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NEW ENZYME IMMUNOASSAY WITH VISUAL DETECTION BASED ON MEMBRANE PHOTOIMMOBILIZED ANTIBODIES

Key words: enzyme immunoassay, dot-analysis, IgG, Shigella Sonnei, thyroxine, human chorionic gonadotropin.

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ABSTRACT

New method for visual enzyme immunoassay of some model antigens in solution by using covalently photoimmobilized antibodies has been developed. This approach is based on the quantitative photoimmobilization of antibodies on the surface of porous matrixes. It is easy to control the dimensions and shape of the activated zones and the quantity of the active groups

on it by this technique. A strip of the membrane impregnated with p-azidobenzaldehyde was illuminated by the light. As a result, the quantity of aldehyde groups developed on the surface of membrane is proportional to the time of illumination. After the covalent immobilization of antibodies, the membrane has separate zones with an exact surface concentration of antibodies. The antigens of different types were assayed: human IgG, human chorionic gonadotropin, *Shigella Sonnei*. The lowest detection limit was 1 μg/ml, 20 U/l, $1x10^4$ cells/ml. The method allows measure of thyroxine concentrations in the range 50 to 200 nM; the precision of replicate measurements has the coefficient of variation 7%. The reasons for the background signal appearance were accurately analyzed. The choice of support was substantiated, the optimal conditions of its pretreatment being defined. This method makes possible the visualization of the results based on comparison the color intensity of zones with the control.

INTRODUCTION

The use of porous supports in the enzyme immunoassay (EIA) resulted in the creation of so called "on-site" or "doctor-office" kits for a fast visual quantification of different substances in biological fluids. When preparing any type of solid phase im-

munoassay, it is important to choose the optimal surface antibody concentration. In this respect, the covalent immobilization of proteins on the matrixes seems most convenient. There are different techniques of covalent binding, for example the use of benzoquinone as activation reagent for filter paper [1] . The application of porous support allows one to detect the result visually. However, the visual detection which takes into account dot size and intensity of staining is rather difficult. For examples, the dot coloring may not be uniform, the dot shape may be vague. Therefore it is complicated to define the exact dot size and to assess its intensity compared to the standard. This article describes the application of photoimmobilization method previously elaborated for enzymephotography processes [2] . The main idea of the method developed is the following. Impregnated with p-azidobenzaldehyde, the membrane is UV-illuminated by constant intensity. As a result on the membrane surface aldehyde groups are developed, the apparent surface concentration of which is proportional to the time of zone illumination. After incubation of this activated membrane in solution of antibodies, the antibodies were covalently immobilized and zones with proportional antibody surface concentration were developed. The analyte can be quantified in sandwich or competitive scheme of immunoassay.

EXPERIMENTAL

Materials and methods

Horseradish peroxidase (HRP), specific activity 829 U/mg, $RZ=A_{403nM}/A_{280nM}=3.0$, was purchased from Biolar. Sodium periodate was obtained from Merck. Potassium 4,4′-diazidostilbene-2,2′-disulfonate and diethylacetate p-azidobenzaldehyde were originally synthesized by A.A.Cshegolev at the division of chemical enzymology, Moscow State University. The following matrixes were used: chromatography paper FN-11 (Filtrak, Germany), regenerated cellulose MFC, regenerated cellulose on nylon net KS-49, nylon membrane (Polymersynthesis, Vladimir, USSR), nuclear (track) filters (Institute for Nuclear Research, Dubna, USSR), nitrocellulose (Chemapol, CSFR). Thyroxine (T4), bovine serum albumin (BSA), morpholinoethyl carbodiimide and diaminobenzidine dihydrochloride (DAB) were purchased from Sigma.

To prepare peroxidase-labeled anti-peroxidase antibodies, antiperoxidase serum was fractionated with ammonium sulfate, followed by ion-exchange chromatography on DEAE-Toyoperl 650M with a subsequent purification of IgG fraction coupled with peroxidase by the periodate method [3]

For sensitization, the matrixes were placed in the solution of photoreagent, than dried in the air at

room temperature in the dark. Two substances were used as photoreagents: potassium 4,4'-diazidostilbene-2,2'-disulfonate (PDAS) (10 mg/ml water solution) and diethylacetate p-azidobenzaldehyde (DABA) (0.45 mg/ml benzene solution). Nonfiltered light of mercury lamp and a Shimadzu CS-9000 deuterium lamp were used for illumination. The matrixes were washed with water or ethyl alcohol, respectively. DABA-activated matrixes were additionally treated with 0.1M HCl for 15-30 min.

IgG immobilization on photoactivated supports.

a) PDAS activated matrixes were placed in morpholinoethyl carbodiimide solution (5 mg/ml) for 30 min at room temperature then replaced into 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and immunoglobulin of different concentrations. Incubation was carried out at room temperature for 30-120 min. Noncovalently bound proteins were extracted by using PBS with 0.05% Tween-20. In other cases, 0.1 M Tris-HCl and pH 9.2 buffer containing 1 M NaCl, 0.5% Triton X-100 and 0.2% potassium deoxycholate were used.

b) DABA activated matrixes were placed into immunoglobulin solution ($1x10^{-6}$ - $1x10^{-8}$ M) in 0.01 M phosphate buffer (pH 7.4; 0.14 M NaCl) (PBS). Incubation was carried out for 3-120 min at room temperature or 12-14 h at $4^{o}C$. Then supports were treated with $NaBH_4$ (1mg/ml) solution in PBS for 15 min at room tem-

perature. The noncovalently bound proteins were extracted as described above.

