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Behaviour of glucose oxidase during formation and ageing of silica gel studied by fluorescence spectroscopy

MAŁGORZATA PRZYBYT*

Institute of General Food Chemistry, Department of Biochemistry and Food Science,
Technical University of Łódź, 90-924 Łódź, ul. Wólczańska 171/173, Poland

Glucose oxidase was entrapped in silica gels obtained by the sol-gel process with the retention of a part of its activity. The stability of the enzyme molecule during the sol-gel transition and ageing of the wet gel was followed by the measurement of fluorescence of tryptophan and flavin adeninedinucleotide (FAD). The results indicate unfolding and denaturation of the enzyme protein caused by the changes of microenvironment inside gel, presence of ethanol, decomposition of FAD and photosensitised oxidation of tryptophan by FAD. Among the products of FAD decomposition, alloxazine derivatives were identified through their specific fluorescence characteristics. The results of the observation of the fluorescence variation and activity assay of the gel are in good agreement. The experiments with the dried gel indicate that illumination is not necessary to sensitise the reaction of oxidation of tryptophane by FAD although in the presence of light the effectiveness of this process is higher. Presumably the decomposition of FAD and tryptophan is not only induced by light but also by the paramagnetic (with free radical character) defects in the gel matrix. This reaction is enhanced by the presence of glucose and depends also on pH of the buffer used in the gel preparation. The products of the enzyme degradation are easily washed out from the gel. Leaching of active enzyme from the gel was also observed.

Key words: *sol-gel; glucose oxidase; fluorescence; flavin adeninedinucleotide; enzyme stability*

1. Introduction

Biosensors for assay of glucose with immobilised glucose oxidase are the most commonly developed and studied ones because of their biomedical, industrial and clinical applications. Since Clark and Lyons [1] have introduced the first concept of glucose sensing electrode, a tremendous variety of different types of biosensors were described [2]. Practically, all types of electrochemical, optical, thermal and other

* E-mail: mprzybyt@snack.p.lodz.pl.

sensors were used to construct glucose biosensors. All methods of enzyme immobilisation were also tested with glucose oxidase. The first glucose analyser was successfully introduced to the market in 1979 [2]. The replacement of a natural redox mediator – oxygen – by synthetic ones [3] allowed introducing in 1987 the first individual glucometer [2] for every-day control of blood glucose by diabetic patients. In last two decades, a substantial progress was made in construction of implantable biosensors for continuous glucose measuring [4]. In the case of *in vivo* application of the glucose biosensors, the immobilisation matrix must be non-toxic, biocompatible and stable. It must also give no immunological response, allow a free diffusion of oxygen and glucose and stabilise the activity of the immobilised enzyme. From that point of view, the silica gel obtained by the sol-gel technique has interesting properties as the immobilisation matrix because of the simplicity of preparation, tunable porosity, chemical inertness, optical transparency and mechanical stability. It shows also no toxicity and is biocompatible.

In 1990, Braun at al. [5] reported for the first time a successful immobilisation of enzymes in silica gel obtained from organic silica precursors via the sol-gel method. Since then the sol-gel technique has been widely used in construction of different types of biosensors and reviewed few times [6–9]. Glucose oxidase can be immobilised in pure silica gel or in composite materials with organic groups introduced into silica network (ORMOSIL) or mixtures of gel and other materials like graphite, colloidal gold, organic polymers and others [6–9]. Materials obtained by the sol-gel technique were mostly applied in electrochemical biosensors – amperometric with oxygen detection [10] or hydrogen peroxide detection [11–19] and with synthetic redox mediators [20–24]. Other types of biosensors with glucose oxidase immobilised in silica gel are optical biosensors with pH-dependent fluorophores [25], fluorophores quenched by oxygen [26, 27], based on optical properties of glucose oxidase prosthetic group (flavin adeninedinucleotide – FAD) [28, 29]; thermal [30]; chemiluminescent [31] and electrochemiluminescent [32].

The glucose oxidase is a very stable enzyme while it is a glycoprotein [2, 33] and many biosensors with this enzyme show a great operational and storage stability deciding about their practical use. Unfortunately, many biosensors with glucose oxidase immobilised in silica gel show rather poor stability [12, 14–16, 24, 32]. Partially the instability of the sensor is ascribed to leaching of the enzyme from the gel [14, 15, 25, 30] or to changes of electrochemical mediator (ferrocene) properties [24]. To avoid the leaching of the enzyme it is sometimes cross-linked inside the gel [15] or covalently bound to the gel lattice [16, 23] but such an approach does not improve very significantly the properties of the biosensor. It is also very characteristic that the retention of the glucose oxidase activity after entrapment in silica gel is very low [26]. Such results indicate that the entrapment of glucose oxidase in silica gel changes the properties of the enzyme, especially its stability.

Generally, the properties of the enzyme molecule inside the gel are changed due to the change in its conformation leading sometimes to a denaturation of protein, restriction of molecule motion and lower accessibility of the entrapped enzyme by the

substrate [6]. As the result, the enzyme immobilised in silica gel has properties different from the native one. Typically the activity of the entrapped enzyme is lower. In general, enzymes immobilised in silica gel follow the Michaelis–Menten kinetics but the Michaelis K_M constants are greater and the catalytic constants k_{cat} lower as compared with the native ones. The stability of the enzyme in the silica gel can either be decreased or increased depending on the nature of protein and composition of the gel [6]. The changes of protein conformation and dynamic motion inside the gel are caused by interactions of the molecule with silica surface inside the pores and different microenvironment. The porosity of the gel and the interactions of the enzyme substrate with the gel surface change the diffusion conditions causing the lower accessibility of the active centre and thus leading to different kinetic properties [6]. Although glucose oxidase is often sequestered in silica gel in biosensor construction, there are only few papers on the properties of this enzyme inside the gel [29, 34, 35]. Practically none of them reports on the changes of the enzyme state during a dynamic sol-gel transition and ageing of the gel.

The research area of our group is the immobilisation of enzymes in silica gel in construction of biosensors. The results for the glucose oxidase immobilised in silica hydrogel on the tip of oxygen electrode indicate a very low stability of the enzyme in the gel partially caused by leaching [36]. The goal of our work is to construct the optode for glucose with glucose oxidase and fluorophore quenched by oxygen entrapped in silica gel. Thus there is a need to study the behaviour of glucose oxidase during the sol-gel process, ageing of hydrogel and drying of gel. A very useful method to examine the state of glucose oxidase molecule inside the gel is the measurement of the fluorescence. In glucose oxidase molecule, there are two fluorophores with different spectral properties: tryptophan in apoenzyme (protein) [35, 37, 38] and prosthetic group – FAD [29, 38] which can be observed independently.

