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## A NEW SOLID-PHASE SYSTEM FOR IMMUNOASSAYS\*

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### Abstract

#### A NEW SOLID-PHASE SYSTEM FOR IMMUNOASSAYS.

The development of a new solid-phase separation system based on silane polymers is described. A  $T_3$  radioimmunoassay (RIA) was optimized using coated tubes with polymer coatings containing hydrophilic surface aldehyde groups for antibody coupling and a  $T_4$  RIA developed on the basis of surface anilino group containing particles using a suspension method. Both RIAs offer very good performances and show the variable usability of the new separation system.

### 1. Introduction

Immunoassays are widely used in medicine and have become an indispensable tool for modern diagnosis. The method is used as a routine test in many clinical laboratories. Commercial test kits for the determination of more than 100 biochemical components are available today. The rapid development is demonstrated by the increasing number of determinations: in the Federal Republic of Germany 1979 a total number of 17 million determinations was performed. The numbers for 1985 are estimated as high as 39 million. Assays for new analytes (hormones, enzymes, vitamins, drugs, virus, bacteria, residues in food and others) are being developed, and on the other hand, methodological improvements are worked out in order to make present analysis more precise and/or more economic. Thus, much work is devoted to mechanizing or automating analysis or to use other than radioactive markers like enzymes or fluorescent dyes. A considerable part of these assays is based on solid phase techniques as separating

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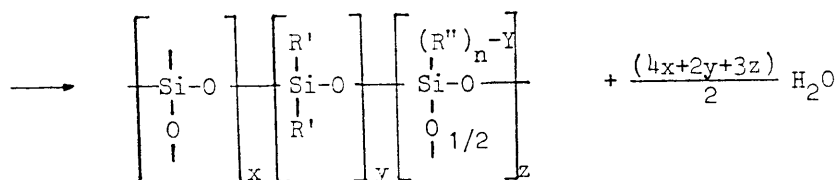
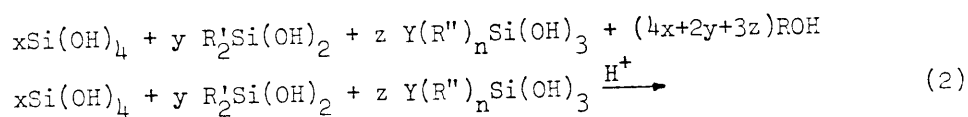
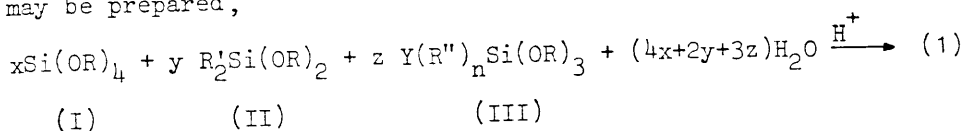
procedure, and in many cases the stability of the immobilized antibody is better than that of antibody in solution. For this reason, further research work in the field of solid phase systems is being carried out in many laboratories.


The coupling (especially covalent coupling) of antibodies to proper solid phases generally requires several reaction steps [1]. On silylation of silicates with suitable silanes and coupling antibody onto them, e.g. separation media are obtained that perform well in RIA [2]. This principle, however, is limited to porous glass and other pre-formed siliceous substrates. The main objective of the present study therefore was to develop a generally applicable solid phase system based on modified silane polymers [3] and to prove its performance in RIA. For practical reasons, a coated tube system was chosen. Other geometric forms have been included in the study later on.

## 2. Results

### 2.1. Development of coated tube T3 RIAs

From [3] it was known that, using the process of hydrolyzation and polycondensation of monomeric silanes, solid materials containing organofunctional groups according to equation (1) and (2) may be prepared,



R = -C<sub>2</sub>H<sub>5</sub>; R' = -CH<sub>3</sub>, -Ph; R'' = -CH<sub>2</sub>-; Y = -NH<sub>2</sub>, -NH<sub>2</sub>; -CHO, -OH; x,y,z = 1-100; n = 3-9

and that these polymers may be used for coating of any mechanically stable material. The polymer surfaces are able to bind antibodies covalently.

Based on these results, the following investigations were aimed at the development of materials suitable for mechanized coating procedures. Therefore, a T3 competitive assay was chosen as a model.