Assay procedures.

Photoactivation (by DABA), pretreatment, immobilization of specific antibodies and removal of noncovalently bound proteins were performed as mentioned above.

a) Human chorionic gonadotropin (hCG) assay. Supports containing immobilized anti-β-subunit of hCG IgG were placed into the standard hCG solution in PBS containing 0.05% Tween-20 (PBST). The incubation time was varied. After washing the supports were placed into anti-hCG (α-subunit) IgG-enzyme conjugate solutions in PBST ($5^{x}10^{-9}$ M) for 10-60 min. After washing, thereof, the substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization. The intensity of dyeing was measured by a Shimadzu CS-9000 instrument.

b) Assay of human and rabbit immunoglobulins G. Matrixes were incubated successively in the solutions of IgG-standards, IgG-enzyme conjugate ($5x10^{-9}$ - $1x10^{-9}$ M) and substrate. The solution of 4-chloro-1-naphtol[4] was used for the detection.

c) *Shigella Sonnei* assay. Matrixes were incubated successively in the solutions of *Shigella Sonnei* standards, protein A-enzyme conjugate (1 ampule in 10 ml

PBST, 1 h, room temperature) and substrate. Diaminobenzidine solution was used for the detection.

d) Thyroxine assay. Matrixes were successively incubated in two solutions: one containing thyroxine standards and horse radish peroxidase labeled antigen (15-30 ng/ml) in PBST and the other containing DAB as substrate.

RESULTS AND DISCUSSION

IgG immobilization on photoactivated supports.

The earlier developed method for protein photoimmobilization allows one to easily control the size and shape of the activated matrix zone as well as the degree of its activation. The prospects for application of photoimmobilization to construct the systems for quantitative and semiquantitative immunodetection (with and without instrument respectively) of some model antigens will be discussed further. We selected the photoactivation reagent, support, light source and the method for pretreatment of matrixes to design the appropriate - to - exploit system possessing the maximum ratio, useful signal-background.

Choice of photocoupling reagent.

Some photoactive compounds known can be applied for photoimmobilization of proteins on solid supports.

Two reagents of these tested in our work were sodium 4,4'-diazidostilbene-2,2'-disulfonate and p-azidobenzaldehyde. The first compound is water-soluble and is characterized by a high rate of photodegradation [5].

A reagent was covalently bound to the matrix, nitrocellulose membranes or chromatographic paper, impregnated with sodium 4,4'-diazostilbene-2,2'-disulfonate by UV-illumination. The proteins were subsequently immobilized by the activation of one or two sulfo-groups of a photo-binding reagent with carbodiimide and by the reaction with amino-groups of protein.

In these conditions, rabbit G-immunoglobulin was immobilized on nitrocellulose matrix. For the quantitative determination of protein binding efficiency, the modified matrix was treated with A-protein marked with horse radish peroxidase (A-HRP). After evaluating the nonspecifically bonded protein (see "Materials and methods") the matrix was allowed to react with the substrate mixture to detect the enzyme label. The intensity of support colour is related to the amount of immunoglobulins in solution analyzed.

It follows from the dependency obtained (Fig.1) that the binding efficiency vs time is linear if the illumination time is about 15 min or less. It should be noted, however, that the rather high background level seems to be due to the activation of some acid groups on the matrix by carbodiimide and to the following

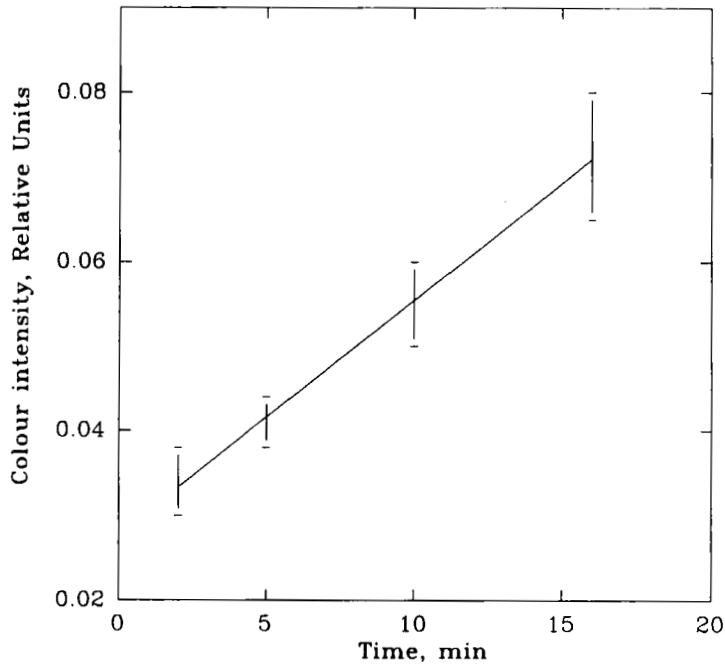

Fig. 1. Support colour intensity vs. illumination time. Nitrocellulose matrix was impregnated with sodium 4,4′-diazostilbene-2,2′-disulfonate. The concentration used was $5x10^{-7}$M. Incubation time was 60 min. Immobilized IgG molecules were quantifyed by using of protein A - HRP conjugate (1 (1 ampule in 10 ml of PBST, 1h, room temperature). Substrate solution containing 0.5 mg/ml DAB + 10^{-3} M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

binding of IgG-molecules. The decrease in immunoglobulin concentration in solution as well as in the immobilization time reduces the colour density of both the background and the exposed sites (table 1).