The paper presents the results of fluorimetric investigations of glucose oxidase physically entrapped in silica gel obtained by the sol-gel method from tetraethoxysilane during the sol-gel transition, ageing of hydrogel and in a dried gel.

2. Experimental

2.1. Reagents

As a gel precursor, tetraethoxysilane (TEOS) (99%, Aldrich Chemie GmBh, Germany) was used. Glucose oxidase (GOD) from *Aspergillus niger* of activity 30 U/mg was purchased from Serva. GOD was dissolved in water (500 mg in 10 ml). Anhydrous glucose (analytical grade) was obtained from POCh (Gliwice, Poland). All other reagents were of analytical grade. Twice distilled water was used throughout.

At pH 5 an acetate buffer was used, at 7 and 9 – the phosphate ones. The buffers were adjusted to desired pH with a concentrated solution of NaOH.

2.2. Apparatus

Fluorescence measurements were done using Fluoromax-2 spectrofluorometer (Jobin Yvon-Spex Instruments S.A., Edison, New Jersey, USA). The fluorescence spectra were measured with 10 mm pathlength closed quartz cells during ageing experiments or with polymethacrylane ones during drying. The excitation and emission slits were set at 2 nm each. The increment was set at 1 nm and integration time at 2 s. The measurements were done at ambient room temperature.

Between measurements the hydrogels were kept at 20 ºC in a homemade air-circulated thermostat.

Oxygen concentration measurements were done using a galvanic silver–zinc oxygen electrode CTN-920.S (MES-EKO, Wrocław, Poland) connected with a microcomputer pH/oxygen meter CPO-551 (Elmetron, Zabrze, Poland). The results of measurements were expressed as the percentage of oxygen concentration in a solution saturated from air at 25 ºC. The constant stirring rate of the solutions was maintained by magnetic stirrer BMM 21 (DHN Wigo, Piastów, Poland). The temperature was maintained with a thermostat U1 (MLW, Medingen, Germany).

pH of the buffers was measured with combined glass electrode connected with a microcomputer pH/oxygen meter CPO-551.

2.3. Preparation of gels

The stock solution of the sol was prepared as follows: 4.5 ml of TEOS, 1.4 ml of water and 0.1 ml of 0.1 M HCl giving the molar ratio TEOS : H_2O: HCl equal to 1 : 4 : 10^{-5} were stirred vigorously at room temperature until transparent homogeneous solution was obtained (3 h). The sol was then cooled in a refrigerator (4 ºC) for about 30 mins.

Table 1. Composition of gels studied and the gelling times

Buffer	pH (estimation)	Gelling time [min][a]
Water	3	~15
Acetate	5	~9
Phosphate	7	~3.3
Phosphate	9	~1.2

[a]Gelling time is the time span from mixing all components until the bulk gel is formed.

The samples were prepared as follows: 1.5 ml of the stock sol was mixed with 0.5 ml of GOD solution and 1 ml of water or 0.03 M buffers (Table 1). In the case of water, pH of the mixture was 3.3, and with the acetate buffer pH = 5.4 was obtained. For phosphate buffers, pH of the mixtures could not be measured due to a short gel-

ling time. Thus, in all the cases when pH of the gel is mentioned, the reported value is the one of the buffer. Two samples of the gel were prepared, and to the second one 54 mg of glucose was added to obtain the final concentration 0.1 M. At such a concentration of glucose, the whole FAD in the GOD molecule occurs in a reduced form, while in samples without glucose added, the oxidised form of FAD is present.

2.4. Measurement procedure

Three different sets of experiments were done:

A. Because at given compositions of initial mixture of sol and enzyme solution the gelling times were short (Table 1), it was impossible to record the fluorescence spectra of tryptophan and FAD during the sol-gel transition; only kinetic measurements were done. The tryptophan fluorescence was excited at 295 nm and measured at 340 nm. The fluorescence of FAD was excited at 370, 396, 438, 450, 467 and 490 nm and measured at 520 nm. In this case, the integration time was 0.5 s. The mixtures were prepared in polymethacrylate cuvettes closed with parafilm. Immediately after preparation, the sample was placed in the spectrofluorimeter and the spectra were recorded for 2 h. After the measurement, the samples were taken out, some pinholes were made in the parafilm and the sample was left to dry at ambient room temperature for 22 days. Then the cuvettes were opened and left to dry for another day. During drying, the samples were placed on a shelf in the laboratory, not being protected from the light. The dimensions of the gel monoliths obtained were measured with the accuracy of 0.1 mm. Then the fluorescence spectra of FAD and tryptophan were recorded and the monoliths were subjected to 3 ml of 0.02 M acetate buffer, pH 5, and left for 2 days in a refrigerator. After that time the buffer was checked fluorimetrically for the presence of tryptophan and FAD.

B. The samples were prepared in closed quartz cuvettes and fluorescence spectra were measured about 0.5 h after a rigid gel was obtained. The measurements were repeated every day in the first week, then more rarely. The whole course of measurements lasted 4 weeks. Only for samples of pH 5, the time regime of measurement was a little different.

C. The samples were prepared as in (A). 1 hour after gelation, the fluorescence spectra were taken. Then the gels were left to dry in the same manner as in (A) but in darkness. The monoliths obtained were measured as in (A). Then they were weighted and powdered in a mortar. 0.3 g of the powder was immersed in 3 ml of 0.02 M phosphate buffer, pH 7, and left overnight in a refrigerator. Next day the samples were filtered and the supernatants were checked fluorimetrically for the presence of FAD and tryptophan and then for the activity of GOD.

2.5. Assay of GOD activity

100 ml of 0.1 M glucose solution in 0.02 M phosphate buffer, pH 6, saturated with oxygen from air was poured into the thermostated vessel. The oxygen electrode was

dipped in the solution and the vessel was closed. The measurements of oxygen were done at 25 ºC at a constant stirring rate of 500 rpm. When the signal of electrode became constant, the sample checked for GOD activity was added, and the oxygen concentration was recorded for 5 min. In the case of native GOD, 10 or 5 μl of the enzyme solution was added, in the case of supernatants (C) – 0.5 ml. For the assay of the GOD activity, 50 mg of the powdered gel was added. Each assay was triplicated.

