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Construction and use of two α-human atrial natriuretic peptide-fragment affinity chromatography columns in the isolation of C- and N-terminal epitope-specific antibodies for use in a prototype α-hANP biosensor☆

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ABSTRACT

Two α-human atrial natriuretic peptide (α-hANP) based affinity chromatography columns were produced by covalently immobilizing the C- and N-terminal epitopes of α-hANP. The stationary phase was made from a controlled-pore-glass bead solid support, which was silanized and treated with sulphosuccinimidyl 4-(maleimidomethyl)cyclohexyl carboxylate before the individual fragments were immobilized by substitution at their thiol groups. These columns were used to isolate α-hANP-specific antibodies from a goat anti-α-hANP serum, which were then further sorted according to their epitope specifity. These C- and N-terminal epitope-specific antibodies were in turn used as components in the construction of an α-hANP biosensor based on an enzyme-linked immunosorbent assay (ELISA) sandwich principle. Initial *in vitro* testing of the sensor using a physiological α-hANP solution showed a reproducible response to the peptide. There is to date no other equally fast, sensitive and precise method available to detect this peptide. This α-hANP sensor may prove to be an invaluable aid in human medicine as a monitor of patient status during transplant surgery, for example, an area inaccessible to radioimmunoassay and normal ELISA techniques.

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INTRODUCTION

The α-human atrial natriuretic peptide (α-hANP, amino acid sequence: SLRRSSCFGGRMDRIGAQSGLGCNSFRY with a disulphide bridge between residues 7 and 23) is a peptide hormone with 28 amino acids, produced and processed in specialized endocrine myocytes in the cardiac atrium [1–7]. Its receptors are distributed throughout the tissues of the human body, at its source in the atrium, the whole circulatory and nervous systems and at its main sinks in the kidneys, liver and lungs [8–11]. Its release is triggered by a stretch-stimulus in the atrium caused by high blood pressure, volume expansion or endothelin [12–15]. This provokes a rapid systematic response, an action taking 3–5 min, starting with the relaxation of smooth vascular muscle and culminating in the excretion of salt and water in the kidneys. It was thus found to be responsible for the regulation of blood pressure and salt/water content [16–25]. This hormone is excreted during the course of its action and forms the basis for the determination of kidney status.

There are various test systems for α-hANP on the market, but only complete antisera or monoclonal antibodies have been used in their construction. The systems used are the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). Both techniques are time-consuming, taking three days (excluding the time required to extract the substance to be tested for from a complex biological matrix) and are cumbersome as well as costly to use. Their viability decreases rapidly with storage time. The problem with an α-hANP RIA is the short half-life of the ^{125}I isotope used to label the peptide. Avoiding the use of radioactive compounds was another incentive to develop an alternative method of detection. The classic ELISA procedure, which employs an enzyme label instead of ^{125}I, does not solve the problems posed by time consumption and ease of use as well as long-term stability. In addition, both traditional tests require a lot of storage space in the laboratory refrigerators and freezers, especially during use. Neither procedure may be used directly, necessitating the extraction of the compound to be detected with methods that are not entirely reproducible and cause significant errors in the determination procedure. The design of these assays is such that samples are stored until a sufficient number have been stockpiled to make use of the assay economically viable. The effects of storage on the analyte content vary and have not yet been sufficiently documented. Thus, a rapid, sensitive, compact, easy-to-use system for the detection of α-hANP has been sought.

The ELISA-type assay was chosen as a starting point for the development. The only means of improving on and adapting the classical ELISA in our group were (i) to introduce a sensor surface instead of the microtitre plate, (ii) a change in the type of enzyme employed (traditionally horseradish peroxidase) to glucose oxidase, and (iii) the use of an easily detectable and perfectly reproducible hydrogen peroxide-induced electrical signal instead of the colour reaction with optical detection, thus eliminating another source of error. The most formidable problem was how to increase the specificity of the assay while maximizing the affinity of the antibodies used for analyte-recognition and labeling. The only source was a polyclonal goat anti-α-hANP serum, because monoclonal antibodies (obtained by cell culture) are usually of lesser affinity than the antibodies present in the antisera, although they are highly specific. Epitope scanning had shown that it contained at least two sets of antibodies, specific to separate epitopes (the site on the antigen recognized by the antibody) of the peptide: the C- and N-terminal sequences. It was decided that the only efficient and effective method of obtaining the separate antibody specificities would be the chromatographic separation of the specific antibodies by use of affinity chromatography matrices specially synthesized for this purpose. This particularly selective technique is passed on to the completed sensor.

A series of difficulties had to be overcome: the custom-synthesized N- and C-terminal sequences of the peptide had to be covalently immobilized onto a chemically well defined solid support. The

solid support had to be chemically and mechanically stable. The costly immobilized sequences and the adsorption blocker were not to bleed out of the column during use. The elution procedure was to supply biologically active antibodies.

The sensor provides the most rapid detection of α-hANP possible, it may be used more than once (*i.e.* one sensor for a series of samples) if so wished, and, once fully optimized, may be stored for longer periods of time without suffering an impaired response, as well as making possible the *in vitro* detection of α-hANP in a complex biological matrix, such as blood or urine.

This study sought to use only those antibodies in the antiserum specific to α-hANP. Many IgGs (immunoglobulin G) in the antiserum respond to the carrier of the synthetic antigen, but are useless for the α-hANP determination procedures, serving only to dilute the specific antibodies present. This carrier and dilution function of non-α-hANP-specific antibodies is not desirable when constructing a biosensor, as the signal intensity is reduced with the decrease in specific antibody concentration and biological activity.

The only feasible method of obtaining specific antibodies is first to immobilize the peptide in a biologically active form on a chemically and mechanically stable affinity chromatography matrix. The synthetic peptide is expensive and thus requires the use of a chemically well defined solid support. The only way to ensure the correct immobilization of α-hANP would be to tailor a matrix according to these requirements [26]. The synthesis and use of such a chemically and mechanically stable α-hANP-affinity chromatography matrix has been described previously [27]. This α-hANP column was used to isolate all antibodies specific to α-hANP from an anti-α-hANP serum.

Epitope scanning had shown that the antiserum used for this study contained antibodies specific for at least two separate epitopes of α-hANP: the C- and N-termini of the peptide. Therefore, it was logical to synthesize the C- and N-terminal fragments of the peptide and immobilise them separately. This produced two new types of affinity chromatography column with which it was possible to separate the antibodies specific for the C-terminal of the peptide from those specific to the N-terminal of the peptide.

This paper describes the synthesis of these new affinity chromatography matrices and the improvement on our previously developed CPG-α-hANP affinity chromatography matrix [27–33] and their use in the isolation of two pools of α-hANP-epitope-specific antibodies from a goat anti-α-hANP serum. The use of the antibody pools in the construction of a prototype ELISA-type α-hANP biosensor and repeated *in vitro* testing is also described [34–37]. It is hoped that this sensor, once fully optimized, will be of use for the *in vivo* detection of α-hANP in human medicine.