The better results were obtained by using p-azidobenzaldehyde as a photoimmobilization agent. It can be used in organic solvent, but not in water, which obviously restricts a possible matrix range. When the impregnated membrane is UV-illuminated the photosensitive molecule transforms into the nitrene biradical which can effectively interact with the matrix.

The protein immobilization was performed in this case by the Schiff's reaction of aldehyde groups on the matrix with amino groups of immunoglobulins.

It follows from the kinetic dependence of IgG binding on the photomodified matrix (Fig.2) obtained that if the immunoglobulin concentration ranges from $3x10^{-6}$ M to $3x10^{-7}$ M the basic amount of protein binds to the support faster than in 5 min. After 10-20 min incubation, the reaction was complete . Figure 2 shows the results correspond to the covalently immobilized proteins only. At all immobilization time intervals, the surface concentration of immunoglobulins deviates from one site of a matrix to the other in accordance with variable activation degrees.

It was reported [6] that in the process of photoimmobilization on the porous supports the ratio of

Table 1. Influence of immobilization time on the colour density of both background ($D^{650}_{R,0}$) and 15 min exposed sites ($D^{650}_{R,15}$) of matrix impregnated with sodium 4,4'-diazostilbene-2,2'-disulfonate.

Immobilization time, hours [IgG]=1mg/ml	$D^{650}_{R,0}$	$D^{650}_{R,15}$
2	0.025	0.070
1	0.010	0.030

aldehyde groups to protein molecules was 500:1. It is obvious that in this case the amount of protein would be the same on all activated sites. It is likely for most aldehyde groups to exist in spatially unapproachable areas of porous matrix or to be protected by already immobilized immunoglobulins. Therefore the matrix with a higher regular porous structure increases the concentration of protein per unit of surface area.

Choice of matrix

The properties of the matrix chosen as solid support have a large influence on the results of enzyme immunoassay. These matrixes should be a neutral polymer with minimal nonspecific interactions with biomolecules and components of staining solution. They are able to absorb qualitatively the final insoluble product of en-

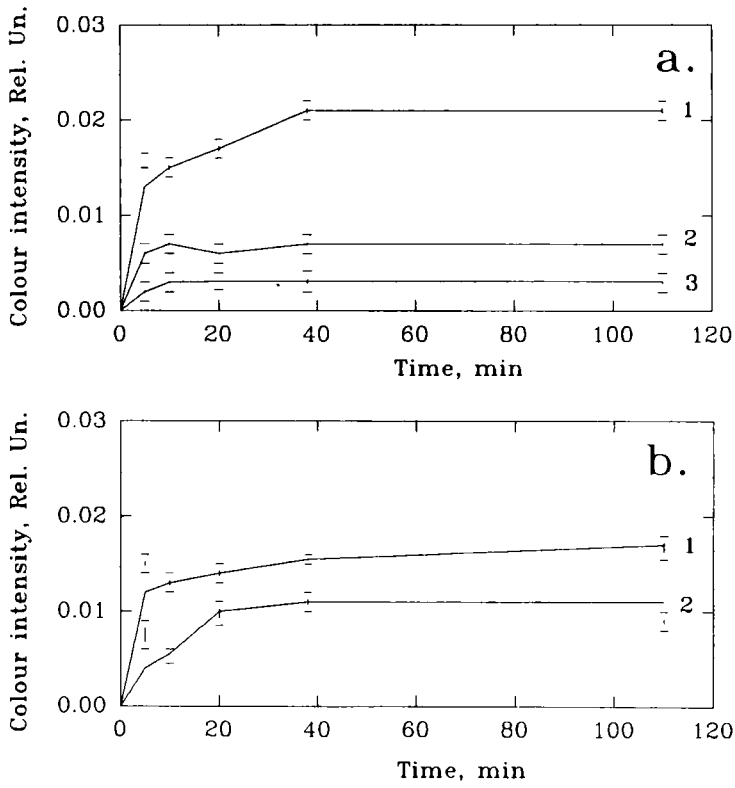

Fig. 2. Kinetics of the immunoglobulin immobilization on the photoactivated support FN-11. Plots were obtained for the following initial concentrations of IgG: a) 3.0×10^{-6} M, b) 3.0×10^{-7} M, and photoactivation times: 1) 10 min, 2) 3 min, 3) 0.5 min. Matrixes were impregnated with DABA. Attached IgG molecules were quantified by using protein A - HRP conjugate (1 ampule in 10 ml of PBST, 1 h, room temperature). Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

zyme reaction. Other requirements are the mechanical strength and the chemical stability during activation and functioning conditions. The porous matrix structure provides high specific surface and therefore high protein capacity.