From the recorded dependencies of oxygen concentration on time, the slope of the initial straight line equal to the initial rate of oxygen consumption was calculated and taken as the GOD activity. The results were corrected for actual atmospheric pressure.

3. Results and discussion

3.1. Fluorescence properties of glucose oxidase in solutions

The glucose oxidase molecule is a homodimer with two FAD molecules as prosthetic group. Each FAD residue is buried in a deep pocket formed in the protein subunit [33]. This pocket is a β-D-glucose binding domain. FAD is not covalently bound with the apoenzyme. Glucose oxidase is the catalyst of the reaction of oxidation of glucose with the use of oxygen dissolved in water. The mechanism of glucose oxidation is well established [2]: it consists of two half-reactions in which FAD is reduced and oxidised. Previously FAD in glucose oxidase was reported as non-fluorescent in both forms [39] but now it is well established that it shows fluorescence [29, 38, 40]. The fluorescence of reduced $FADH_2$ (in the presence of glucose) is reported to be higher than that of the oxidised form both in solution and when it is immobilised in an organic gel [29, 40] but lower when GOD is entrapped in silica gel [29]. The changes of FAD fluorescence caused by glucose were used as a basis for optical biosensors [29, 40].

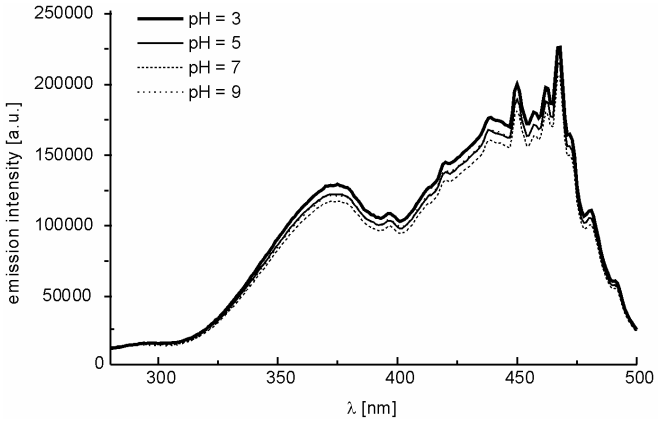

Fig. 1. Excitation spectra of FAD in oxidised glucose oxidase at different pH. The emission was collected at 520 nm

Figure 1 shows the excitation spectra of oxidised FAD in glucose oxidase at different pH values in solution. The intensity of emission is practically independent of pH; only at pH 3 it is a little higher. For free FAD, the emission intensity is decreasing with the pH increase. The excitation spectrum of FAD consists of two bands: one with the maximum at about 450 nm and vibronic structure corresponding to the HOMO-LUMO singlet $\pi \rightarrow \pi^*$ transition and the second with the maximum at about 370 nm corresponding also to singlet $\pi \rightarrow \pi^*$ transition but some results suggest mixing with an $n \rightarrow \pi^*$ transition [41]. In the presence of 0.1 M glucose, the fluorescence intensity of FAD increases slightly at pH 3 but at higher values of pH it decreases, especially in alkaline solutions (pH 9).

Because of the existence of two excitation bands, the emission spectra were excited at 370 nm and at 450 nm, and recorded between 460 nm and 600 nm. The wavelength of the maximum is practically independent of pH, red-ox state of GOD and excitation wavelength (Table 2). Only at pH 9, the maximum of the reduced form is shifted by 5 nm to higher values when excited at 450 nm (Fig. 2).

Table 2. Characteristics of FAD fluorescence in glucose oxidase

pH	Form	Ratio of emission intensity at 450 and 370 nm I_{450}/I_{370}	Maximum of the lower excitation band [nm]	Maximum of emission [nm]	
				λ_{exc} = 370 nm	λ_{exc} = 450 nm
3	ox	1.778	375	524	524
	red	1.841	373	524	525
5[a]		1.666	375	524	523
5	ox	1.789	372	525	526
	red	1.789	376	525	524
5[b]	ox	1.670	370	522	519
	red	1.583	371	518	520
7	ox	1.786	372	527	526
	red	1.594	375	524	522
9	ox	1.796	372	525	524
	red	1.501	372	522	530

[a]Free FAD (oxidised).
[b]In the presence of ethanol (6.74 M).

During the hydrolysis of TEOS and formation of gel, ethanol is produced. Assuming a complete hydrolysis 4 moles of ethanol would be produced from 1 mole of TEOS yielding the concentration in the final gel equal to 6.74 M. The presence of ethanol could influence the conformation of glucose oxidase molecule and change the fluorescence properties of FAD and tryptophan. Because of that, the spectra were recorded at pH 5 and at ethanol concentration equal to 6.74 M when the precipitation of glucose oxidase did not occur. Because of that, the silica gel with entrapped GOD

cannot be produced in the process in which ethanol is present in the initial mixture at a high concentration allowing the slow gelation, with gelling time measured in days [42]. The presence of ethanol caused the change of FAD fluorescence properties – increase of the emission intensity and blue shift of the maximum (Table 2).

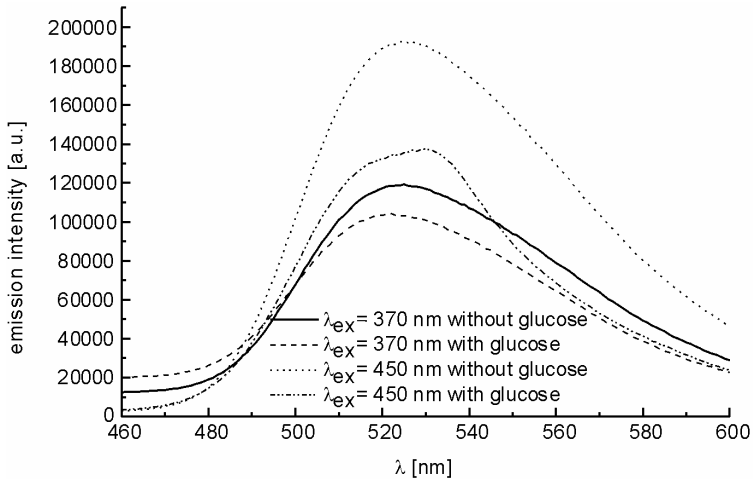

Fig. 2. Emission spectra of FAD in glucose oxidase at pH 9

The spectral parameters of tryptophan fluorescence are dependent on the dynamic and electronic properties of its environment. Thus the measurement of steady-state fluorescence of tryptophan can be used to obtain informations about the conformation of protein molecule, its dynamics and folding. There are 10 tryptophan residues in each monomer of GOD and 4 of them are located in the FAD binding domain [33]. The fluorescence of tryptophan in GOD molecule depends on the red-ox state of FAD prosthetic group and these changes can be interpreted as due to the Förster energy transfer from tryptophan residues to the FAD moiety [37]. In a reduced GOD molecule, the fluorescence of tryptophan is increased and the efficiency of the energy transfer from the apoenzyme to FAD is decreased indicating the greater freedom of FAD inside the pocket formed by the apoenzyme. The tryptophan fluorescence in GOD is also pH-dependent [38]. The properties of tryptophan residues in GOD at the pH under investigation and in the presence of ethanol at pH 5 are summarized in Table 3.