EXPERIMENTAL

Materials

Synthetic α-hANP and its custom-synthesized C- and N-terminal fragments were obtained from Cambridge Research Biochemicals (Norwich, UK); [^{125}I]-α-hANP tracer was purchased from ANAWA (Wengen, Switzerland); controlled-pore-glass (CPG) beads (44741) were obtained from Serva (Heidelberg, Germany); glucose oxidase was ordered from Boehringer Mannheim (Vienna, Austria); bovine serum albumin was obtained from Sigma (St. Louis, MO, USA); the disodium salt of EDTA (99% pure), powdered charcoal (highest purity, 2184), Triton X-100 (for scintillation measurement), chloranil and 1,4-diaminobenzene (for synthesis), amino- and thiosilane, sodium nitrite, citric acid monohydrate, cysteine, disodium hydrogenphosphate and all other buffer salts used, glutaraldehyde, glycylglycine and all of the solvents used, were purchased from Merck, (Darmstadt, Germany); the RIAzid radioimmunoassay was obtained from Henning (Berlin, Germany); the Eiken RIA was purchased from Behring (Vienna, Austria); Sulfo-SMCC and BS3 were ordered via Pierce (R. U. P. Margaritella Biotrade, Vienna, Austria); donkey anti-goat second antibody reagent and keyhole limpet haemocyanin were purchased from Sorin Biomedica, via Biomedica (Vienna, Austria); Sephadex G25 produced by Pharmacia (Uppsala, Swe-

den) and Spectra/Chrom desalting cartridges produced by Spectrum (Houston, TX, USA) were used; protein A Affigel Maps II was obtained from Bio-Rad (Vienna, Austria); α-hANP "for injection" was delivered by Bissendorf Peptides (Wedemark, Germany). The highest available reagent quality was used where possible, mainly for analysis unless stated otherwise.

Diazo-coupling onto CPG beads using an adapted method

The CPG beads were treated with amino- [27] or mercaptosilane, chloranil and 1,4-diaminobenzene [36]; 2 ml of 2 *M* HCl were mixed with 400 μl of a 2% (w/v) $NaNO_2$ solution, added to 1 ml of degassed, derivatized glass beads and shaken for 20 min at 8°C. The beads were washed with 10 m*M* sodium phosphate buffer (pH 7.0). α-hANP (0.25–10 mg) was dissolved in 1 ml of methanol and 2 ml of buffer, added to the glass beads and incubated at 8°C for 12–72 h. A sample of the supernatant was tested for unchanged α-hANP. Eiken and Henning RIA kits were used; each process was carried out according to the instructions supplied by the vendor. The CPG–α-hANP beads were washed with phosphate-buffered saline (PBS) containing 1 *M* NaCl followed by PBS to remove unchanged α-hANP.

Adsorption blocking and column packing

A 0.01 *M* solution of HCl followed by normal goat serum diluted 1:3 with PBS was used for the α-hANP matrix. Cysteine and glycylglycine (0.01 g/ml each in PBS) were combined for the C- and N-terminal fragment matrices. The columns were packed in McIlvaine buffer (pH 7.6) or PBS. Equilibration took at least 24 h before use. The columns were pre-run without loading a sample onto the matrix.

Immobilizing the C- or N-terminal fragment using Sulfo-SMCC

Aminosilanized CPG beads (1 g) were added to 10 m*M* sodium phosphate buffer (pH 8) (reaction volume 13.9 ml). Then 19.55 mg of Sulfo-SMCC were added, and the mixture was shaken at 30°C for 1 h. The beads were washed with 30 ml of Tris–HCl buffer (1.0 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, pH 7) and equilibrated in 10 m*M* phosphate buffer (pH 7) (reaction volume 9 ml). The C- or N-terminal fragment (0.0364 g) in 20 μl of methanol and 980 μl of 10 m*M* phosphate buffer (pH 7) was added to the CPG beads and incubated at 4°C for 20 h. The matrix was washed with (i) PBS spiked with 1 *M* NaCl (10 ml), (ii) PBS and (iii) 10 ml of PBS containing 5 μl each of mercaptoethanol and ethylenediamine, followed by repeated washes with PBS until no odour was detectable.

Treatment of the polyclonal serum prior to affinity chromatography

An ammonium sulphate precipitation or an Affigel Protein A MapsII column (used according to the manufacturer's instructions) was used to obtain the antibody fraction. The precipitated antibodies were desalted by use of either a Sephadex G25 column equilibrated in PBS buffer or a Spectra Chrom desalting cartridge (according to instructions supplied by the manufacturer). This raw antibody fraction was loaded onto the affinity chromatography columns designed during this study. The specific antibodies were eluted using McIlvane buffer at pH 2.6.

Treatment of affinity chromatography eluates

The acidic fractions were neutralized. A 50-μl aliquot of each fraction was mixed with a constant amount of [^{125}I]-α-hANP tracer and incubated at 4°C overnight. The eluates were stored at 4°C until the results of the adapted RIA were known.

Version 1. A 200-μl volume of precycled charcoal suspension [27] was added to all vials, except the total-counts vials (containing tracer only; total counts *T* used for the test), vortex-mixed and incubated at 4°C for 30 min along with vials containing buffer instead of sample (non-specific binding, NSB). The charcoal particles were centrifuged off at 2000 *g* and 4°C for 15 min. The supernatants were decanted into fresh tubes, and all tubes were counted for 1 min each in a gamma counter. The %NSB was determined by expressing the average NSB counts as a percentage of

the average T counts. This should be less than or equal to 5% T. The precycled charcoal may be used with an NSB up to 10% T without altering the significance of the test results. The cpm values obtained were plotted against the eluate fraction number next to the pH of the eluate. The eluate fractions containing the antibody peak were pooled according to the elution profile.

Version 2. A 200-μl volume of commercial second antibody reagent were added to each vial, except the total-counts vials, vortex-mixed and incubated at room temperature for 20 min. The immune complex was centrifuged off at 2000 *g* and 4°C for 15 min. The supernatant was aspirated, and 1 ml of RIA buffer [27] was added to wash the pellet, which was centrifuged off as before. The supernatant was aspirated and the pellets were counted as above.

Determination of the antibody titre of the eluate pool

A 200-μl volume of PBS was pipetted into all but the second of twenty vials, then 200 μl of the antibody pool were pipetted into the second and third vials only. The contents of the third vial were vortex-mixed for 10 s, and half the volume (200 μl) was pipetted into the next vial. This was repeated until the last vial was vortex-mixed, when half its contents were discarded. The procedure was continued as for the treatment of the eluate fractions, versions 1 and 2. The graph of cpm *versus* dilution was used to determine the antibody titre of the pools investigated by reading the dilution at double the background (NSB) counts.

Determination of the optimum immobilization pH for α-hANP

The matrix used was CPG–Si-NH–glutaraldehyde–1,4-diaminobenzene–NH_2; aliquots of 10 mg were used in a 10 mM phosphate buffer at pH values of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The Bio-Rad Bradford Reagent microassay was employed.

