

## Immobilization of 1-[N-(M-Nitrobenzoyl)- $\alpha$ -D,L-Asparagyl]-2-Benzylbenzimidazole on Gellan

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**Abstract:** This paper studies the dicyclohexylcarbodiimide activated coupling reaction through ester-type covalent bonds of an oxazolone derived from N-(*m*-nitrobenzoyl)-L-asparagic acid, ring opened with 2-benzylbenzimidazole, on gellan. Based on a centered, rotatory, composed, second order experimental design, the regression equation describing the dependence of the amount of drug chemically bonded to the support on the reaction parameters is obtained. The efficiency of the coupling reaction over the variation domain established is maximum when the parameters' highest values are used. The kinetics of the drug's release from the support are studied under alkaline hydrolysis conditions.

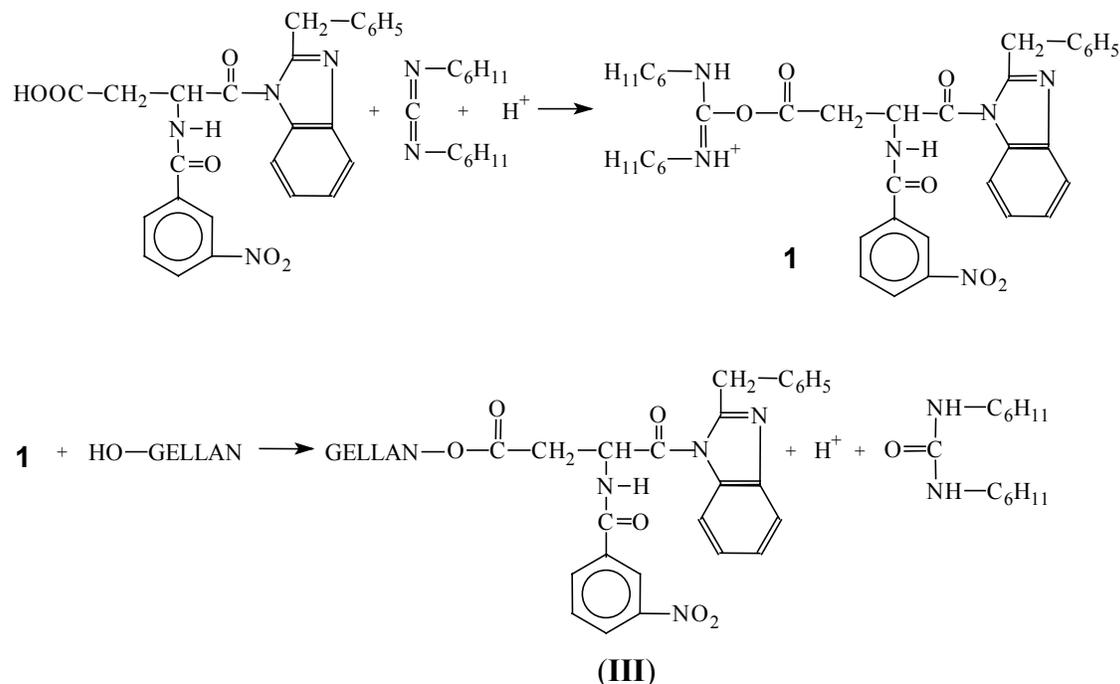
**Keywords:** Gellan, polymer-drug system, controlled release

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Coupling of OxBBI to gellan is based on the esterification reaction of the active principle's carboxylic groups, with the support's hydroxylic ones, activated by DCCI, according to Scheme 2:

Scheme 2



Formation of the OxBBI ester with gellan is evidenced by the IR spectra, which attest to the presence of the ester groups at  $1735\text{ cm}^{-1}$ , as well as of the functional groups specific to the oxazolone moiety:  $1080\text{ cm}^{-1}$  (C-O-C group),  $1360\text{ cm}^{-1}$  and  $1540\text{ cm}^{-1}$  (symmetric and asymmetric  $\text{NO}_2$  group stretches),  $1620\text{ cm}^{-1}$  (C=N group) and  $880\text{ cm}^{-1}$  (disubstituted aromatic ring C-H).