The results obtained are similar to those reported earlier [37, 38]. For the reduced form of GOD, the increase of tryptophan fluorescence as compared with the fluorescence of the oxidised form is observed at all pH values studied. This increase is the greater the higher pH is. The presence of ethanol caused an increase of fluorescence of both forms of the enzyme accompanied with practically no change of the λ_{max} suggesting a weaker contact of the tryptophan residues with FAD.

Table 3. Steady state tryptophan fluorescence characteristics in glucose oxidase (λ_{exc} = 295 nm)

pH	Form	λ_{max} [nm]	$\Delta\lambda$[a] [nm]	$I_{R\,max}$[b] [%]	I_{red}/I_{ox}[c]
3	ox	338	60.4	102.5	1.210
	red	342	62.2	124.0	
5	ox	339	60.7	100	1.245
	red	342	61.9	124.5	
5[d]	ox	338	62.5	141.6	1.367
	red	340	63.6	193.7	
7	ox	340	61,1	106.2	1.272
	red	342	62.8	135.1	
9	ox	341	62.1	101.2	1.365
	red	344	63.0	138.2	

[a] $\Delta\lambda$ bandwidth at a half intensity of the maximum intensity.
[b] $I_{R\,max}$ relative maximum intensity (intensity of oxidised GOD at pH 5 taken as 100%).
[c] I_{red}/I_{ox} ratio of intensities of reduced and oxidised forms of GOD.
[d] In the presence of ethanol (c = 6.74 M).

3.2. Kinetic fluorescence measurements during sol-gel transition

Because the longest gelling time was 15 min at pH 3 for the initial gel compositions studied, only kinetic measurements were done. Changes of fluorescence were studied continuously during first 2 h after all components had been mixed.

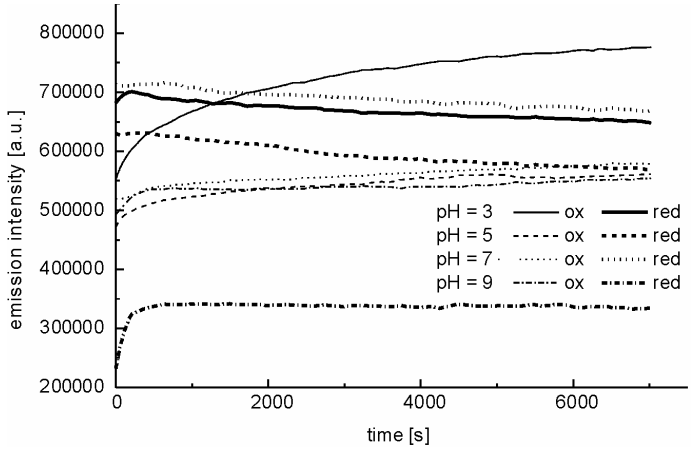

Fig. 3. Intensity of tryptophan fluorescence vs. time during gel formation (λ_{exc} = 295 nm; λ_{em} = 340 nm)

The tryptophan emission was excited at 295 nm and measured at 340 nm. The results are shown in Fig. 3. For the oxidised form of GOD, the tryptophan fluorescence intensity increases, the increase being most significant at pH 3. For the reduced form at pH 3, 5 and 7, a very slow decrease of fluorescence is observed. Only at pH 9 the

tryptophan fluorescence increased to about 145% of its initial value during first 7 min and then it was slowly decreasing.

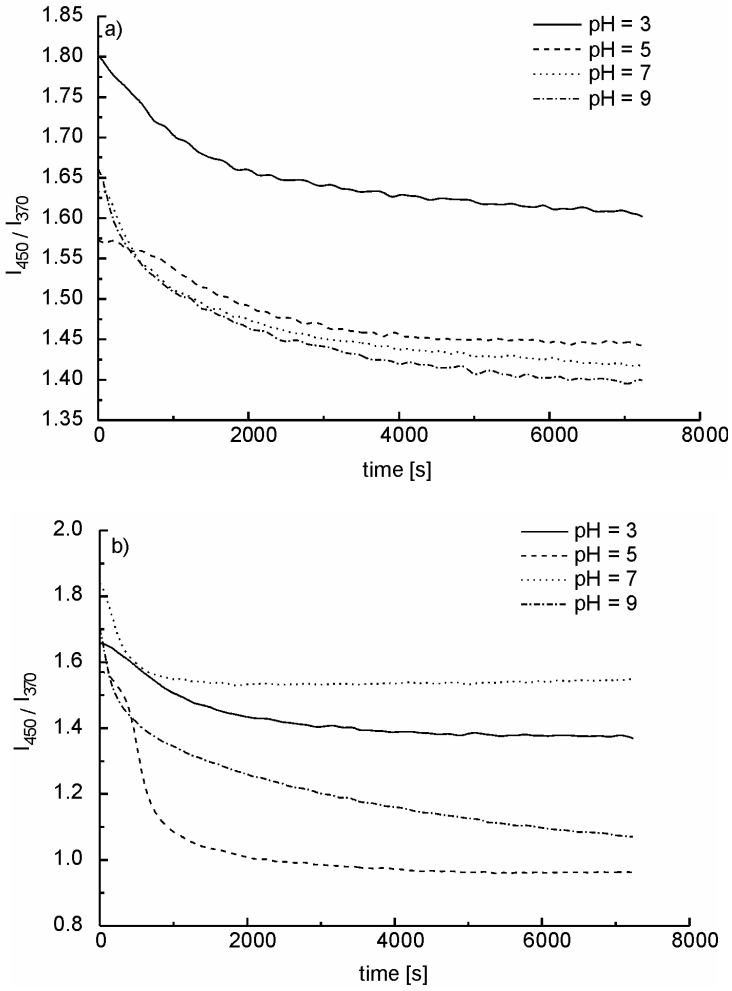

Fig. 4. Time dependences of I_{450}/I_{370} of FAD emission measured at 520 nm during the gel formation: a) oxidised enzyme, b) reduced enzyme

