IMMOBILIZATION OF THE ENZYME *L*-ASPARAGINASE FROM *E. coli* ON POLYSACCHARIDES. VI. PREPARATION AND PROPERTIES OF POLYMERIC DERIVATIVES BASED ON DEXTRAN CARBONATES

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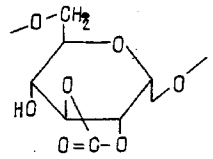
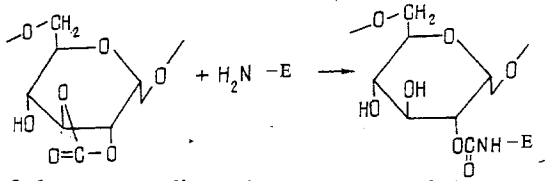
Methods have been developed for obtaining soluble and insoluble dextran carbonates. The addition of the latter to E. coli L-asparaginase has given water-soluble, gel-like, and insoluble forms of the immobilized enzyme. The influence of the bound polymer on some physicochemical properties of L-asparaginase has been determined. The antileukemic action of soluble dextran derivatives of L-asparaginase has shown that their efficiency is greater than that of the native enzyme.

We have previously reported the immobilization of *E. coli* *L*-asparaginase on hydroxypropyldextran and dialdehyde-dextran [1, 2]. In the present paper we give information on the covalent combination of the enzyme with cyclic dextran carbonates.



Methods are known for introducing *trans*-cyclic carbonate groups into *β-D*-glycopyranose residues of insoluble polysaccharides such as starch and cellulose by the action of ethyl chloroformate on them in the presence of triethylamine, followed by binding with certain enzymes [3-5]. We have used this method for binding the *ε*-amino groups of *L*-asparaginase with dextran carbonates to form a urethane bond between the enzyme and the polysaccharide.



In addition to the formation of the *trans*-cyclic carbonate groups of the polysaccharides, giving a characteristic absorption maximum of 1810 cm^{-1} in the IR spectra, partial replacement of some hydroxycarbonyl groups by ethoxycarbonyl groups giving an ester band at 1760 cm^{-1} usually takes place. A comparative study of the IR spectra of dextran modified in this way has permitted a qualitative estimate of the amount of carbonate groups necessary for subsequent binding with *L*-asparaginase and for selecting the conditions for modifying the polymer with the maximum content of cyclic groupings. By changing the standard conditions for activating polysaccharides with ethyl chloroformate we obtained water-soluble and water-insoluble dextran carbonates. The factor responsible for the solubility of the latter, with the optimum choice of the reaction time (10 min) proved to be the amount of ethyl chloroformate added. The use of 3.5 ml gave dextran carbonates having high solubility, while an increase in the amount of ethyl chloroformate (3.7 and 4.0 ml) led to a lowering of the solubility, and at 4.5 ml no solubility was observed.

In its IR spectrum, native dextran has a single characteristic absorption band in the 1640 cm^{-1} region. The IR spectra of samples of modified dextran (Nos. 1-4) showed, in addition, the presence of strong absorption bands of cyclic carbonate

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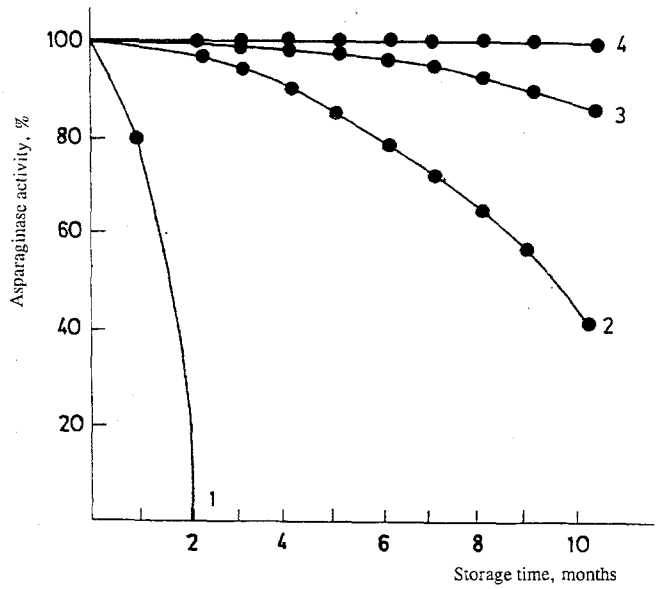

Fig. 1. Stability on storage of native *L*-asparaginase (1) and of samples of immobilized *L*-asparaginase: soluble (2), gel-like (3), and insoluble (4).

