To obtain covalent complexes of the collagen–heparin type we investigated four procedures for immobilizing heparin, which are shown in the scheme [3-5]. As can be seen from the scheme, in method I (reactions A, B, and C) the immobilization of the heparin on the collagen is preceded by modification of the film with epichlorohydrin followed by the opening of the oxide ring with ammonia. Amino acid analysis of the modified film showed that the lateral functional groups of the hydroxyproline, serine, tyrosime, lysine, histidine, and arginine residue of the collagen took part in the reaction with epichlorohydrin, their amount decreasing appreciably after modification and complete hydrolysis (Table 1). As a result of this reaction we obtained a collage film enriched with free NH_2 groups separated from the polypeptide chain by an additional three CH_2 units.

TABLE 1. Amounts of Amino Acids and Glucosamine, nmole/mg of Collagen

Amino acid	Initial collagen	After treatment with epichlorohydrin and ammonia	After reaction I-A*	After reaction I-B*	After reaction I-C*	By method II*
Hyp	747	443	506	—	498	—
Asp	354	374	357	—	341	—
Thr	130	128	127	—	119	—
Ser	262	115	183	—	170	—
Glu	629	640	595	—	594	—
Pro	1018	1268	1364	—	1231	—
Gly	2624	2725	2678	—	2544	—
Ala	851	927	851	—	814	—
Cys	18	22	16	—	18	—
Val	158	161	147	—	139	—
Met	40	—	—	—	—	—
Ile	85	81	87	—	81	—
Leu	198	209	193	—	183	—
Typ	10	—	—	—	—	—
Phe	97	93	92	—	86	—
Lys	163	9	17	—	14	—
His	37	—	—	—	—	—
Arg	358	90	219	—	222	—
Glucosamine	—	—	55	20	18	36

*See scheme.

TABLE 2. Amount of Immobilized Heparin on a Collagen Film after Covalent Binding, mg/g

Form of the collagen film	Reaction			Method II*
	A*	B*	C*	
Without modification by epichlorohydrin	15,2	1,5	6,85	—
After modification with epichlorohydrin and ammonia	47,1	17,4	13,5	—
After activation with a 0,1 N solution of caustic soda	—	—	—	30,5

*See scheme.

Modification of a collagen film with epichlorhydrin followed by the addition of amino groups permitted immobilization of heparin to be performed with the aid of CMEC (reaction I-A), by succinylation (reaction I-C), and also by reductive amination in the presence of sodium cyanotrihydroborate (I-B). In the case of method II, the activation of the carboxy groups of the collagen with the aid of CMEC was performed on a film that had previously been kept in an 0.1 N solution of NaOH at 37°C.

Table 2 gives comparative results on the amount of heparin on collagen film with various methods for its immobilization. The two-stage modification of a collagen film first with epichlorohydrin and then with ammonia substantially increased the amount of immobilized heparin. It is quite likely that after such treatment "bridges" are created which eliminate the steric hindrance to the condensation reaction and to which the heparin may be covalently attached. Such a mobile "bridge," as it is assumed, permits the heparin to exhibit the properties of an anticoagulant more completely, since its functional groups are remote from the protein substrate and are capable of interacting with the proteins of blood plasma — fibrinogen, thrombin, fibrinolysin, albumin.

The preliminary activation of collagen films in a weak solution of alkali or acid by method (II) (see scheme) also increased the amount of immobilized heparin. It may be assumed that as the result of such treatment the number of reactive groups of the protein was increased through the partial breakdown of the microstructure of the fibrils of the surface layer of the film, and also by the hydrolysis of $CONH_2$ groups to free COOH groups, which were then activated by CMEC.

EXPERIMENTAL

Industrial samples of collagen film with a thickness of 0.1 mm were used. To identify the collagen its amino acid composition was determined. The results of the amino acid analysis agreed basically with literature figures [6].

For immobilization, a domestic preparation of heparin (from lungs) with an activity of 140 units/mg was used. The individuality of the preparation was determined by chromatography and with the aid of the amino acid analysis of samples of the heparin.

The water-soluble carbodiimide 1-cyclohexyl-3-[2-(N-methylmorpholino)ethyl]carbodiimide p-toluenesulfonates (CMBC) (Sigma, USA) was used to activate the carboxy groups of the collagen.

In the reductive amination method, sodium cyanotrihydroborate ($NaBH_3CN$, Serva) was used.

The completeness of succinylation was checked by the reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS, Serva) [7].