Table 2 shows the results demonstrating the possibilities of the maximal binding of immunoglobulins by various porous supports. Some supports (nitrocellulose membranes, for example) can not withstand the treatment with a photoreagent solution in organic solvent. Others have a sufficiently large specific surface for the high efficiency binding (for instance, nuclear filters with a pore diameter of about 0.2-2 μm). For such membranes, the summary section area of the pores obtained is about a few percent of the whole film surface[7]. The highest ratio of signal to background was obtained with the matrixes manufactured from the regenerated cellulose (MFC) and cellulose matrix FN-11.

The absence of necessary mechanical strength of MFC membranes hinders their application in analysis. Application of regenerated cellulose matrix onto nylon network (KS-49) provides a tensile or bending strength to the matrix, a stability upon photochemical modification and EIA. The ratio of signal to background is high enough for KS-49 too (table 2).

Satisfactory results were obtained with chromatography paper support Filtrak FN-11, a highly porous cel-

Table 2. Application of the various supports for photoimmobilization of immunoglobulins.

Support	Photoreagent used	$D^{650}_{R,0}$	$D^{650}_{R,15}$
Chromatography paper FN-11	DABA	0.05-0.1	0.6-1.2
Regenerated cellulose MFC	-"-	0.01-0.02	0.6-0.9
Regenerated cellulose on nylon net KS-49	-"-	0.02-0.05	0.6-0.9
Nylon membrane	-"-	0.3-0.5	1 - 2
Nuclear filters	-"-	0.01	0.03

lulose material. However, such matrix can not be successfully used in all cases, as will be shown below.

Choice of UV-light source.

Any source of UV-light can be applied for the support photoactivation with the help of DABA. Mercury lamp and deuterium lamp of CS-9000 were used in this case. It should be noted that the differences in the dependence of the matrix activation degrees from the UV-illumination time can be caused both by the consequence of light source change and by the change of the matrix. For example, larger UV-illumination times are required for regenerated cellulose matrix activation

compared to FN-11. This should be caused, in particular, by glycerine widely used as stabilizer with some types of membranes, which can effectively interact with DABA upon its photoactivation. As follows from the dependence of surface protein concentration on FN-11 matrix versus illumination time (Fig.3), the most effective binding of immunoglobulin density on the matrix takes place during the period of UV-illumination by this source for about 4-5 minutes. After 20 min illumination, the surface protein concentration is highest ($>2x10^{-12}$ mole/cm^2). The quantity of covalently bound protein on the matrix was determined by subtraction of noncovalently bound protein from its total quantity.

Increase of signal/noise ratio.

The availability of the internal control section is important for verifying the total nonspecific binding in any type of immunoassay. The method of photoimmobilization permits combination of internal control with signal section on one matrix. The ratio of colour intensities for signal and internal control sections corresponds to signal/noise value. Influencing factors are considered below.

The quantity of noncovalently bound antibodies. This binding can be realized as a result of hydrogen, electrostatic, hydrophobic and dispersion bonds. For

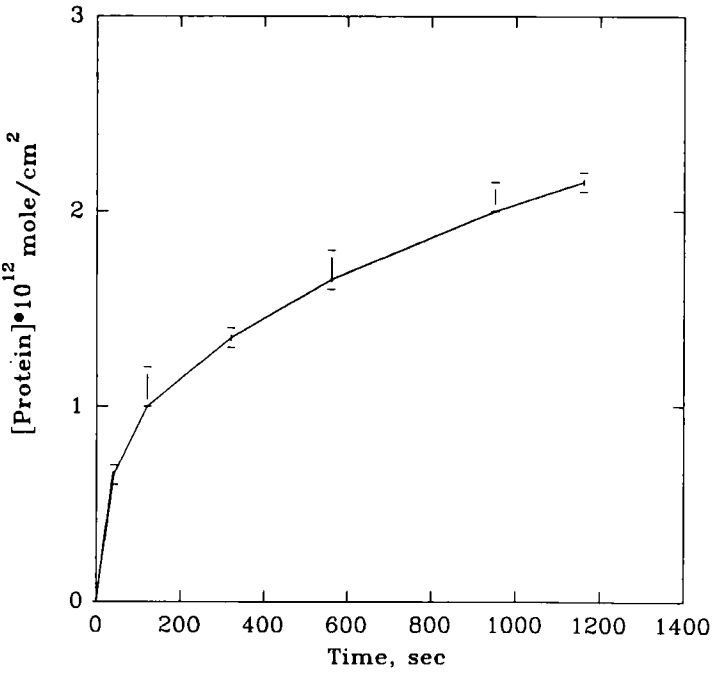

Fig. 3 Surface protein (conjugate IgG - HRP) concentration on FN-11 matrix versus illumination time. Deuterium lamp of CS-9000 was used in this case. Matrixes were impregnated with DABA.

Table 3. Composition of solutions for removing noncovalently bound immunoglobulins from different porous supports.