At all studied pH values, the fluorescence of reduced form of FAD decreases dramatically during first 10 min when excited at the wavelengths belonging to the band of HOMO-LUMO transition (450 and 467 nm). For the oxidised form of FAD the direction of changes depends on pH. At pH 3, the FAD fluorescence intensity increases, at pH 5 initially it increases then decreases, and at pH 7 and 9 it slowly decreases. When the fluorescence is excited at 370 nm, the changes are not so substantial and in some cases the direction of changes is different. Because of that the ratio of the emission excited at 450 nm and 370 nm (I_{450}/I_{370}) was taken into account

as the parameter characterising the FAD fluorescence. The changes of I_{450}/I_{370} during gel formation are shown in fig. 4. At every studied pH value and for both forms of enzyme (oxidised and reduced) this ratio is decreasing during gel formation. The rate of this decrease is the highest at the beginning (after mixing of all components) before gel is formed with some exception at pH 5. For the reduced form of FAD, the period of rapid decrease of I_{450}/I_{370} is well correlated with the gelling time.

The initial changes especially of FAD fluorescence during the gel formation can generally be attributed to the changes of the environment of a GOD molecule and the increase of ethanol concentration. These changes can alter the conformational motions of the whole molecule, the strength of FAD binding and thus its dynamics inside the GOD molecule [29].

3.3. Ageing of wet gels

Wet gels were prepared as described in the experimental part and kept in closed cuvettes, to prevent the gels from drying. During the ageing, a syneresis was observed. For every gel, the excitation and emission (excited at 370 nm and 450 nm) spectra of FAD and emission spectra of tryptophan were recorded. A characteristic example of the changes of FAD excitation spectra during ageing of gel is shown in Fig. 5.

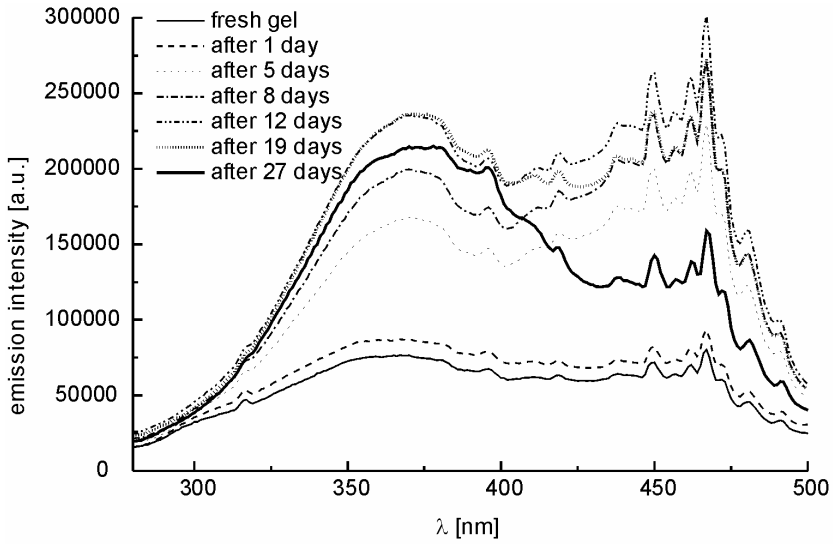

Fig. 5. Excitation spectra of FAD in reduced glucose oxidase during ageing of a wet gel prepared with the acetate buffer, pH 5

At all pH values studied, the intensity of FAD fluorescence initially increases (in the case of oxidised form only on the first day) and then decreases (Fig. 6). For freshly prepared gel, the intensity of FAD emission is much lower in the presence of

glucose (reduced form) as compared with the oxidised FAD. After 4 weeks of ageing the relation is quite opposite (with the exception of pH 3, at which the intensities tend to be equal).

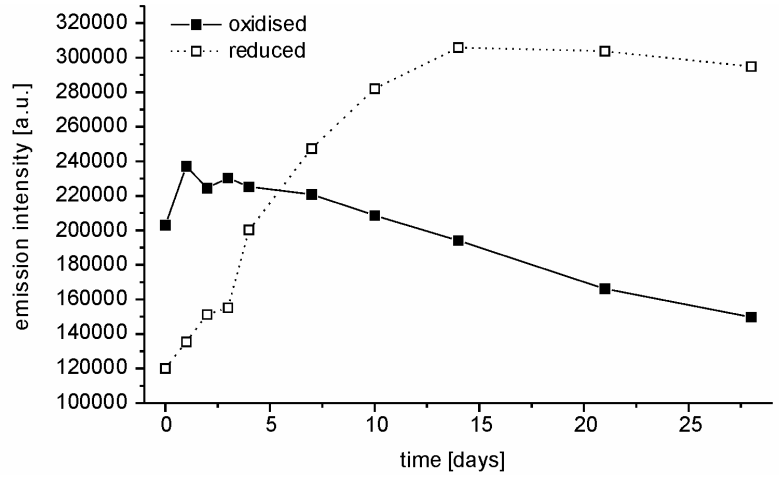

Fig. 6. Changes of FAD in glucose oxidase fluorescence intensity during ageing of a wet gel prepared with a phosphate buffer, pH 7, λ_{exc} = 450 nm, measured at the maximum of emission – 520 nm

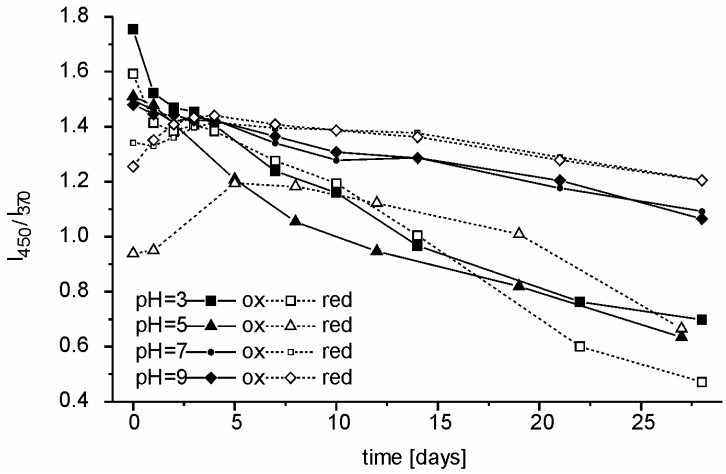

Fig. 7. Changes of I_{450}/I_{370} of FAD emission measured at 520 nm during wet gel ageing