Processing of the experimental results led to the following regression equation:

$$\text{OxBBI (\%)} = 19.95 - 2.47x_1 + 1.103x_2 + 2.024x_3 - 0.186x_1^2 + 0.022x_2^2 - 0.843x_3^2 - 0.032x_1x_2 - 0.053x_1x_3 + 0.159x_2x_3$$

By specifying two of the variables at the center of the experimental domain, information on the influence of the third one may be obtained, as indicated in the curves plotted in Figures 1-3.

The amount of coupled OxBBI decreases with the gellan/OxBBI molar ratio. It is obvious that to achieve maximum coupling yields, a minimum amount of gellan should be used. The high number of hydroxylic groups contained in one mole of polysaccharide (10 -OH groups) assured a sufficient number of reactive sites for the drug's esterification.

**Figure 1.** Influence of the gellan/OxBBI molar ratio on the coupled drug ratio, at  $t=21$  hours, 1 – DCCI/OxBBI=1.1 mol/mol; 2 – DCCI/OxBBI=1.25 mol/mol; 3 – DCCI/OxBBI=1.4 mol/mol.

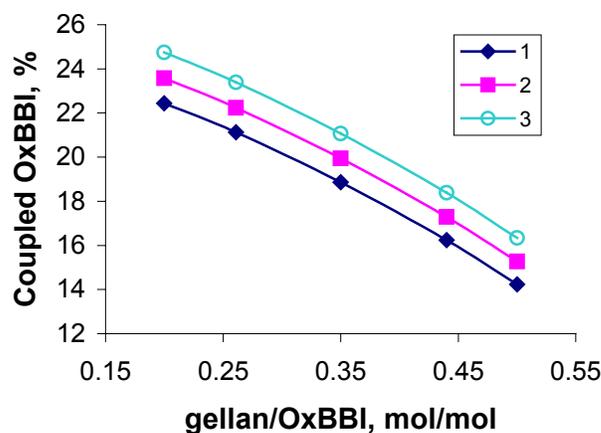
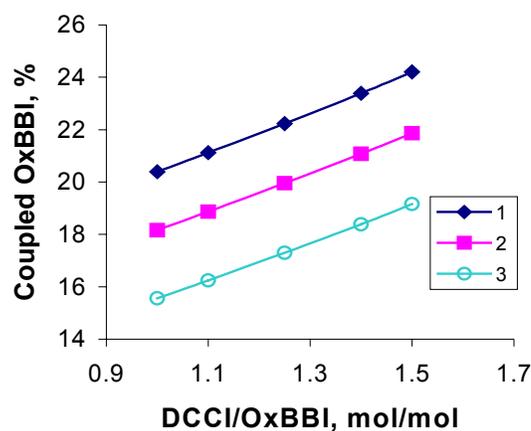


Figure 2 presents the influence of the amount of activator, expressed as the DCCI/OxBBI molar ratio, on the efficiency of the coupling reaction. The observation to be made is that increase of DCCI amount induces a continuous increase of the OxBBI ratio in the reaction product, over the variation interval of this parameter.

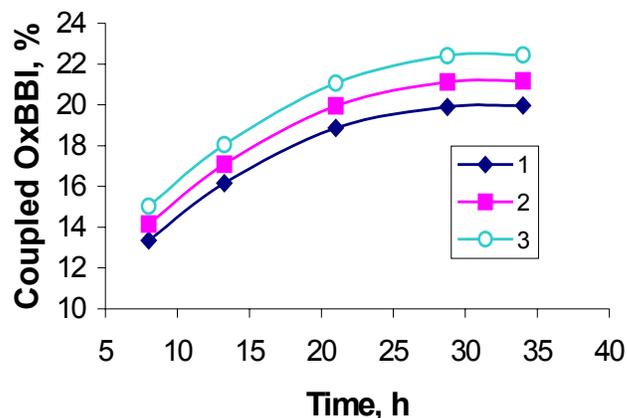
**Figure 2.** Influence of the dccI/oxbbi molar ratio on the coupled drug ratio, at  $t=21$  hours, 1 – gellan/oxbbi=0.261 mol/mol; 2 – gellan/oxbbi=0.35 mol/mol; 3 – gellan/oxbbi=0.44 mol/mol.