Matrix	Composition
Chromatography paper	1M NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, TRIS-HCl buffer pH 9.2
Nylon membrane	-"-
Regenerated cellulose	0.05% Tween-20, 0.01 M phosphate buffer, pH 7.2
Regenerated cellulose on the nylon net	0.1% Triton X-100, 0.01M phosphate buffer, pH 7.2

removal of antibodies noncovalently bound with matrix, the detergent solutions (Tween-20, Triton X-100, sodium deoxycholate) were used. The compositions of solutions to obtain the best results for different porous supports are presented in Table 3.

The antigen studied as well as the double antibody conjugate with an enzyme (in sandwich assay) could be adsorbed on the non-illuminated sites of the matrix. To decrease this type of interaction we blocked the matrix surface using BSA solutions (0.1-1.2%). The dependence of a reference signal value for FN-11 matrix is shown in Fig.4. It is obvious that 0.5% BSA solutions could be successfully used. To shorten the treatment duration, we used 2-5% protein solutions.

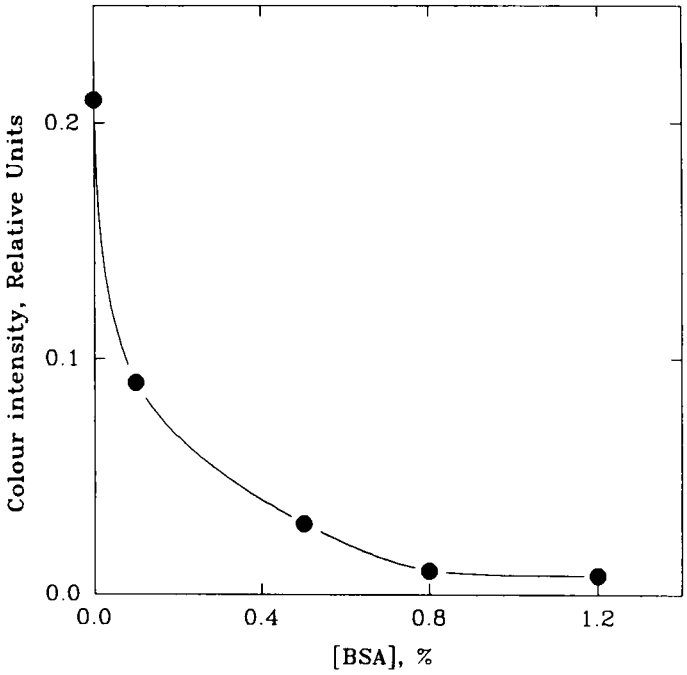

Fig. 4. Reference signal values versus concentrations of blocking BSA solution for FN-11 matrix. Matrixes were impregnated with DABA. Blocking time was 30 min. Unoccupied sites for sorption were titred by IgG - HRP conjugate ($5x10^{-7}$M), incubation time was 30 min. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

Table 4. Application of FN-11 matrix in hCG assay. Investigation of background signals.

	[hCG]=0 U/l	[hCG]=500 U/l
$D^{650}_{R,0}$	0.36	0.38

This method was used for creation of enzyme immunoassay systems for detection of different model antigens in solutions. Antigens of different types were analyzed: high molecular weight antigens - human IgG, human chorionic gonadotropin, cells of *Shigella Sonnei* and low molecular weight analyte thyroxine.

Immunoenzymatic determination of human chorionic gonadotropin.

As follows from the results of Table 4, the signals of high (500 U/l) and zero concentrations are practically equal for FN-11 support. The best results were obtained with the regenerated cellulose MFC matrix, no nonspecific protein sorption taking place actually.

The calibration curve for hCG determination in solution is presented on Figure 5. In practice the critical concentration of hCG is about 50 U/l. It follows from Fig.5 that this concentration can be detected with ease. This appears from the kinetic dependences of the hormone binding to the specific an-

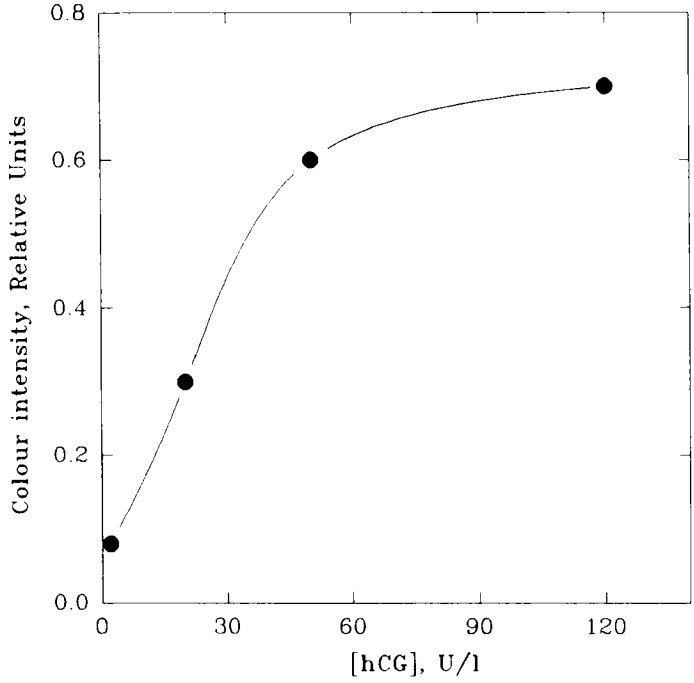

Fig.5 Standard curves of human chorionic gonadotropin. Time for first incubation was 40 min. Time for second incubation was 45 min. PBST was used as a washing solution. Time for colour development was 5 min. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

tibodies immobilized on the porous support (Fig.6) and to the specific antibodies conjugated with HRP via immunological complex on the porous support (Fig.7). The maximal signal develops in 20-30 minutes depending on variable concentrations (480 U/l, 48 U/l) of original protein under study. Thus, the whole analysis time was estimated to be 1.5-2 hours.