The sensor substrate

The sensor was a 100–300 μm sodium silicate glass sheet, partially coated with a 30-nm titanium adhesion layer and a 50-nm platinum thin-film electrochemical electrode. Structuring of these thin films was performed by the lift-off technique. The layers were insulated by a 2 μm siliconenitride (Si_3N_4) layer and structured by dry etching [34,35]. The platinum surface was cleaned by chemical etching or sputtering, and oxidized by use of plasma oxidation in CF_4. The finished product was washed with acetone in a sonicator for 10 min and stored in a microcentrifuge tube until used [38,39].

Derivatization of the sensor substrate

The immobilization area was reduced with dithionite, oxidized with chromate, and aminosilanized [33].

Preparation of the N-terminal antibodies

The volume of the antibody solution was determined, and $NaIO_4$ added to a final concentration of 0.02 M. The mixture was vortex-mixed and incubated in a water-bath at 23°C for 1 h in the dark. The reaction was stopped by the addition of ethylene glycol to a final concentration of 0.36 M. The solution was dialysed against 0.1 M phosphate buffer (pH 6.0). Ethylene diamine was added to a final concentration of 0.1 M and incubated at 35–40°C for 1 h. The solution was treated with 0.2 mg/ml $NaBH_4$ and dialysed against 10 mM sodium acetate buffer (pH 4.8).

Coupling of the N-terminal antibodies to the sensor substrate

A 0.6-ml volume of antibody solution was added to 2.4 ml of 0.1 M $NaHCO_3$ buffer (pH 8.3) and vortex-mixed; 125 μl were added to each substrate and incubated at 4°C overnight.

Derivatization of the C-terminal antibody

The antibody concentration was determined by a Bradford assay. The procedure was analogous to the preparation of the N-terminal antibodies. A glucose oxidase (GOD) stock solution was prepared to give a 10.5-fold molar excess when added to the antibody solution. Bis-sulphosuccinimidylsuberate (BS3) was added to a final

concentration of 1 m*M* and incubated at 35–40°C for 1 h. The reaction was stopped by the addition of 10 μl of PBS containing 2.5 μl of ethylene diamine per millilitre. A 1-ml volume of the reaction mixture was cleaned up by gel permeation in PBS-equilibrated Spectra desalting column. The procedure was monitored by adsorption at 280 nm.

Loading the sensor

A Bissendorf α-hANP vial and solvent were used. The sensor was washed in 0.1 *M* phosphate buffer (pH 7.0). α-hANP solution (125 μl) was added to each sensor and incubated at 37°C for 10 min. The sensor was washed in 0.1 *M* phosphate buffer (pH 7.0), incubated in 125 μl of the second antibody-GOD marker at 37°C for 20 min, and washed before measurement.

Measuring principle

The sensor was operated in a constant flow cell with a 20 m*M* glucose solution in 0.1 *M* sodium phosphate buffer (pH 7.0). A standard H_2O_2 solution, 10 μl of 30% H_2O_2 in 100 ml of sodium phosphate buffer (pH 7.0), was used for calibration purposes.

RESULTS AND DISCUSSION

The biological recognition element exploited throughout was the antibody–antigen immune reaction between synthetic α-hANP or two of its fragments and antibodies specific to it. The polyclonal goat anti-α-hANP serum was raised using a synthetic antigen consisting of synthetic α-hANP haptenized onto a bovine serum albumin (BSA)–keyhole limpet haemocyanin (KLH) carrier using glutaraldehyde.

The study was divided into two phases. The object of phase 1 was the development and optimization of α-hANP and α-hANP-fragment affinity chromatography columns, and their use in the isolation and concentration of α-hANP-specific and α-hANP-epitope-specific antibodies from the polyclonal serum. Phase 2 was concerned with the development and preliminary testing of a prototype biosensor for the detection of α-hANP, using the antibody pools isolated in phase 1. It is hoped that the system may be applied to the detection of α-hANP in human urine and plasma.

Epitope scanning results using the antibody eluate of one of the first α-hANP columns synthesized (glutaraldehyde spacer) [27] showed that there are three sectors of the peptide that may be considered to be epitopes. So far, antibodies corresponding to two of these have been isolated.

The antigen/antibody affinity is high, because very short adapted RIA incubation times, as little as 1 h, sufficed to form the immune complex for *e.g.* tracer testing (only the biological activity of the tracer was of importance here), because the elution pH for antibodies from the column was rather low (pH 2.6), and because the determination of the immune complex was also possible by use of high-performance capillary electrophoresis (HPCE) [30].

Two types of affinity chromatography matrix were tailor-made to isolate antibodies of known specificity from this serum.

CPG beads have previously been tested for their suitability for the site-directed covalent immobilization of α-hANP [27]. The C-terminal tyrosine of α-hANP was the only residue available for this purpose, without altering the conformation and thus the biological activity of the peptide. The identity of the peptide was checked by plasma desorption mass spectrometry and high-performance liquid chromatography before use (results not shown).

The surface of the CPG beads had to be chemically altered to suit this application, as shown in Figs. 1–3. Surface silanol groups were formed, which served as anchors for the spacer moiety onto which α-hANP was immobilized. The following generations of functional groups introduced shared one feature: they formed the flexible spacer onto which α-hANP was immobilized. They were specially chosen for their hydrophobic/hydrophilic interactions with the surroundings of the finished spacer, so as not to interfere with the biological recognition of immobilized ANP in the finished matrix. Amino- and thiosilanes were used to coat the surface with 15–800

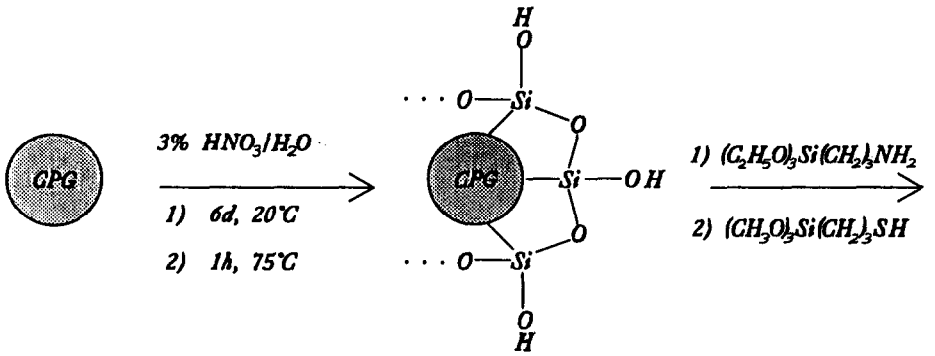

Fig. 1. CPG beads were treated with an acidic aqueous solution to produce silanol groups and allow the solid support to swell. The silanol groups were treated with either amino- or mercaptosilane to produce amino- and thio-glass beads, respectively.