The effect is normal, being due to the activation of a higher and higher number of functional carboxylic groups and drug molecules, respectively. Another important observation is that, to attain high coupling yields, one should employ an excess of the activator.

Another important parameter in the synthesis is the duration of the esterification reaction (Figure 3). One may observe that, regardless of the other parameters of the process, maximum yields are obtained for about 30 hours of reaction.

**Figure 3.** Influence of the reaction time on the coupled OxBBI ratio, at a gellan/OxBBI molar ratio = 0.35 mol/mol: 1 – DCCI/OxBBI = 1.1 mol/mol; 2 – DCCI/OxBBI = 1.25 mol/mol; 3 – DCCI/BBI = 1.4 mol/mol.



A possible explanation might be that, for the whole duration of the synthesis, an intermolecular esterification reaction of the polysaccharide takes place at the time as the OxBBI esterification to gellan's carboxylic groups. DCCI activates the support's carboxylic groups too, being partially consumed in this reaction, which results a slight crosslinking of the gellan. It may happen that, at reactions times exceeding 30 hours, the amount of activator would be wholly consumed in the two esterification reactions.

Figures 4 and 5 confirm the results plotted graphically in Figures 1-3, which illustrate, in three-dimensional representation, the influence of each two parameters on the efficiency of the coupling (estimated by the ratio of OxBBI in the coupling products).

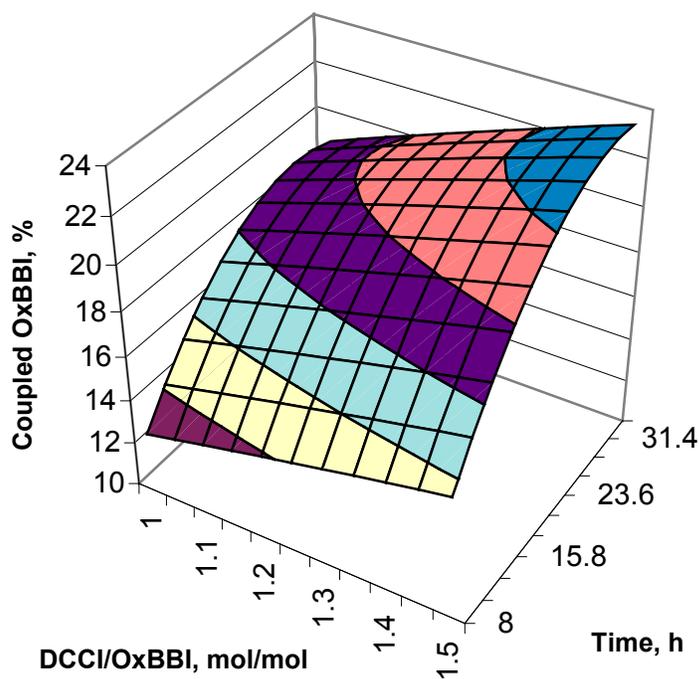
Analysis of the above discussed data shows that, to achieve a maximum content of biologically active product in the coupling compounds, the synthesis should be developed in the following conditions:

Gellan/OxBBI = 0.2 mol/mol;

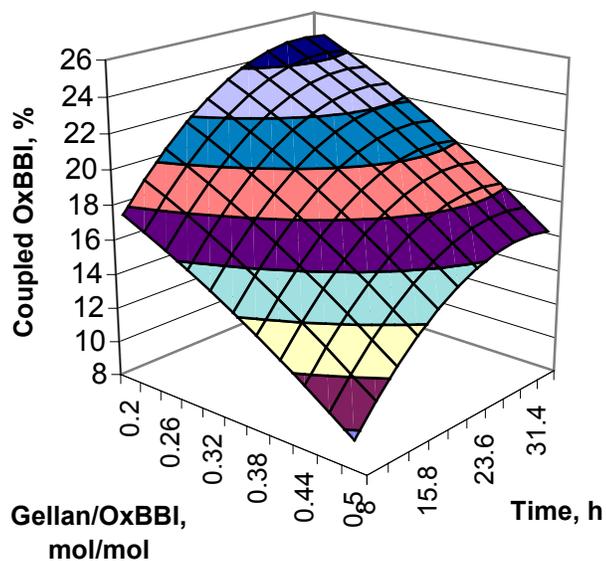
DCCI/OxBBI = 1.5 mol/mol;