To simplify the analytical procedure and to decrease its time, the immobilized antibodies to β-subunit of the hormone containing strip were treated with the mixture of the hormone and conjugate solutions. The dependences of the signal versus the incubation time of the strip in this mixture are shown in Fig.8. Curves a, b, c, d correspond to hCG concentrations of 480, 120, 48, 0 U/l, respectively.
The analysis of these kinetic data allows us to consider that 20-30 min incubation time is sufficient to obtain the signals differing from the background even if the antigen concentration is significantly low. Therefore, the analysis time was reduced to 30 minutes as a result of optimization of the steps.

Enzyme immunoassay of human IgG.

IgG enzyme immunoassay was carried out using chromatography paper FN-11 as a solid matrix. The matrix was photoactivated in a manner, which allows obtaining the maximal concentration of immobilized antibodies.

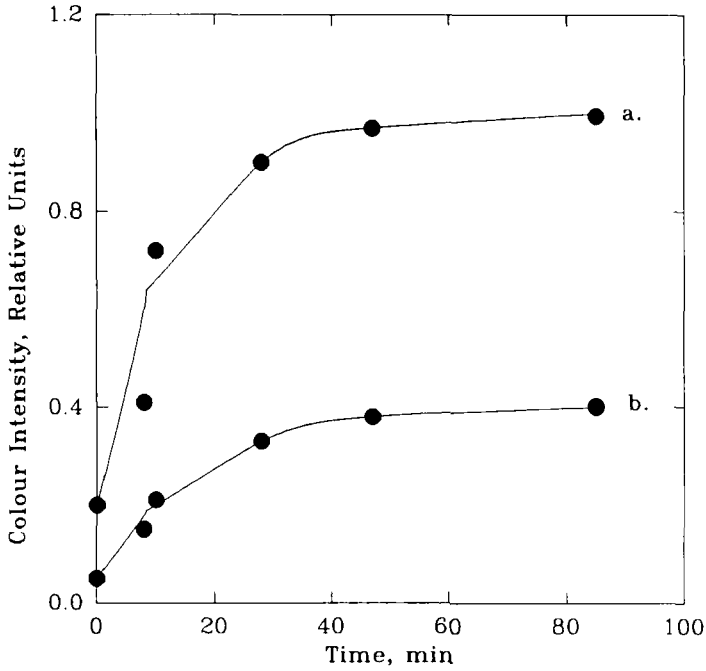

Fig. 6. Kinetics of the interaction between antigen (hCG) and immobilized antibodies. Plot was obtained for the following initial concentration of antigen a) 480 U/l, b) 48 U/l/. After washing the supports were placed into anti-hCG (α-subunit) IgG-enzyme conjugate solutions in PBST ($5x10^{-9}$ M) for 60 min. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

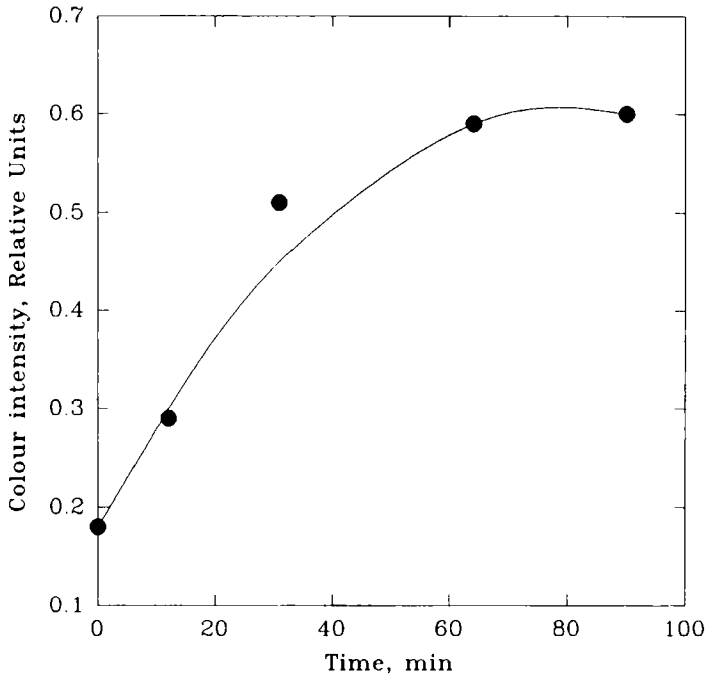

Fig. 7. Kinetics of the interaction between antibody-enzyme conjugate (5.0×10^{-9} M) and immobilized antigen (hCG) - antibody complex. The first step of the analysis carried out with 1 h incubation time. Initial concentration of antigen was 480 U/l. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