TABLE 1. Characteristics of *L*-Asparaginase Bound with Various Forms of Dextran Carbonate

Number of the dextran carbonate sample	Solubility of the immobilized form of L-asparaginase	Retention of enzymatic activity, %	Amount of enzyme bound with 1 g of dextran, g
1	Soluble	25	0,20
2	Soluble	24	0,25
3	Gel-like, insoluble	21	0,11
4	Insoluble	20	0,09

groups, with a rise in the peak intensity of the bands for the soluble samples (Nos. 1 and 2), and also very weak absorption bands of acyclic ethoxycarbonyl groups. This presupposed the possibility of the subsequent immobilization of *L*-asparaginase. In actual fact, the addition of the corresponding dextran carbonates to the enzyme gave water-soluble, gel-like, and insoluble forms of polymeric conjugates of *L*-asparaginase, the activities of which were between 20 and 25% of the initial activity of the native enzyme (Table 1).

The weight ratios of dextran carbonates and *L*-asparaginase were selected here on the basis of the complete binding of the components, which was monitored by electrophoresis in polyacrylamide gel and by gel chromatography, as described in [6], and, for the insoluble products, from the maximum achievable amount of enzyme added to the support [7]. The fact of the chemical binding of the carbonate groups of the dextrans with the *L*-asparaginase was also confirmed by the IR absorption spectra of the immobilized forms of the enzyme, which showed the complete disappearance (sample Nos. 1 and 2) or a marked decrease to a minimal value (sample No. 3) of the absorption bands of free cyclic carbonate groups.

As was expected, the immobilization of the *L*-asparaginase led to a considerable increase in the stability of the enzyme on storage in buffer systems (Fig. 1). At the same time, at elevated temperatures, the stabilization effect was less pronounced (Fig. 2). In all cases the greatest stability was possessed by the insoluble form of the immobilized *L*-asparaginase.

The pH ranges of the enzymatic action of the native and dextran-bound enzymes differed little qualitatively from one another (Fig. 3), although a more pronounced peak of the enzymatic activity of the isomeric form of *L*-asparaginase in the pH 4.5-5.5 region was characteristic for the immobilized form. However, neither these slight changes nor the fall in the enzymatic activity of *L*-asparaginase as a result of immobilization lowered the biological activity of the enzyme. As shown in Table 2, the antileukemic action of the immobilized *L*-asparaginase (which was studied experimentally on mice) was more considerable than that of the native enzyme. This made it possible to increase the therapeutic effect as far as 100% cure of the experimental animals even at small therapeutic doses, and this means that the method described above for the chemical modification of *L*-asparaginase by dextran will permit a soluble stabilized form with an increased antileukemic activity or an insoluble form of the enzyme necessary for performing various enzymatic and biological studies to be obtained selectively.

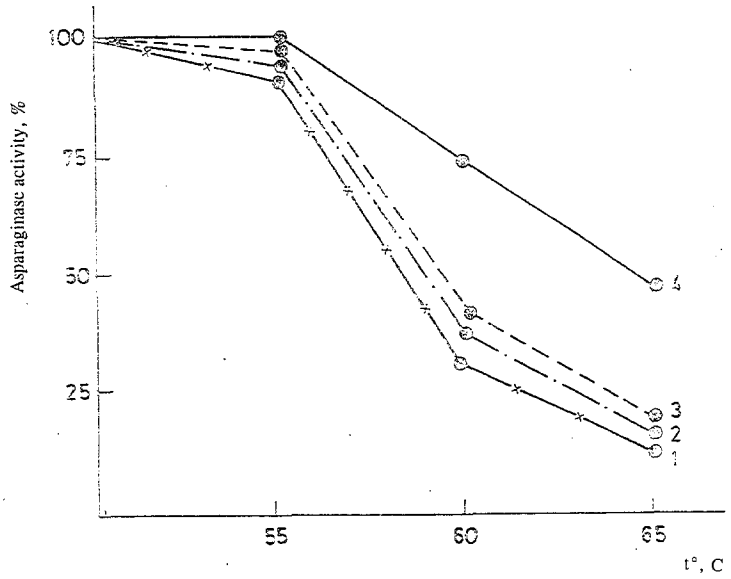

Fig. 2. Thermal stability of native *L*-asparaginase (1) and of samples of immobilized *L*-asparaginase: soluble (2), gel-like (3), and insoluble (4).