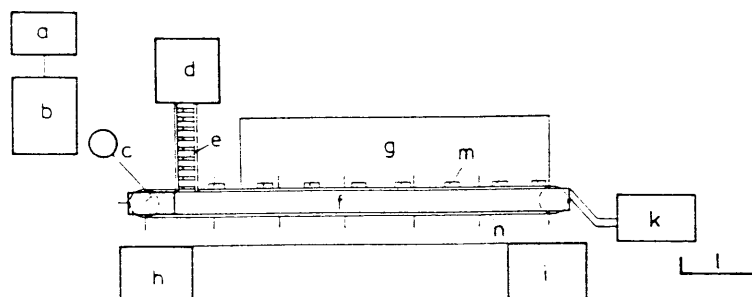
Glass tubes were used, since they are stable against temperature treatment and might later be used as a cuvette for colorimetric measurements, if necessary.

For the preliminary experiments amino and anilino groups were chosen as active functional groups for coupling. With the amino group glutardialdehyde was taken as coupling agent, while with the anilino group antibodies were bound via diazo bonds.

First of all, the influence of the starting components in the hydrolyzation and condensation reaction according to the equations (1) and (2) on the properties of the condensed material, especially with regard to the coating experiment, was studied. The result shows that, if understoichiometric amounts of water are used in the hydrolyzation process, oligomeric products will be formed which are soluble in organic solvents like ethanol or acetone. After the introduction of a proper amount of a diluted solution of these oligomers into the inside of the tubes a homogeneous distribution of the solution over the inner wall of the glass tube was achieved by rotating the tubes in a horizontal position in a way that the centrifugal force exceeds the gravity. On heating the solvent is evaporated and the coating remained as a thin film of some  $\mu\text{m}$  thickness. The whole procedure was completely automated.

Fig.1 shows a schematic drawing of the coating machine. The machine is controlled by two microprocessors. One controls the whole coating procedure including positioning the tubes on the rotating cylinders, introducing the solution and transporting the tubes through the heating channel. The second one controls the homogeneity of the film by optical means. A light source emits light to a photo cell, which measures the transmission of the light through the film. 3,000 measurements per tube are carried out using a scanning procedure. It is possible to define the measuring points so that the homogeneity of each coating can be controlled. The apparatus is able to produce 10,000 coated tubes per day.

Amino group containing coatings were produced by cohydrolyzation and cocondensation of tetramethoxysilane (I), dimethyldiethoxysilane (II), and  $\gamma$ -aminopropyltriethoxysilane (III) according to the equations (1) and (2), using HCl as catalyst as described in [3,4]. The experiments show that the time of condensation affects strongly the hardness of the film. Condensation



- |                       |                                       |
|-----------------------|---------------------------------------|
| a: coating solution   | h: microprocessor for coating control |
| b: dosing pump        | i: microprocessor for quality control |
| c: injection nozzles  | k: quality control                    |
| d: tube stock         | l: coated tube stock                  |
| e: tube supply        | m: tubes under treatment              |
| f: rotating cylinders | n: conveyor                           |
| g: heating channel    |                                       |

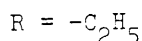
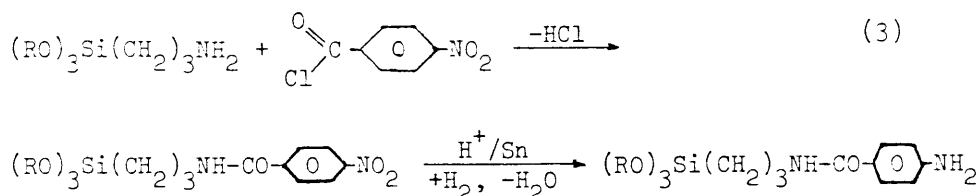
FIG.1. Scheme of the coating apparatus.