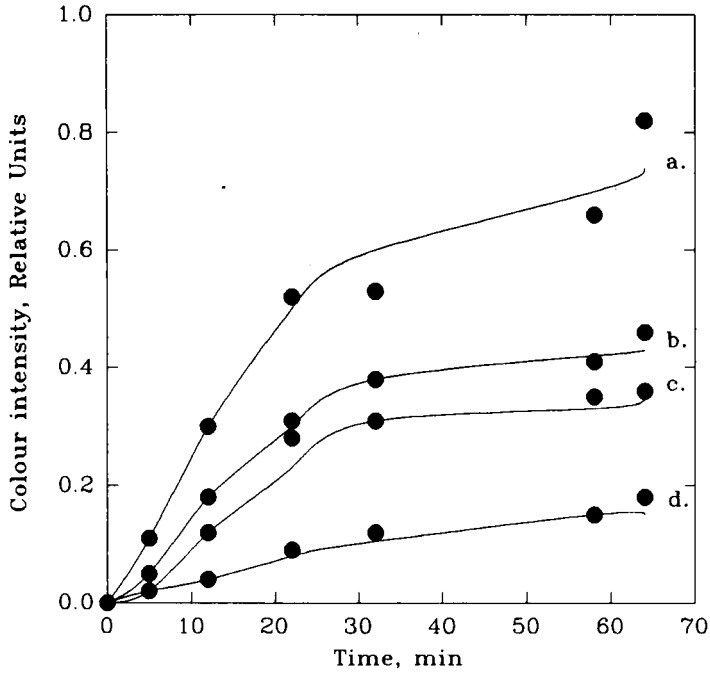

Fig. 8. Kinetics of the interaction between antigen (hCG), antibody-enzyme conjugate (5.0×10^{-9} M) and immobilized antibodies. Matrixes were incubated in the solution containing hCG and anti-hCG (α-subunit) IgG-enzyme conjugate solutions in PBST (5×10^{-9} M). Plots were obtained for the following initial concentration of antigen: a) 480 U/L, b) 120 U/L, c) 48 U/L, d) 0 U/l. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

The calibration curve obtained is shown in Fig.9. The lower detection limit was defined as a concentration corresponding to the signal differing from the background level on the double value of the absolute error in observation (the variation coefficient varied from 6 to 9 % (n=6) in this method). It was 0.5 μg/ml in this case. The linear part of the dependence is from 2 to 60 μg/ml.

Enzyme immunoassay of *Shigella Sonnei* cells.

From the calibration curve of the dependence of the matrix staining versus antigen concentration (measured by the appropriate number of cells) is shown in Fig.10. The fact follows that the concentration range for *Shigella Sonnei* cells analyzable is from $1*10^3$ to $1*10^6$ cells/ml. It should be noted that a low background signal (about 10% of the maximal) was observed only with the regenerated cellulose as porous support. In the experiments with nylon membrane and chromatography paper FN-11 extremely high values of the background signal were observed.

Competitive enzyme immunoassay of thyroxine.

For the choice of optimal surface antibody concentration, the influence of activation conditions on the calibration curve was studied. Support (FN-11) was UV-illuminated using a mercury lamp for 1, 4 and 6 sec. After that, the immobilization of specific antibodies, the incubation of the matrixes in solutions containing

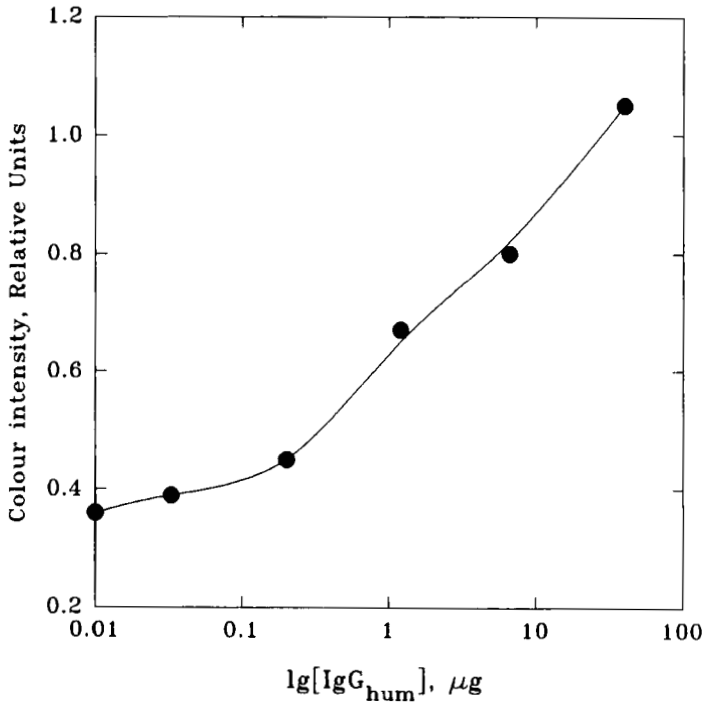

Fig. 9. Standard curves for human IgG. Initial concentration of antibody-enzyme conjugate was 5.0×10^{-9} M. Time for first incubation was 45 min. Time for second incubation was 45 min. PBST was used as a washing solution. Time for colour development was 20 min. The solution of 4-chloro-1-naphtol [4] was used for the detection.