The relative intensity of the band with its maximum intensity at 370 nm is higher as compared with the intensity of the HOMO-LUMO transition band (Fig. 7). Also the band at 370 nm is characteristically broadened after 3 weeks of ageing (Fig. 5). All these changes are less pronounced at pH 7 and 9. When the FAD emission was excited

at 450 nm, its maximum was situated at about 519–520 nm and showed no shift during ageing. The wavelength at the maximum is lower than that of the native enzyme in pure buffer but practically the same as for the enzyme in the presence of ethanol (Table 2). The FAD emission spectra show a blue shift when excited at 370 nm. At pH 3 and 5 the shift was about 20 nm. After 2 weeks the emission at the maximum disappeared and only a shoulder at 503 nm could be seen. At pH 7 and 9, the blue shift is less pronounced – only about 10 nm and the maximum of fluorescence does not vanish.

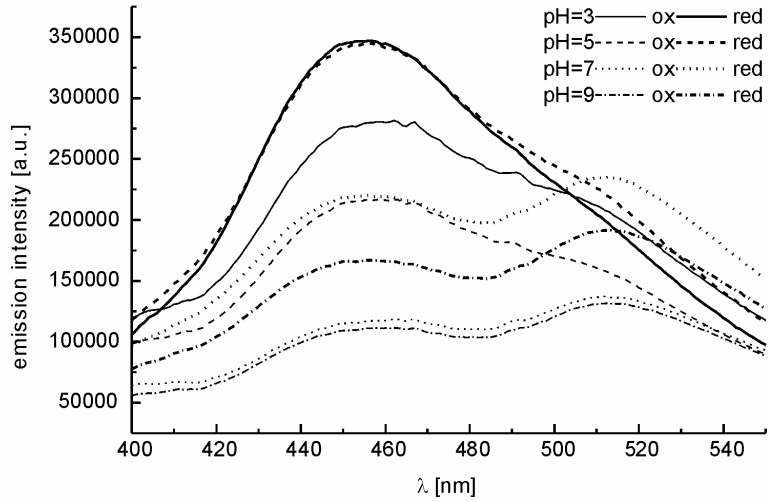

Fig. 8. Emission spectra of wet gels after 4 weeks of ageing, excited at 360 nm

Such pronounced changes of the FAD fluorescence spectra during ageing of the gel cannot only be attributed to changes of the microenvironment and molecular dynamics but indicate that a new species is formed during the ageing. To recognise this unknown species, the emission spectra excited at 340, 350, 360, 370, 380 and 390 nm were recorded between 400 and 550 nm (Fig. 8). It could be seen that a new maximum appeared at about 460 nm for the oxidised GOD and at 456 nm for the reduced GOD. The wavelength of the maximum is independent of the excitation wavelength in the absence of glucose but shows a red shift (about 10 nm) when the excitation wavelength increases from 340 to 390 nm for gels with glucose added. The intensity of this maximum decreases with increasing pH. The second band with the maximum at 510 nm is visible only at pH 7 and 9. At pH 3, there is no evidence of this maximum and at pH 5 it is only a shoulder. Also the excitation spectra of the unknown species were recorded (λ_{em} 460 nm for the oxidised GOD; λ_{em} 455 nm for reduced GOD in the presence of glucose). They showed one maximum at about 355 nm, a shoulder at 380 nm and a second maximum at 396 nm. The fluorescence properties of the unknown species are very similar to those of alloxazine derivatives like lumichrome and 1-methyllumichrome in water [43]. It is well known that isoalloxazine derivatives like

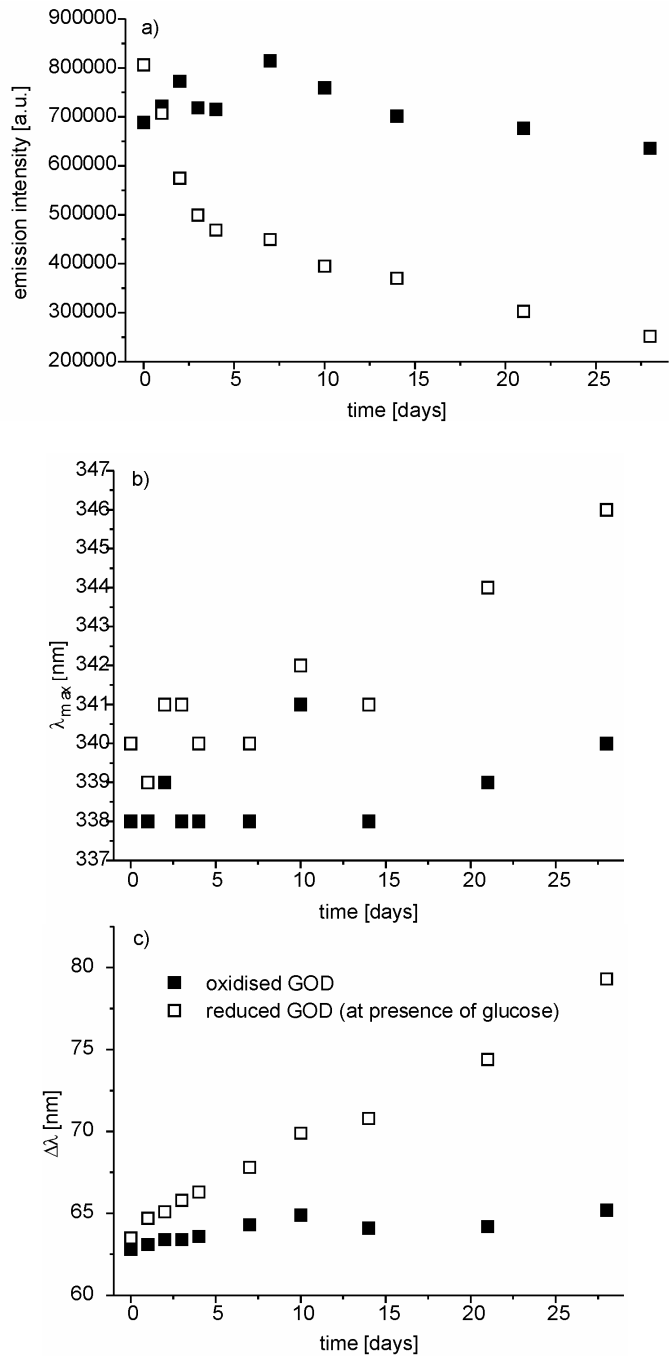

Fig. 9. Changes of tryptophan emission excited at 295 nm during ageing of wet gel prepared with a phosphate buffer, pH 7: a) intensity of the emission at the maximum, b) maximum wavelength, c) bandwidth at a half intensity of the maximum intensity; ■ – oxidised glucose oxidase; □ – reduced glucose oxidase (in the presence of glucose)

riboflavin, flavin mononucleotide (FMN) and FAD are not photostable and can undergo a photodegradadion [41, 44, 46]. The UV light irradiation caused first debranching of flavin derivative by removal of the ribityl moiety, then the reconfiguration of the ring structure, oxidation or reduction.