t = 28 h

**Figure 4.** Influence of the DCCI/OxBBI ratio and the reaction time on the amount of OxBBI bound in the coupling product, for a gellan/OxBBI ratio=0.35.



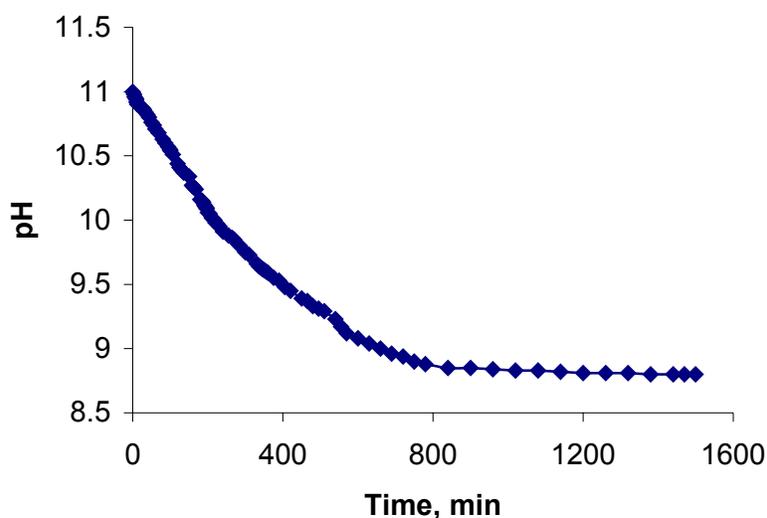
**Figure 5.** Influence of the gellan/OxBBI ratio and the reaction time on the amount of OxBBI bound in the coupling product, for a DCCI/OxBBI ratio=1.25.



Mention should be made nevertheless of the fact that, compared to the situation in which oxazolone had been immobilized on the same support and under similar conditions [22], in the case of OxBBI immobilization lower amounts of drug are fixed. A possible explanation might be the steric hindrance created by the bulky OxBBI molecule.

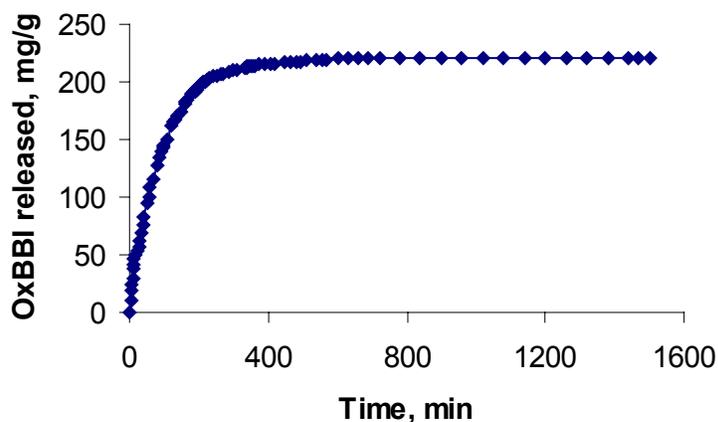
In order to study the active principle's release capacity, hydrolysis of the ester groups in a basic medium has been performed, based on the hypothesis that pH variation represents a suitable method for estimating the kinetics of drug release. The results obtained are shown graphically in Figures 6-7.

**Figure 6.** Time variation of pH for the release, in a basic medium, of OxBBI from the OxBBI-gellan system (OxBBI content = 22.29 %).



Based on this curve, the time variation of the amount of OxBBI in the process of drug release could be calculated and represented graphically (Figure 7).