μmol of amino or thio groups per gram while blocking access to the support structure underneath, producing so-called "amino" and "thio" glass beads, respectively. They were stable in this form when dried, and formed the basis of all the matrices produced. The amino glass beads were further treated with chloranil and 1,4-diaminobenzene as described in Experimental. The diazo-coupling procedure to attach α-hANP to the spacer, developed previously [27–33], was modified so as to reduce the reaction time and decrease exposure of the matrices acidic solutions. RIA kits (Henning RIAzid and Eiken) were used to test for excess unreacted peptide and showed

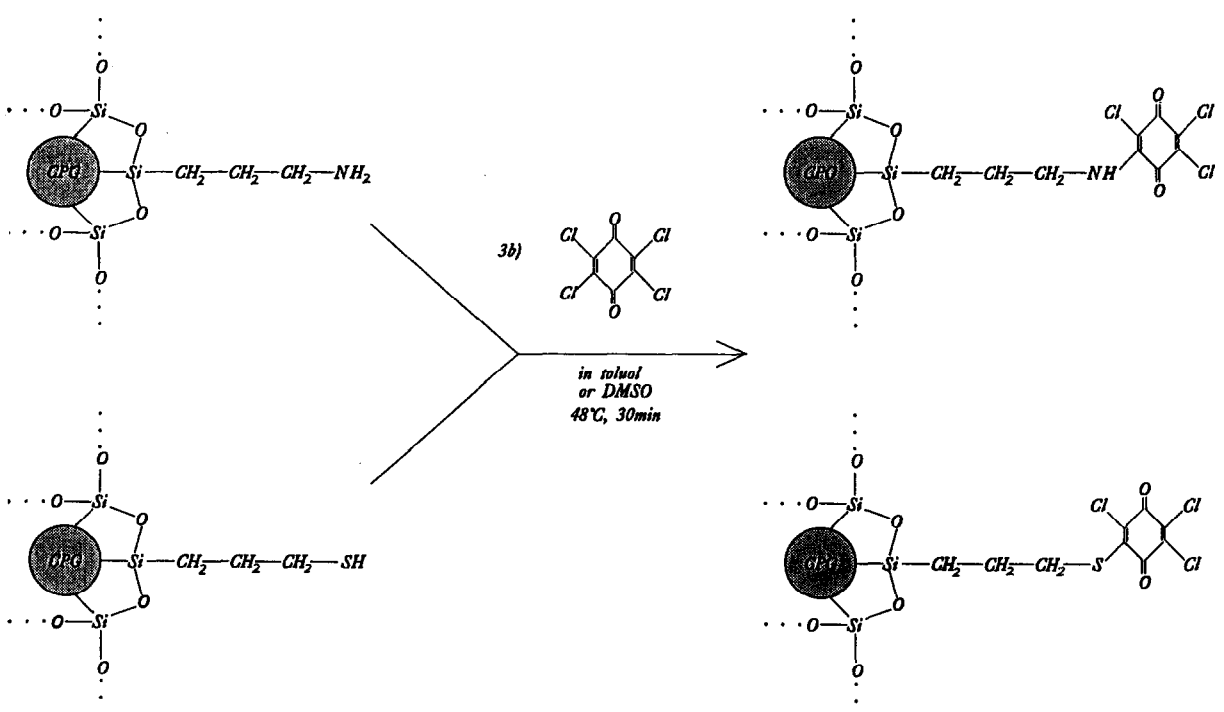

Fig. 2. Amino- and thio-glass beads were treated with a solution of chloranil in toluene and DMSO in preparation for the reaction with 1,4-diaminobenzene.

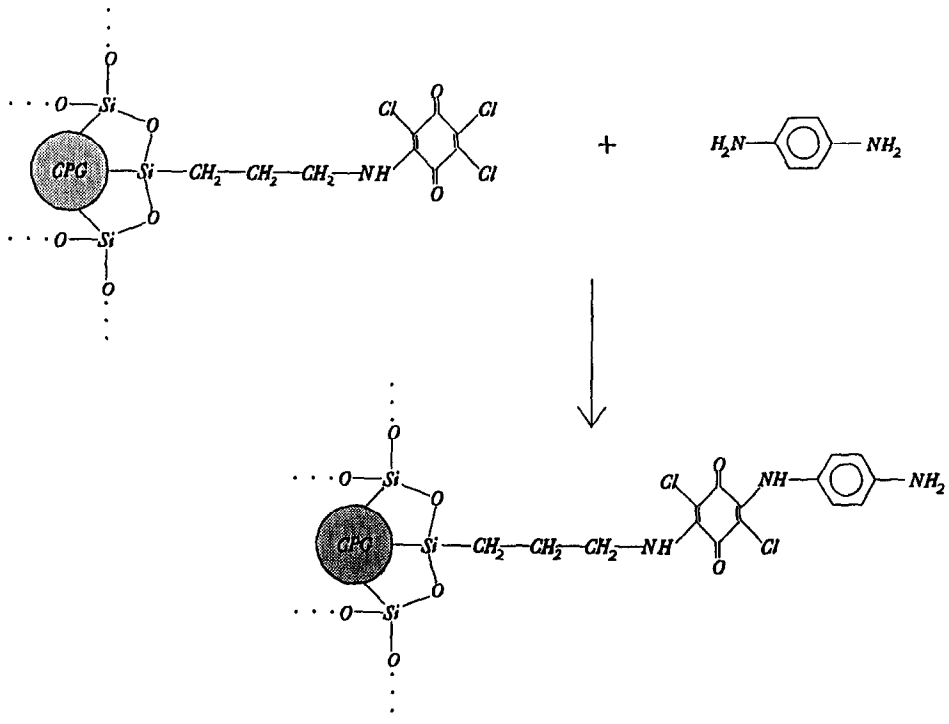

Fig. 3. Chloranil-glass beads were treated with 1,4-diaminobenzene to introduce an aromatic amino group suitable for the immobilization of α-hANP.