Modification of Collagen Films with Epichlorohydrin and Ammonia According to the Scheme (Method I). Collagen films with dimensions of 3 × 5 cm were placed in a solution containing 90 ml of water, 39 ml of 2 M HaOH, and 9 ml of epichlorohydrin and were thermostated with shaking at 90°C for 7 h. Then the films were repeatedly and carefully washed with water and were kept in 25% of ammonia at 40°C for 5 h. The excess of ammonia was washed off with water to a neutral reaction. The modified films obtained were dried. A sample of film (6 mg) was hydrolyzed under standard conditions (6 N HCl, 105°C, 24 h) and its quantitative amino acid composition was determined on a Hitachi amino acid analyzer 835 (Table 1).

Immobolization of Heparin on a Collagen Film by the CDI Method Using Reaction I-A. Two collagen films each weighing 278 mg that had been modified with epichlorohydrin and ammonia as described above were placed in a solution containing 12 ml of water and 2 g of heparin; the pH was adjusted to 4.75 with 0.1 N hydrochloric acid and then, with continuous stirring, a 2.8% solution of CMEC was added in three 12-ml portions, the pH being maintained at 4.75 with a 0.1 N solution of HCl. The films were kept at 4°C for 12 h and were then washed with water free from the excess of reagents. Samples of dried films (4-6 mg) were hydrolyzed under standard conditions to determine their quantitative amino acid compositions and glucosamine contents (Table 1).

Immobilization of Heparin by the Method of Reductive Amination using Reaction I-B. Two collagen films weighing 278 mg that had been modified with epichlorohydrin and ammonia were treated with 30 ml of a 0.2 N solution of K_2HPO_4 (pH 8.5) containing 1 g of heparin and 0.29 g of $NaBH_3CN$. The films were kept at room temperature for 16 days and were then washed with water free from adsorbed heparin and the reagents. A part of the dried films (6-7 mg) was hydrolyzed under standard conditions to determine the glusosamine content (Table 1).

Succinylation of Collagen Films (Reaction I-C). To four collagen films each weighing 278 mg that had been modified with epichlorohydrin and ammonia and that were immersed in 100 ml of NaCl solution was gradually added 5.33 g of dry succinic anhydride, a pH of 6.0 being maintained with a 20% solution of NaOH. The films were shaken in the resulting solution at 20°C for 12 h, and the completeness of succinylation was checked by the reaction with TNBS. Because of the only partial succinylation of the free NH_2 groups, another portion (5.33 g) of succinic anhydride was added and the reaction was continued for four days. The films were washed with a 0.1 M solution of NaCl and then with water until the adsorbed succinic anhydride had been completely eliminated. Part of the dried samples (7-8 mg) was hydrolyzed under standard conditions and its quantitative amino acid composition was determined (Table 1).

Immobilization of Heparin on Succinylated Collogen Films Using Reaction I-C. Two succinylated films, each weighing 278mg and preliminarily modified with epichlorohydrin and ammonia (gas), were placed in 12 ml of water containing 2 g heparin (the pH of the water was adjusted to 4.75 with 0.1 N hydrochloric acid). Three portions (12 ml each) of a 2.8% solution of CMEC were then added — the pH being maintained at 4.75 with 0.1 N HCl solution. The films were left to stand at 4°C for 12h and were then washed with water to remove excess heparin and CMEC. The dried samples (4-6 mg) were then hydrolized under the standard conditions for determining the glucosamine content (Table 1).

Activation of Collagen Films and Immobilization of Heparin by Method II (see Scheme). Six samples of collagen films each weighing 278 mg were placed in 100 ml of a 0.1 N solution of NaOH and the mixture was thermostated at 37°C for 2 h, after which the films were washed with water to neutrality and were placed in 140 ml of a 0.02 M solution of CMEC, the pH being maintained by 4.6 by means of a 0.1 N solution of hydrochloric acid with continuous stirring. After 8 h, the films were carefully washed with water and were kept in 50 ml of water containing 2 g of heparin at 4°C for 14 days, after which they were carefully washed with water from adsorbed heparin and reagents. Part of the dried samples (4-5 mg) was hydrolyzed under standard conditions for determining the glucosamine content (Table 1).

CONCLUSIONS

1. Collagen preparations containing immobilized heparin have been obtained.

2. It has been shown that the preliminary modification of collagen with epichlorohydrin and ammonia in one case, and also preliminary activation with alkali in another case, appreciably increased the amount of immobilized heparin.

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