**Figure 7.** Time variation of the amount of OxBBI released from the coupling product, in a basic medium (OxBBI content = 22.29 %).



During the first 200 minutes, approximately, the release rate of OxBBI is higher; over the 200-400 minute interval, the release rate diminishes and beyond 400 minutes it stabilizes, becoming practically constant, up to an almost total elution of the immobilized OxBBI (22.12 % OxBBI). This evolution of the OxBBI released amount with time allows one to conclude that the gellan-OxBBI system can be viewed as a controlled-release drug system.

Among the compounds involved in the therapy with non-steroid antiinflammatory agents, special mention should be made of some benzimidazole derivatives. The toxicity and antiinflammatory activity of 1-*m*-nitrobenzoyl- $\alpha$ -asparagyl-2-benzylbenzimidazole on a polysaccharidic support has been investigated, for the establishment of a possible structure – biological activity relation. The coupled products were tested from the viewpoint of their antiinflammatory activity on rats. The results obtained are presented in Table 1.

**Table 1.** Investigation of the antiinflammatory activity of gellan-OxBBI on rats.

Product	Experimental inflammatory edema		Antiinflammatory activity (inhibition, %)
	Control group average $\pm$ DS	Treated groups average $\pm$ DS	
gellan-OxBBI	0.134 $\pm$ 0.073	0.033 $\pm$ 0.029	78.6

Analysis of the data obtained showed that gellan-OxBBI displays a remarkable antiinflammatory activity, which may be explained through the intensification of the antiinflammatory effect by the benzimidazole derivative. The prolonged action is probably caused by the gradual scission of 1-*m*-nitrobenzoyl-asparagyl-2-benzylbenzimidazole.

## Conclusions

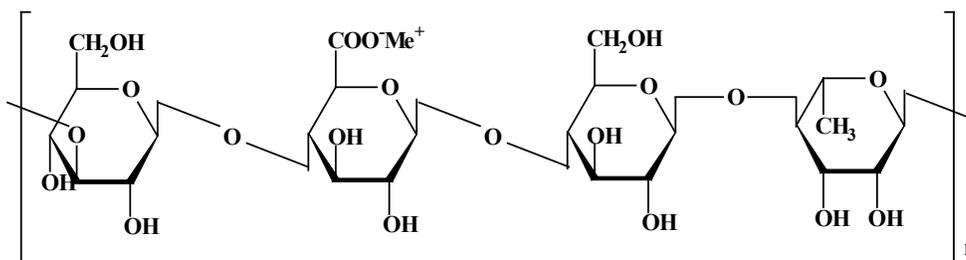
Aminoacid based oxazolones, ring opened with benzylbenzimidazole, may be coupled on natural polymers such as gellan through esterification, in the presence of dicyclohexyl carbodiimide as activator.

- The amount of drug bound in the coupling product increases with the increase of the activator/active principle ratio and the reaction duration, and decreases with the increase of the gellan/active principle ratio.
- The drug is released from the coupling products through basic hydrolysis, according to a first order kinetics, which allows them to be considered as a controlled release system for the biologically active principle.
- The coupling product displays antimicrobial activity against certain microorganisms.

## Experimental

### Materials

*2-(m-Nitrophenyl)-4-(β-carboxymethyl)-Δ<sup>2</sup>-oxazolin-5-one* (Ox, I) – was obtained through treatment of the *n-m*-nitrobenzoyl-*l*-asparagic acid with acetic anhydride, according to the method described elsewhere [8]. *1-[n-(m-Nitrobenzoyl)-α-d,l-asparagyl]-2-benzylbenzimidazole*, (OxBBI), was obtained through opening of the oxazolone ring with benzylbenzimidazole (bbi), described below. Gellan – provided by the KELCOGEL Company, is a polysaccharide obtained from a microbial culture [20], with the following formula:



Dicyclohexyl carbodiimide, (DCCI), was obtained from Merck.