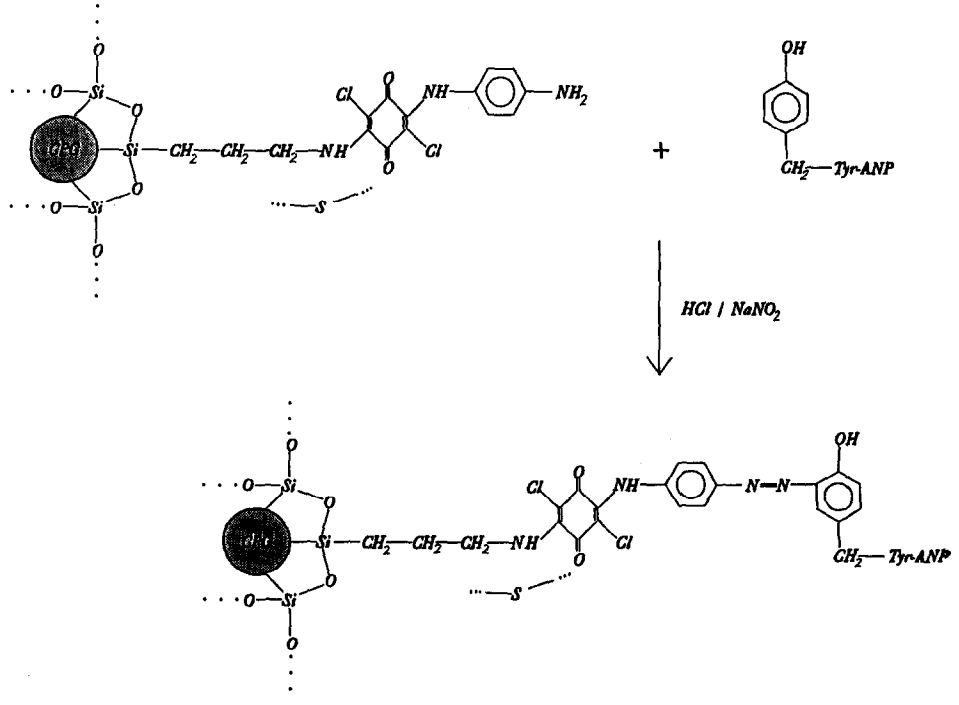

Fig. 4. Chloranil/diaminobenzene glass beads were treated with HNO_2 to form the diazonium ion, which reacted with α-hANP at the tyrosine residue marked in Fig. 1 on the aromatic ring, *ortho* to the hydroxy group.

that more than 99% had reacted. Possible unreacted or incomplete spacers, as well as patches of unsilanized glass bead surface, were blocked using dilute HCl and normal goat serum. Other blockers, such as human haemoglobin, L-tyrosine, 0.01 *M* ethylenediamine, were found to be less effective or to block antibody access to the immobilized peptide. Every matrix was run under optimized conditions before being loaded with antibodies to open a "window" for change in pH and ionic strength during its use. The columns were packed in McIlvaine buffer (pH 7.6) or PBS. Equilibration took at least 24 h before use. Glutaraldehyde, previously used as a spacer moiety [27], is well known for its side-reactions. Its substitution by chloranil (*cf.* Fig. 2) resulted in increased stability and reproducibility of the matrices. This reagent reacts specifically with the amino or thio groups present and facilitates the reactions with 1,4-diaminobenzene (*cf.* Fig. 3).

The α-hANP may be immobilized directly onto the chloranil, but this did not result in a specifically oriented peptide and its biological activity was reduced slightly, so that oriented diazo coupling to the aromatic amine was preferred (*cf.* Fig. 4). A pH series showed that α-hANP was best immobilized at pH 7.0.

The following procedure was the result of extensive screening. A batch procedure was used for the matrices during the optimization procedure, but thereafter only thermostatted columns, Amicon or minicol (8°C), were used. Antibodies applied to the matrices were cycled through the columns for 1–12 h during loading in PBS or McIlvaine buffer (pH 7.6). The columns were then washed with McIlvaine buffer (pH 7.6) to remove excess antibody, followed by PBS to counter reversible adsorption. The antibodies were eluted at pH 2.6 as soon as required. Both the acidic fractions and the columns were neutralized with McIlvaine buffer (pH 7.6) to accelerate the neutralization procedure. The antibodies were found to be stable on column for at least three months. The eluates of the α-hANP columns were stable at 4°C in McIlvaine buffer (pH 7.0) for at least one year, their titre was not significantly reduced (results not shown). It was noted that the CPG bead matrices can be recycled in batch and in column form even after drying.

α-hANP labelled with ^{125}I on the tyrosine residue was used as a tracer in a RIA designed to monitor immune-reactive antibodies in the eluate aliquots. Two versions were used: (i) excess tracer was adsorbed onto precycled charcoal: use of RIA-grade BSA reduced the NSB; (ii) a second antibody reagent was used to precipitate the immune complex (*cf.* Fig. 5): the concentration of a second antibody in the reagent must be in excess of that of the first antibody–antigen complex.

The spacer used for the stationary phase was long enough to allow antibody access to both the N- and C-termini of the immobilized peptide. Either the C-terminal tyrosine residue was not essential to the epitope or else its covalent bond to the spacer moiety had not sufficiently altered the epitope to be able to cause significant interference to antibody recognition. This necessitated the design of a second column type. These polyclonal anti-α-hANP antibodies were separated according to their epitope specificity using the next generation of columns.

The custom-synthesized C- and N-terminal fragments, each up to and including the cysteine residue (CNSFRY and SLRRSSC, respectively) were immobilized separately onto amino glass beads using the heterobifunctional coupling re-

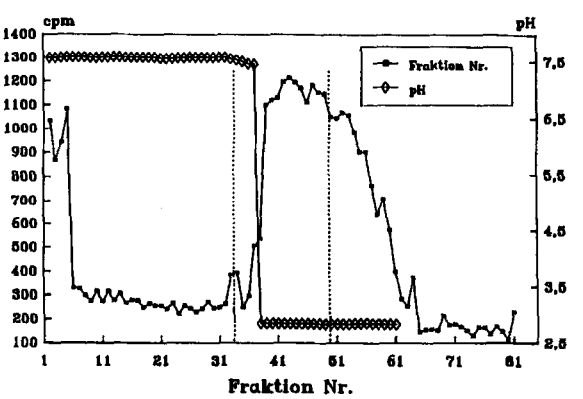

Fig. 5. Complete elution profiles of CPG-α-hANP, CPG-C-terminal and CPG-N-terminal affinity chromatography columns. The activity (cpm) bound by the antibodies present in the eluate and the pH value of the individual eluate fractions were plotted against the eluate fraction number.