The mechanism of the photoreactions of flavins and detailed final products depend on the pH of the solution, the presence of oxygen and reducing species, temperature, environment, etc. [41]. The final products are: lumichrome (alloxazine derivative) in neutral and acidic solutions and lumiflavin (isoalloxazine derivative) in alkaline solutions [44]. It could be concluded that during ageing of gels the prosthetic group of glucose oxidase – FAD undergoes decomposition and as the result mainly alloxazine derivative is produced at pH 3 and 5, while at pH 7 and 9 both tautomeric forms are present. The ratio of the isoalloxazine form to alloxazine one increases with increasing pH (Fig. 8). The samples of hydrogel were not isolated from the daylight during ageing. Also the analysing light during the measurements could cause the FAD decomposition. The changes of tryptophan fluorescence are very similar at all pH studied but depend on the presence of glucose. The summary of fluorescence variation of tryptophane during ageing is shown in Fig. 9 (at pH 7 as an example).

For the oxidised form of enzyme, the intensity of fluorescence slightly increases during the first week and then decreases with a very small red shift of the maximum wavelength and some broadening of the emission band. Such changes of the tryptophane fluorescence are characteristic of conformational changes of the protein leading to unfolding and denaturation [47]. In the presence of glucose, the intensity of tryptophane emission decreases. At pH 3, after 4 weeks of ageing only 16.2% of initial intensity was retained, at pH 5, 7 and 9 – 18.9%, 31.2% and 37.7%, respectively. Also the red shift is more significant, after 4 weeks it amounts to 11 nm at pH 3 and 5 nm at other pH values. The emission band is broadened especially at the red edge of the spectrum. These changes indicate that the denaturation of glucose oxidase protein in the presence of glucose during ageing of the hydrogel is very pronounced. It could be supposed that the denaturation is not only caused by physical changes of the protein (apoenzyme) but also by some chemical changes. Aromatic amino acids, when irradiated with UV light in the presence of riboflavin or other flavin derivatives, undergo photodegradation and the rate constant of this reaction is greater in anaerobic conditions [46, 48–50]. The reaction is initialised by excited flavin in a triplet state [48, 49] and as the intermediate product the flavin semiquinones (radicals) are formed [49]. The photodegradation of tryptophane in enzyme protein molecules in the presence of riboflavin causes its deactivation [46].

3.4. Fluorescence of glucose oxidase in dried gels

Characteristics of fluorescence of glucose oxidase entrapped in silica gel dried in contact with the daylight is summarised in Table 4.

Table 4. Fluorescence properties of glucose oxidase entrapped in gels dried at the daylight,

Property	pH = 3		pH = 5		pH = 7		pH = 9	
	Ox	Red	Ox	Red	Ox	Red	Ox	Red
FAD λ_{max} (λ_{exc} = 450 nm) [nm]	511	509	516	516	524	515	521	517
I_{450}/I_{370}	0.614	0.209	0.596	0.289	0.596	0.325	1.116	0.330
Tryptophan λ_{max} (λ_{exc} = 295 nm) [nm]	344	441	338	348	339	433	342	433
$\lambda_{max\ exc}$ of unknown species [nm]	366	363	366	363	363	361	370	363
$\lambda_{max\ em}$ of unknown species (λ_{exc} = 360 nm) [nm]	467	461	467	461	460	460	467	462

As could be seen from Table 4, the fluorescence properties of GOD dried silica gel are very different from those of the native one, especially in the presence of glucose. Intensity of the HOMO-LUMO transition band in the excitation spectrum of FAD is very low as compared with that of the band peaking at 370 nm. In the presence of glucose, the value of I_{450}/I_{370} is extremely low; at pH 3 it is 9 times less than that for the native enzyme and at pH 9 – only 5 times. In the presence of glucose, the typical emission of tryptophan vanishes completely and a new maximum at 430 –440 nm can be observed. For gels without glucose this band can be observed as a shoulder. Also the new unknown species with fluorescence characteristics typical of alloxazine could be observed in dried gels, being the dominant fluorophore in the dried gel. These results indicate that in gels dried in the contact with the daylight, both the apoenzyme and prosthetic group – the FAD in glucose oxidase undergoe a photodestruction. The process is much more enhanced in the presence of glucose when there are practically anaerobic conditions inside the gel. This is in good agreement with the conditions of degradation of tryptophan photosensitized by riboflavin [46]. The degradation of glucose oxidase in gels depends also on pH and is the greatest at acidic environment.

When the gels were dried in darkness, the fluorescence properties of glucose oxidase were also changed as compared with those of the native enzyme but to a lesser extent. The direction of changes and the influence of pH as well as of the presence of glucose are comparable with those determined for wet gels and dried at the contact with the daylight. When the emission was excited at 370 nm, in the fluorescence spectrum of glucose oxidase a maximum at about 520 nm could be observed characteristic of FAD but also a little broad maximum or a shoulder at about 460 nm characteristic of alloxazine is present (Fig. 10).

Both types of dried gels were tested for leaching of the protein. They were soaked in the buffer as is described in the experimental part and then the buffers were tested for emission of FAD and tryptophane. The results are collected in Table 5.