### Methods

#### Preparation of of OxBBI (II)

*2-(m-Nitrophenyl)-4-(β-carboxymethyl)-Δ<sup>2</sup>-oxazolin-5-one* (0.1 moles), 2-benzylbenzimidazole (0.1 moles) and anhydrous dioxane (60 mL) are placed in a round bottomed flask equipped with a reflux refrigerator, The mixture thus obtained is refluxed on a glycerin bath for 4 hours, until a homogeneous solution is formed. Dioxane is eliminated through distillation under reduced pressure and at a temperature of 45-55 °C, up to a volume of 8-10 mL. Addition of an anhydrous ethyl ether - anhydrous petroleum ether mixture (1:2) leads to the separation of an oily product which, through repeated washings with ethyl acetate and then with anhydrous ethyl ether, is transformed into a fine, yellow powder. Recrystallization from freshly distilled ethyl acetate affords a colourless product. It is dried in vacuum, for 8-10 hours, at a temperature of 45-55 °C. The melting point of the product is of 203-205 °C. Yield = 78.5%.

#### OxBBI immobilization on gellan: preparation of III.

OxBBI (0.1416 g,  $3 \times 10^{-4}$  mole) is dissolved in DMSO (5 mL), then gellan is added (in the amounts established by the experimental design) and the mixture is stirred for 30 minutes. Separately, DCCI (in thw amounts recommended in the experimental design, washed with 0.5 mL portions of solvent) is

dissolved in DMSO (2 mL), the solution thus obtained being added to the one containing the OxBBI and the polysaccharide. This is *zero* time of the reaction. After the reaction's completion, to separate the product the reaction mixture is precipitated in methanol (50 mL), under stirring, for 5 minutes, and then centrifuged for 10 minutes, at 5,000 rpm. For the first washing acetone (10 mL) is introduced into the centrifugation vial, the contents are stirred with a spatula and again centrifuged. This sequence of operations is repeated twice, using additional acetone (5 mL) is each instance. For the complete removal of excess dicyclohexyl carbodiimide, the dicyclohexyl urea formed and, eventually, the unreacted OxBBI, the coupling reaction product is extracted in a Soxhlet apparatus with acetone for 24 hours. Towards the end of the extraction 5 mL of the extract are withdrawn and deposited on a watchglass. After solvent removal at 40°C, no trace of solid residue remains on the watchglass, and the weight of the watchglass remains practically constant, indicating that the coupling product does not contains traces of DCI, DCU or OxBBI, and is therefore very pure.

### Experimental Design

Preliminary studies [11, 21], which made use of polysaccharidic supports (xanthan and gellan, respectively) [20, 22], had indicated that the efficiency of the coupling reaction is influenced by several factors. For the present system, the following parameters have been selected as showing a prevailing influence: the gellan/ active principle ratio, the DCCI/ active principle ratio and the process' duration. For the obtention of information on the manner in which coupling's efficiency (expressed by the amount of OxBBI bound chemically) is influenced by these factors, an experimental, centered, rotatory, composed, second order design – which reduces considerably the number of experiments and finally permits optimization of the process - was utilized.

To facilitate processing of the results, the design involves (assures) codification of the variables listed in Table 2, together with the limits of their variation domain.

**Table 2.** Codification of variables and their variation domain.

real variable	coded variable				
	-1.682	-1	0	1	1.682
Gellan/OxBBI (mol/mol) – $x_1$	0.2	0.261	0.35	0.44	0.5
DCCI/OxBBI (mol/mol) – $x_2$	1	1.1	1.25	1.4	1.5
Time (h) – $x_3$	8	13.25	21	28.75	34

The equation proposed for describing the dependence of the amount of coupled OxBBI ( $y$ , %) on the parameters considered has the form:

$$y = a_0 + \sum a_i x_i + \sum a_{ij} x_i x_j, \text{ with } i \leq j$$

- where: -  $a_0$  - free term  
 -  $a_i, a_{ij}$  – regression coefficients  
 -  $x_i, x_j$  – variables expressing the process' parameters

The experimental results listed in Table 3 have been processed by the multiple regression method.