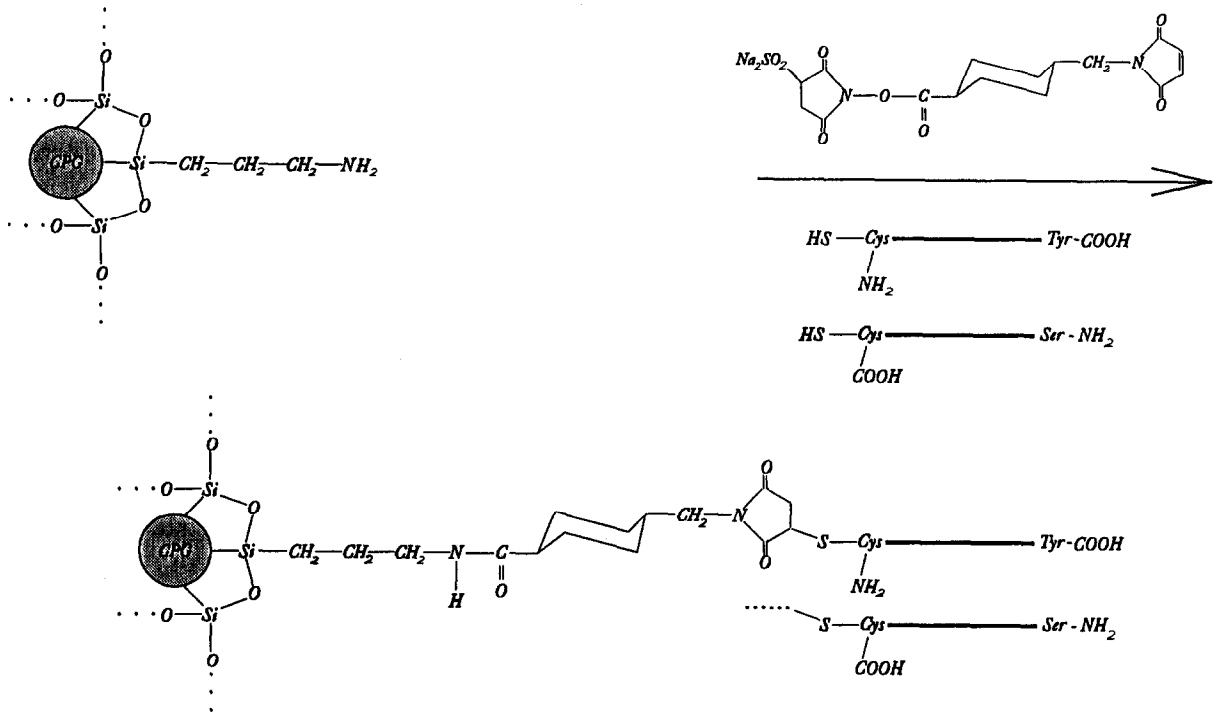

Fig. 6. Amino glass beads were treated with Sulfo-SMCC in preparation for the reaction with either the C- or the N-terminal peptide fragment of α-hANP.

agent Sulfo-SMCC (*cf.* Figs. 6 and 7). Adsorption blocking for these matrices made use of normal goat serum (NGS) or a mixture of cysteine and glycylglycine. Running conditions were identical with those of the α-hANP affinity chromatography columns. These columns enabled the isolation of separate IgG pools, specific to two different epitopes of the peptide hormone. The eluates were referred to as C- and N-terminal antibody pools, respectively.

Fig. 8 shows the elution of anti-α-hANP, anti-C- and anti-N-terminal epitope-specific antibodies form their respective affinity chromatography columns after one week to one month of storage at 8°C in the constant-flow mode. The antibodies were eluted using McIlvaine buffer (pH 2.6). The biologically active antibody content was monitored by use of the adapted RIA procedure described in Experimental.

A biosensor combines the specificity and sensitivity of biological systems with the computing power of the microprocessor, providing a powerful analytical tool. An amperometric device was used to transduce the biorecognition signal. The biological element of this prototype was an ELISA sandwich made up of the C- and N-epitope-specific anti-α-hANP antibody pools isolated above. α-hANP was the chemical to be detected, and GOD [36] was the marker enzyme used to produce a chemical signal, which was converted into a digital electronic signal by an integrated transducer. This was fed to a computer and processed to give a proportional graphical display.

The only reason for constructing an ANP biosensor was to reduce the time required for an α-hANP assay. The advantage of speed of detection, combined with the ideal size of the sensor, make it possible to use in investigating metabolic disorders and heart insufficiency and in monitoring transplant surgery. The derivatization of the sensor–substrate immobilization area is shown in Fig. 9.

The Woodward procedure [40] was adapted to

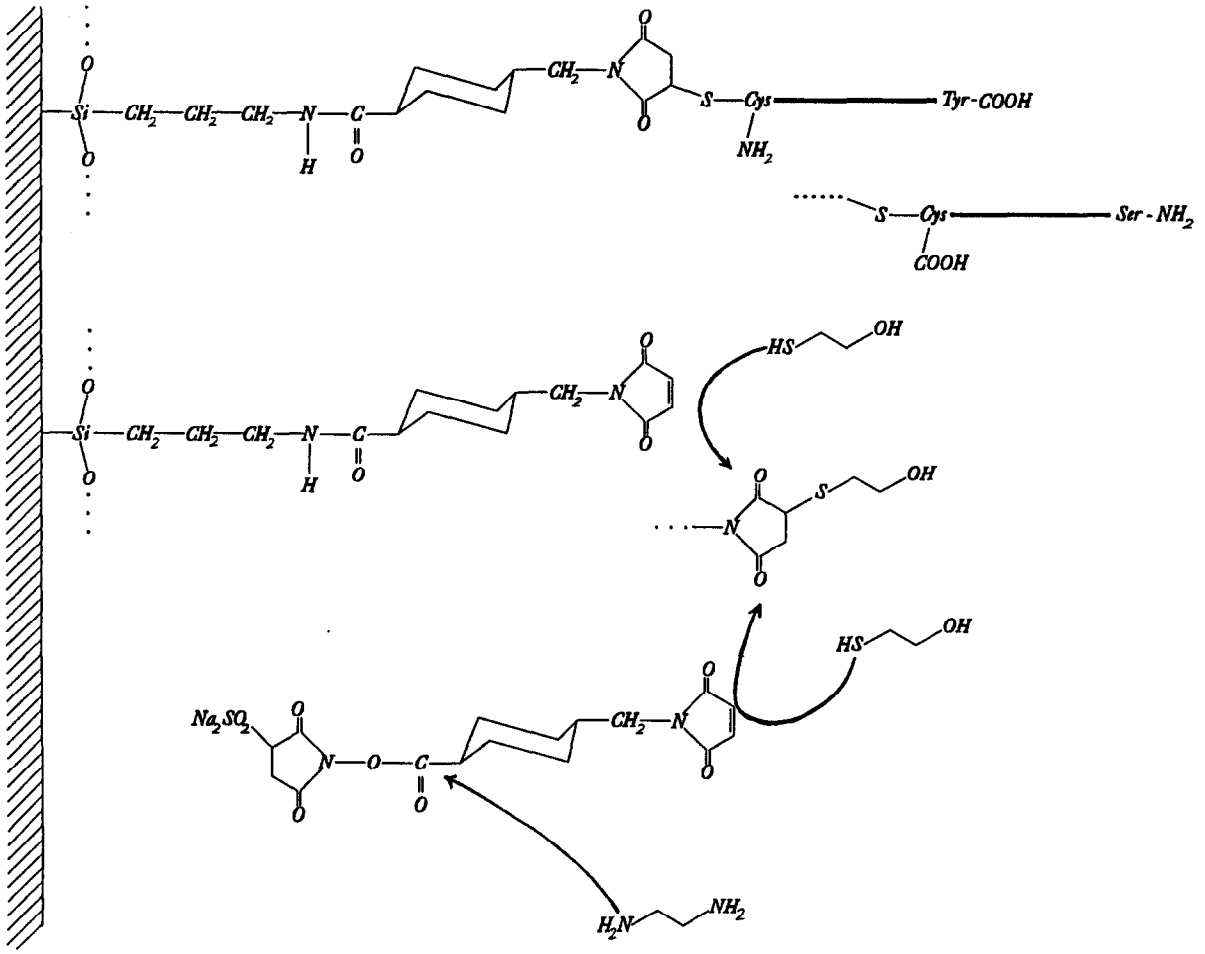

Fig. 7. Glass beads carrying the C- and N-terminal α-hANP fragments were treated with ethylenediamine and mercaptoethanol to remove any residual or partially reacted Sulfo-SMCC before adsorption blocking took place.