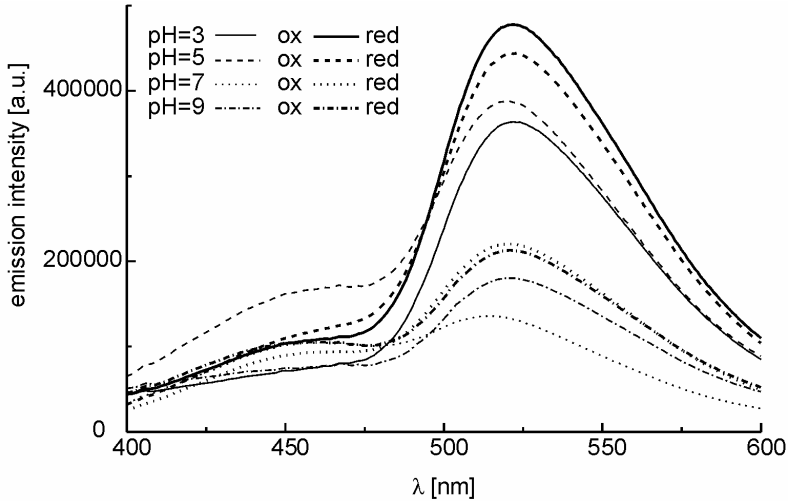

Fig. 10. Emission spectra of glucose oxidase entrapped in silica gels dried in darkness excited at 370 nm (The intensity of emission was corrected for a volume contraction of the gel after drying)

It could be seen that the species washed out from the dry gel have the fluorescence properties characteristic of the products of photodestruction of GOD. The emission spectra excited at 370 nm are similar to those of alloxazine derivatives [43].

Table 5. Characteristics of emission of buffers after soaking the dried silica gels in them

Property		pH = 3		pH = 5		pH = 7		pH = 9	
		Ox	Red	Ox	Red	Ox	Red	Ox	Red
λ_{max} [nm] (λ_{exc} = 450 nm)	a	530 (515^{s})	532	531	531	531	531	526	530
	b	502	509	496	504	494	506	500	505
λ_{max} [nm] (λ_{exc} = 370 nm)	a	466	465	–*	–*	459 (511^{s})	458	464 (506^{s})	456
	b	451	454	454	451	453	453	452	450
λ_{max} [nm] (λ_{exc} = 295 nm)	a	345 (419$^{s)}$	409	343 (414^{s})	414	343 (406^{s})	409	409 (344^{s})	411
	b	429	431	443	443	441	447	434	429

a – gels dried in contact with the daylight, b – gels dried in darkness, s – shoulder, * – not measured.

In supernatants from soaking of gels dried in daylight, species with emission similar to isoalloxazine derivative with the maximum at 530–531 nm (λ_{exc} = 450 nm) can be observed, different from that of FAD. It could be 4′,5′-riboflavin cyclic phosphate which is known as the first intermediate product of FAD photolysis [45]. Also the spectra excited at 295 nm indicate that in supernatant the protein is in a denatured form (red shift of the maximum emission of tryptophan) or the products of oxidation of tryptophan photosensitized by FAD (species with the maximum of emission higher

than 400 nm) are present. This result is very important from the practical point of view. If the sol-gel process is applied to produce a glucose biosensor working *in vivo*, the products of photoreaction of flavins and tryptophan could be washed out. The final products of this reaction (especially lumiflavin and lumichrome) are toxic [48, 50].

3.5. Activity of glucose oxidase entrapped in gels

While the experiments with gels dried at the daylight indicated that the destruction of GOD occurred to a great extent, the activity was tested only for the gels dried in the darkness. The results are presented in Table 6. The greatest retention of activity was observed for the gel prepared with phosphate buffer, pH 9, in the absence of glucose. This result is in good agreement with those of fluorimetric measurements indicating the least degree of decomposition of glucose oxidase. When the gels were prepared with addition of glucose, only traces of activity in dried gels can be found.

Table 6. Retention of the glucose oxidase activity in gels dried in darkness and in buffer after soaking the gel

pH	Form	Retention of GOD activity in gel	% of GOD activity washed from gel
3	ox	13.2%	14.3%
	red	0.3%	0
5	ox	11.8%	32.4%
	red	0.2%	0
7	ox	15.0%	26.2%
	red	0.1%	~ 66%
9	ox	17.6%	> 100%
	red	0.3%	~ 48%

Although the fluorimetric measurements indicated that mainly the products of GOD destruction are washed out, the supernatants showed enzyme activity indicating that also GOD in a native form is washed out from the gel. The greatest leaching was obtained for the gel with the best retention – prepared with buffer at pH 9. The activity of the supernatant at pH 9 for oxidised GOD is higher than that of the gel (Table 6). The reason for this surprising result could be the inaccessibility of a part of the enzyme enclosed for example in closed pores to the substrate [6].

The problem is what is the reason for the decomposition of glucose oxidase in gels dried in darkness with the products similar to those obtained by photodestruction. It is known that the intermediate species produced during the photosensitised oxidation of tryptophan are free radicals [48, 49] and hydrogen atoms are involved in the reaction mechanism [48]. Recently, it was shown that various types of paramagnetic defects are formed in pure silica gels obtained in the sol-gel process from TEOS [51]. In silica gels there were identified entrapped electrons, protons in O^{2-} vacancies, peroxy centres and others. All these defects in the presence of water can produce free electrons

or hydrogen atoms – very reactive red-ox species causing cytotoxicity of some silica gels [51]. It could be supposed that these paramagnetic defects in a gel could initialize the reactions between FAD and tryptophan through formation of free radicals by addition of electrons or protons.

4. Conclusions

Changes of the fluorescence properties of glucose oxidase during ageing of gels indicate unfolding and a partial denaturation of the apoenzyme and a chemical decomposition of tryptophan in the apoenzyme and FAD. The decomposition of glucose oxidase is more pronounced in dried gels, at lower pH and in the presence of glucose. Among the products of the reaction, the alloxazine derivatives were identified. The destruction of the enzyme is caused by photooxidation of tryptophan by FAD. Also FAD itself undergoes a photoreaction leading to dealkylation and tautomerisation to alloxazine. In the darkness, the decomposition of GOD is probably initialised by the annihilation of the paramagnetic centres formed spontaneously during the sol-gel process with water.

The results obtained indicate that the optimum pH of gel production by two step procedure from TEOS is 7 (neutral environment). They also indicate that the main problems in application of the sol-gel process in fabricating an optical biosensor for glucose would be a decomposition of the enzyme enhanced by light and the presence of glucose. The products of the GOD decomposition are easily washed out from the gel and are (especially alloxazine) potentially toxic which could be a problem at applications *in vivo*. If glucose oxidase was used in optical biosensors, it would be advantageous to use fluorimetric indicators excited by the light with the wavelength greater than 500 nm to avoid photodestruction of the enzyme.

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