**Table 3.** Experimental results of the content of coupled OxBBI.

Nr.	coded variable			% bonded OxBBI	
	$x_1$	$x_2$	$x_3$	experimental	theoretical
1	-1	-1	-1	17.025	18.358
2	1	-1	-1	13.654	13.589
3	-1	1	-1	19.301	20.313
4	1	1	-1	15.087	15.415
5	-1	-1	1	20.818	22.197
6	1	-1	1	16.52	17.215
7	-1	1	1	23.01	24.783
8	1	1	1	19.301	19.673
9	-1.682	0	0	25.96	24.63
10	1.682	0	0	15.171	16.321
11	0	-1.682	0	19.132	18.157
12	0	1.682	0	22.952	21.867
13	0	0	-1.682	14.834	14.161
14	0	0	1.682	22.588	20.969
15	0	0	0	19.807	19.95
16	0	0	0	19.975	19.95
17	0	0	0	19.975	19.95
18	0	0	0	19.794	19.95
19	0	0	0	20.05	19.95
20	0	0	0	19.875	19.95

#### *Release of the active principle from the support*

In order to establish capacity for the controlled release of the active principle from the system prepared, a product with a 19.29 % content of OxBBI was selected. This product (0.5 g) was suspended, with stirring, in a solution of NaOH (50 mL, pH = 11), at a temperature of  $37 \pm 0.5^\circ\text{C}$ . The variation of pH with time of the system was followed, based on the principle that the hydroxide from the reaction medium is partially consumed through the hydrolysis of the ester groups, a reaction which determines the release of the biologically active product. To prove that assumption, two witness samples were examined under the following conditions:

(a) Establishment of the pH variation with time of a NaOH solution (50 mL, initial pH = 11), at  $T=37\pm 0.5^{\circ}\text{C}$ ;

(b) Establishment of the pH variation with time of a NaOH solution (50 mL, pH = 11) in which gellan (0.5 g) was suspended, at  $T=37\pm 0.5^{\circ}\text{C}$ .

In the case of the first sample, it was observed that during the 0-600 min. time interval, the pH value remains practically constant. In the case of the second sample, a 0.45 units variation of the pH value was observed during the first 15 min; after that, the pH value remained practically constant at a pH of 10.55, up to the 600 min. point. This pH variation indicates a consumption of NaOH, probably due to its reaction with some carboxylic groups of gellan, which are not already in the sodium salt form. As a consequence, the pH variation curve of the OxBBI-gellan system was corrected, on the time interval of 0-15 min, with the values established from the pH variation curve of the witness sample (b).

The curves obtained permit the conclusion that over the interval time considered, only the hydrolysis of the ester groups which bond the OxBBI on gellan takes place. The biologically active product (OxBBI) is not affected by the alkaline medium, a fact proven by the previously reported chemical stability tests [10].

#### *Antiinflammatory activity of 1-m-nitrobenzoyl- $\alpha$ -asparagyl-2-benzylbenzimidazole*

Toxicity has been determined by *per os* administration of OxBBI-gellan, as a suspension, in 0.1% sodium carboxymethylcellulose, to groups of 3 rats (20-25 g in weight), according to classical laboratory methods [23, 24]. The animals were monitored and their mortality recorded after 7 days. It was observed that the product has acceptable toxicity, namely 3,200 mg/Kg body weight

The antiinflammatory activity was evaluated with the experimental rat's paw inflammatory test, according to the method of Winter and Levy [25]. Groups of 7 white rats of both sexes, 25-30 gr in weight, have been injected, in the tissue of the leg region of the left posterior paw, with a 2% carrageenan solution (0.03 mL), after first measuring the paw's initial volume by the pletismometric method. Two hours after the injection, the compound under consideration was administered *per os*, as a suspension in 0.1% sodium carboxymethylcellulose in 20 mL of carrier. The control batch received only the carrier.

Four hours after the injection of the inflammagenous agent, the paw's final volume was measured and the difference between the initial and the final volume, representing the experimental inflammatory edema, expressed in mL/100 g body weight, was calculated. There followed the determination of the average values of this parameter for the group of treated, which were then compared with those of the control group. The antiinflammatory activity was expressed as percentage inhibition versus the reference.

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*Sample availability:* Samples of compounds **I**, **II** and **III** are available from MDPI.