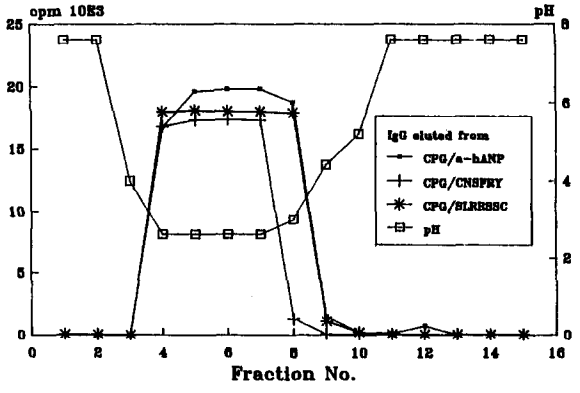

Fig. 8. Elution of anti-α-hANP, anti-C- and anti-N-terminal epitope-specific antibodies from their respective affinity chromatography columns after one week to one month of storage at 8°C in the constant-flow mode. The antibodies were eluted using McIlvaine buffer (pH 2.6). The pH of the fractions was monitored using a miniature pH electrode suitable for volumes of less than 1 ml. The biologically active antibody content was monitored by use of the adapted RIA procedure described in Experimental.

derivatize the N- and C-terminal antibodies at their glycosyl residues, so that they could be immobilized on the sensor or treated with marker in a biologically active conformation, respectively. The bis-sulphosuccinimidylsuberate (BS3, Pierce) used is a homo-bifunctional cross-linking reagent specific for primary amino groups. (*cf.* Figs. 10,11 and 12). Here, the N-terminal-specific antibodies were used to select and bind α-hANP to the sensor. The C-terminal-specific antibodies were labelled with GOD and used to detect the bound α-hANP.

The sensor was incubated in a standardized pyrogen-free physiological α-hANP solution, which is normally used for therapeutic purposes. The second antibody–GOD reagent was used in excess relative to the other reagents, to ensure that

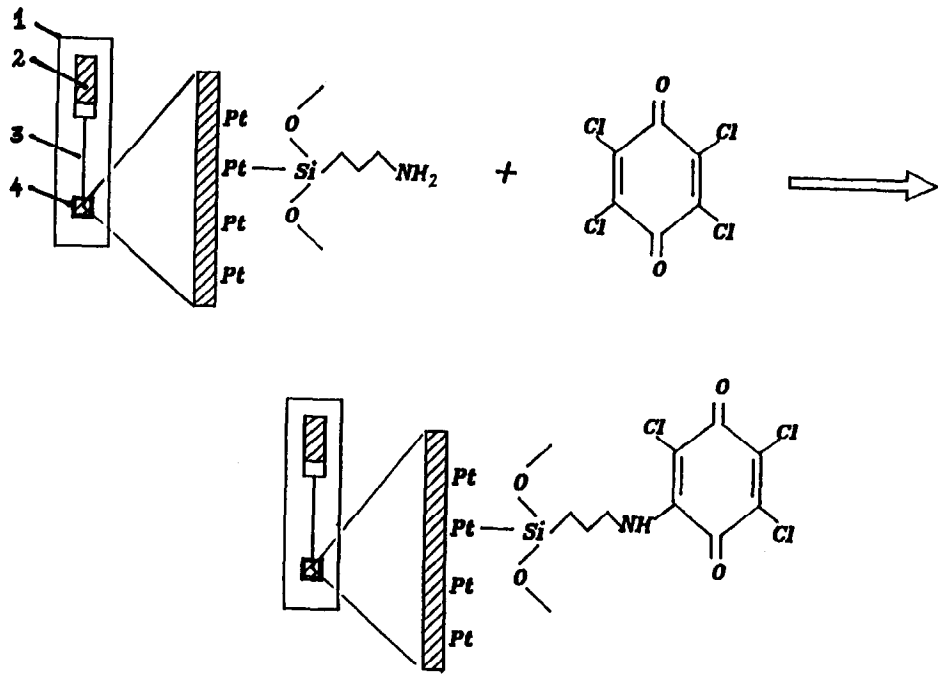

Fig. 9. The sensor substrate was a platinum–titanium thin film sandwich on a glass substrate: 1 = the sodium silicate glass sheet; 2 = the contacting zone (not insulated); 3 = the platinum conductor band; 4 = the 0.64 mm^2 immobilization area, the edges of which were insulated with Si_3N_4. The following reaction is demonstrated: the oxidized platinum surface of the sensor substrate derivatized with 3-aminopropyltriethyoxysilane further reacted with chloranil at the amino groups, thus activating the chloranil residue (in the *para*-position 4) for reaction with primary amines.

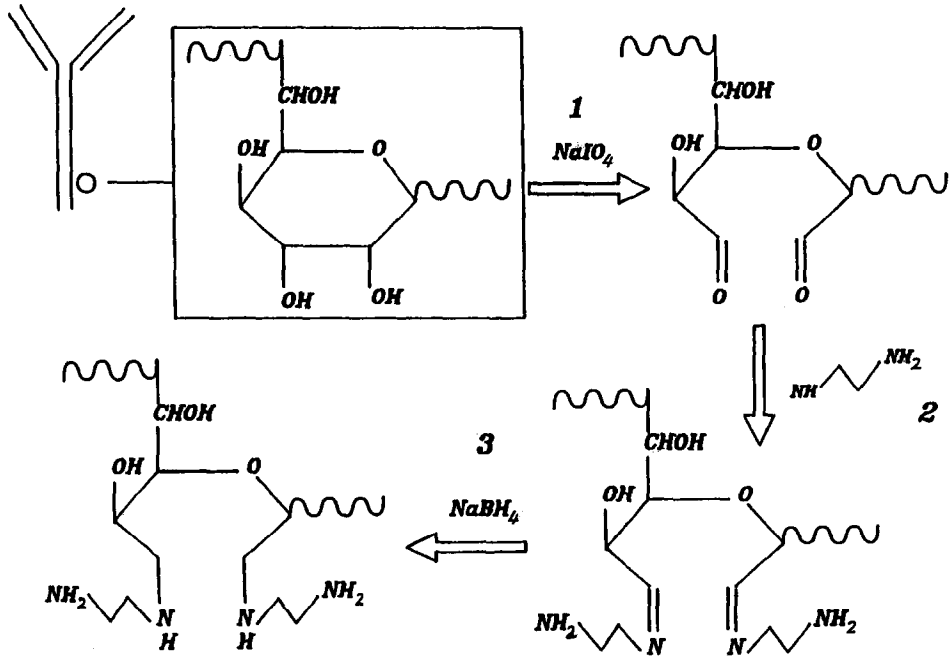

Fig. 10. Schematic representation of the reactions involved in the preparation of the first antibody (N-terminal) for immobilization onto the sensor surface. (1) Oxidation of vicinal OH groups in a sugar residue which is part of the glycosyl moiety of the antibody in question. (2) Reaction of the aldehydes formed with ethylenediamine. (3) Reduction of the amino group introduced by the previous reaction by use of $NaBH_4$.