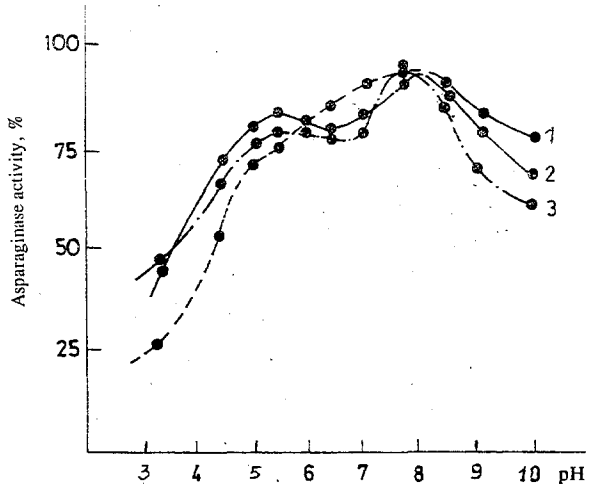

Fig. 3. Dependence of enzymatic activity on the pH of the medium: 1) *L*-asparaginase; 2, 3) samples of immobilized *L*-asparaginase Nos. 1 and 4, respectively.

EXPERIMENTAL

In this work we used *E. coli L*-asparaginase produced by the experimental factory of the Institute of Organic Synthesis of the Latvian Academy of Sciences, with a specific activity of 180 IU/mg of protein, and dextran with a molecular mass of 20,000 from Pharmacia (Sweden).

The enzymatic activities of the soluble forms of *L*-asparaginase were determined as described in [8], and those of the insoluble forms as in [9]. Protein content was determined by Lowry's method [10].

The methods of gel chromatography and electrophoresis used for monitoring the density of binding of the *L*-asparaginase with the dextran were performed in accordance with [2, 6].

We used 0.05 M universal buffer to study the pH dependence of the enzymatic activities of the samples of *L*-asparaginase.

The thermal stability of the samples was studied by incubating buffer solutions and suspensions (200 IU/ml) at the corresponding temperatures for 1 h followed by determining residual activities. The same concentrations of enzyme were used in studying stability on storage at 5°C.

TABLE 2. Antileukemic Action of Native and Soluble Immobilized *L*-Asparaginase

Enzyme	Dose, IU/kg	Prolongation of the life of mice, %	Conditions for the administration of the sample	Pure, %
L-asparaginase	1000	46	Once on the 3rd day	
Immobilized *L*-asparaginase	1000	96		
L-asparaginase	1000	36	Once on the 7th day	
Immobilized *L*-asparaginase	1000	70		
L-asparginase	200	116	Five times from the 3rd to the 7th days	75
	500	120		84
	1000	122		84
Immobilzied asparaginase	200	137	Five times from the 3rd to the 7th days	100
	500	137		100
	1000	137		100

IR spectra were taken in tablets with KBr on a UR-10 spectrophotometer. The antileukemic action of the samples of *L*-asparaginase was studied on the ascitic form of lymphoid leukemia L 5178y, which was inoculated intraperitoneally. As the criterion of the efficiency of the action we used the prolongation of the lives of experimental mice in comparison with untreated control animals. The time of observation was 60 days. The percentage of completely healed mice was determined simultaneously.

Synthesis of the Dextran Carbonates. A solution of 2 g of dextran in 20 ml of dimethyl sulfoxide was treated with 3 ml of p-dioxane and 10 ml of triethylamine. The mixture was cooled in an ice bath and, with constant stirring, ethyl chloroformate was added dropwise over 10 min, and then the solution of the pH was brought to 6 with 5 M HCl. The resulting mixture was precipitated in 400 ml of 90% ethanol and the product was centrifuged at 6000 rpm for 20 min and was washed on the filter with 90% ethanol, absolute ethanol, and ether, and was dried.

IR spectra of samples Nos. 1-4 (ν_{max}, cm^{-1}): 1640, 1760, 1810.

Binding of *L*-Asparaginase with Dextran Carbonate. To 20 ml of a 0.05 M K phosphate buffer solution, pH 6.5 containing 15 mg of *L*-asparaginase, cooled to 4-5°C, was added 200 mg of the appropriate dextran carbonate, and the mixture was stirred for 3 h. The soluble immobilization products were subjected to ultrafiltration in FMO1 cells of domestic production with XM-100A membranes (Amicon, USA) at a pressure of 2 atm and were lyophilized. The insoluble products were washed on the filter with water until the wash-waters no longer contained *L*-asparaginase and were stored in the form of aqueous suspensions at 5°C.

IR spectra (ν_{max}, cm^{-1}): samples Nos. 1 and 2 — 1640, 1760; sample No. 3 — 1640, 1760, 1810.

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