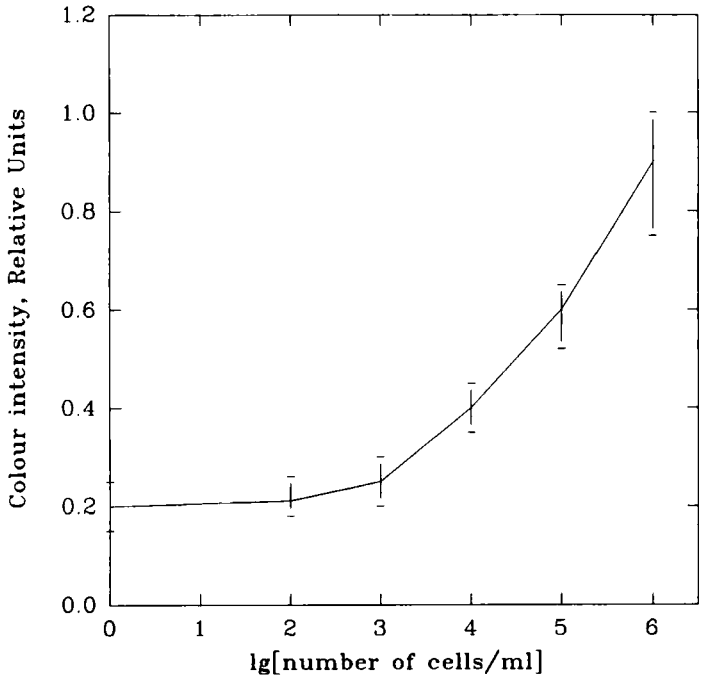

Fig. 10. Standard curves for *Shigella Sonnei* cells. Time for first incubation was 60 min. Time for second incubation was 45 min. PBST was used as a washing solution. Time for colour development was 5 min. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

labeled and analyzed antigens, the washing of matrixes and the detection enzyme label in substrate solution were performed successively. It follows from the calibration curves presented in Fig.11 that the increase in matrix activation degree owing to its longer exposition at the photoactivation step leads to displacement of the detectable concentration range to higher values.

The same result was reached at large illumination times when the inert protein (BSA) in different concentrations was introduced into IgG solution under immobilization. The lower detection limit was 50 nM, the variation coefficient varied from 4 to 9 % (n=8) in this method.

Thus, the possibilities of photoimmobilization technique in regulation of quantity of surface-immobilized protein quantity was used for the development of T4 competitive analysis systems in different concentration ranges.

CONCLUSION

The immunoenzymatic systems for quantitative instrumental determination of several model antigens have been developed on the basis of antibodies photoimmobilized on porous membrane-like supports. This technique can be effectively applied for semiquantitative visual detection. The prospects of using the photoactive reagent - p-azidobenzaldehyde in

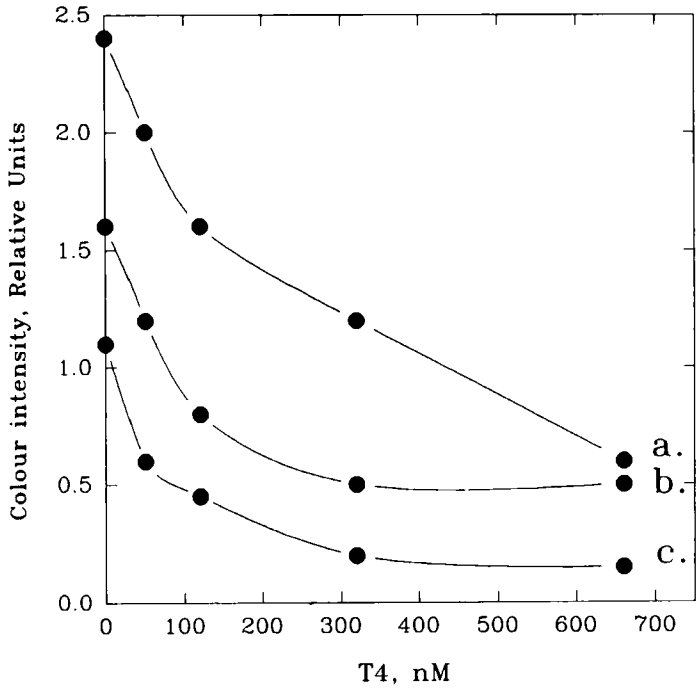

Fig. 11. Standard curves for T4 determination. Matrixes were impregnated with DABA. Support (FN-11) was UV-illuminated using a mercury lamp for a) 6 sec, b) 4 sec, c) 1 sec. Incubation time was 60 min. PBST was used as a washing solution. Time for colour development was 5 min. Substrate solution containing 0.5 mg/ml DAB + 10^{-3}M H_2O_2 + 0.02% Co^{+2} in PBS was used for visualization.

enzyme immunoassay were shown. The reasons for the background signal appearance were analyzed in detail. The best results were observed with the regenerated cellulose matrixes as support. The method was verified on different model objects: low-molecular antigen - thyroxine (the concentration range determined 50-200 nM); high-molecular antigens - human IgG, human chorionic gonadotropin, *Shigella Sonnei* cells (lower detection limit being 1 μg/ml, 20 U/ml, $1x10^4$ cells/ml respectively).

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