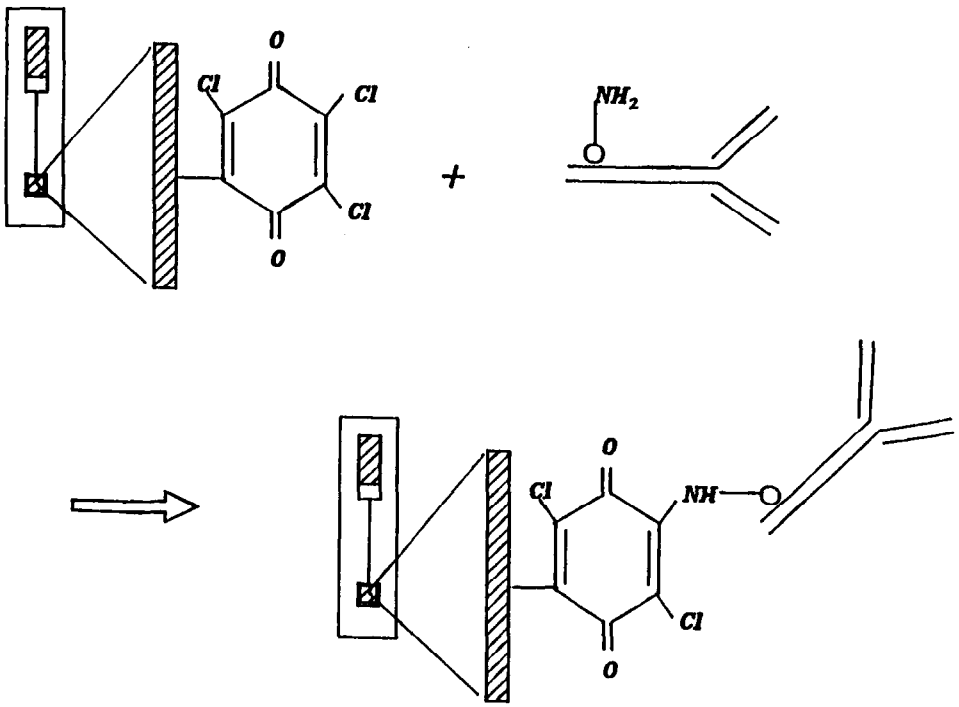

Fig. 11. The derivatized first antibody reagent was immobilized on the sensor surface via the amino group(s) formed.

the maximum signal is obtained. The loaded sensor (*cf.* Fig. 13) was mounted in a measuring cell where phosphate buffer was pumped past it, providing a zero baseline. Switching to a glucose solution, gluconolactone and hydrogen peroxide were produced by the GOD marker enzyme if there was any α-hANP present to form an immune complex with. The H_2O_2 was electrochemically oxidized at the platinum surface, the change in current over time was registered by a computer producing the sensor signal. The GOD reaction was proportional to the bound second

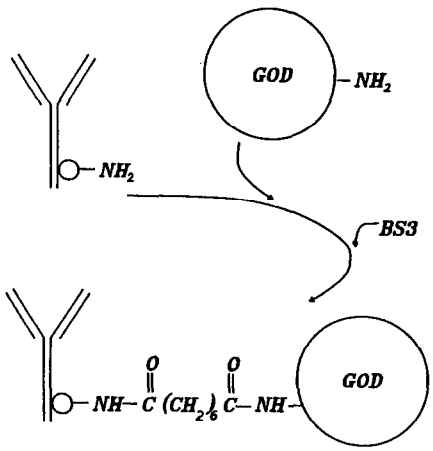

Fig. 12. Production of the second antibody reagent, as described in Experimental. The C-terminal antibody was oxidized, treated with ethylenediamine and reduced at its Fc-glycosyl residue. GOD and BS3 were added to form the biologically active second antibody reagent used to determine the presence of α-hANP immunologically bound to the first antibody on the sensor surface.

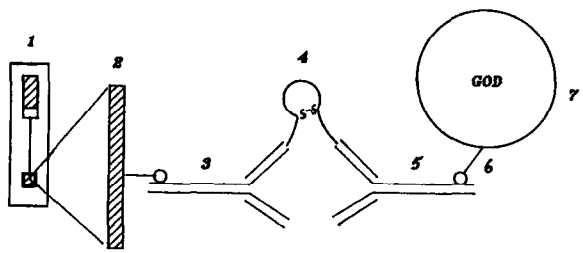

Fig. 13. Schematic representation of the sensor array: 1 = The sensor substrate; 2 = an enlarged view of the immobilization area; 3 = the first antibody, covalently immobilized via its glycosyl residue and, therefore, biologically active; 4 = immunoaffinity bound α-hANP (a standardised pyrogen-free α-hANP solution was used); 5 = the second antibody reagent carrying the marker enzyme; 6 = spacer; 7 = enzyme.

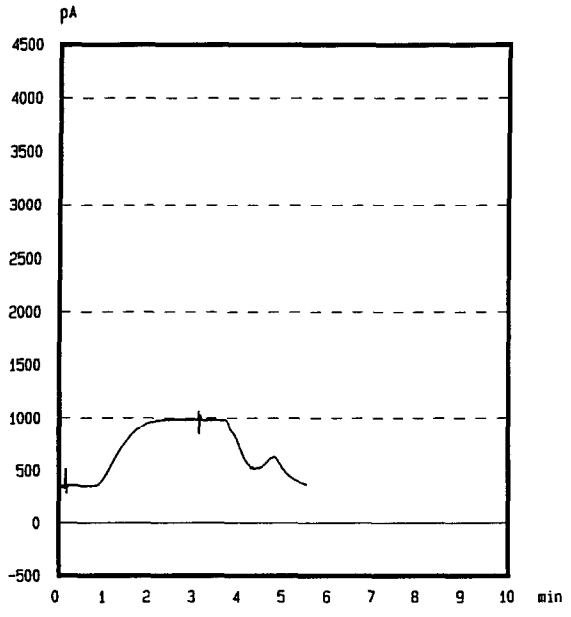

Fig. 14. Typical sensor response obtained by dosing the fully loaded sensor, mounted in the measuring cell, with a standardized glucose solution. Up to 55 s elapsed between the introduction of the glucose solution and the first monitored response (reaction and diffusion time).

antibody, which was in turn proportional to α-hANP bound to the sensor (*cf.* Fig. 14). The response to the presence of α-hANP was no longer detectable when the immune complex was "eluted" from the sensor, but once the immobilized first antibody was reloaded, the response was back to normal.

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