TABLE I. Schematic test procedure for the T3 RIA

standard 100 $\mu\text{L}$	patient samples 100 $\mu\text{L}$
↓	↓
1000 $\mu\text{L}$ $^{125}\text{I}$ -T3 solution	
↓	
incubation: 2 hours at 37°C	
↓	
flushing	
↓	
determination of the radioactivity	
↓	↓
B/B <sub>0</sub> for construction of the standard curve	B/B <sub>0</sub> for determination of the serum concentration from the standard curve

times of more than three hours (30°C, ethanol as solvent) lead to oily films which could not be used. With high amounts of tetramethoxysilane brittle films cracking during the heating procedure were obtained. The number of surface amino groups was determined by using a colorimetric method [5]. Depending on the type of coating, between 10 and 40 amino groups per 100 nm<sup>2</sup> were analyzed. No significant difference with regard to antibody coupling could be observed.

Amino group containing tubes were treated with a 2.5% (w./v.) solution of glutardialdehyde (4 hours; room temperature) and with T3 antiserum (16 hours; room temperature; dilution 1:150 000; white New Zealand rabbits). The reproducibility of the following T3 RIA (B<sub>0</sub>/T = 25%; D = 70%; CV = 15%; T = total binding capacity; D = discrimination; CV = coefficient of variation) was not acceptable. This might be due to irreproducible cross-linking reactions of glutardialdehyde. Therefore, we bound the antibodies with diazo bonds to the surface. The silane with an anilino group was synthesized according to the following scheme (3):



The structure of the compound was proved with IR and mass spectroscopy. Hydrolyzation and condensation reactions led to products with very poor solubility in most organic solvents, even if the reaction time was very short. With a diphenyl silane instead of a dimethyl silane (equation (1), II) products were obtained which were very soluble in acetone, even if the condensation reaction was carried out with the stoichiometrical amounts of water. Thus, it was possible to prepare products not sensitive to air. The coating procedure could be performed by the same method as described above.

The anilino coated tubes were treated with NaNO<sub>2</sub>/HCl at 0°C and then incubated with T3 antiserum. Table I shows the optimized RIA procedure and fig.2 the standard curve. Generally the following assay characteristics were achieved: 27 < B<sub>0</sub>/T < 36%; D = 65-75%; CV < 10%; uptake: 40% < B<sub>0</sub>/T < 50%; CV < 3.5%; recovery = 93-110%; range: 25-800 µg/dL.

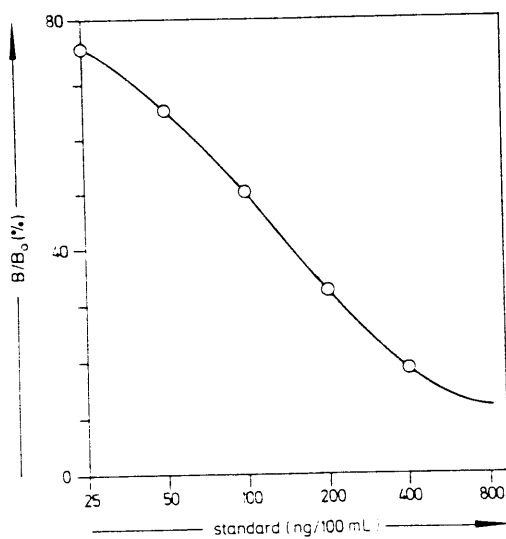


FIG. 2.  $T_3$  RIA based on anilino group containing coated tubes; antiserum dilution: 1:150 000; amount of standard: 100  $\mu$ L; buffer: 0.08 mol/L barbital and 0.03 mol/L sodiumsalicylate, pH 8.6; incubation conditions: 2 h, 37°C.

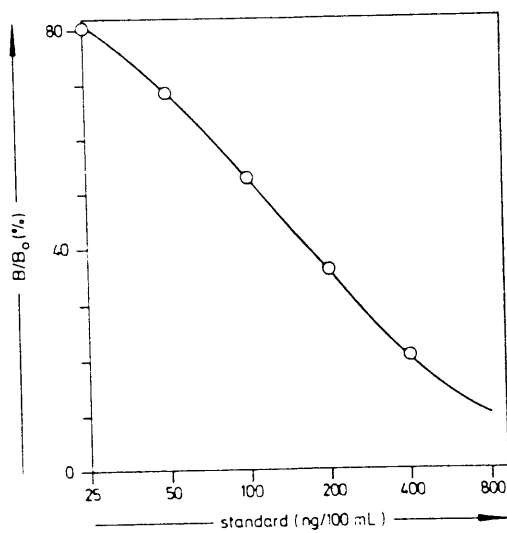
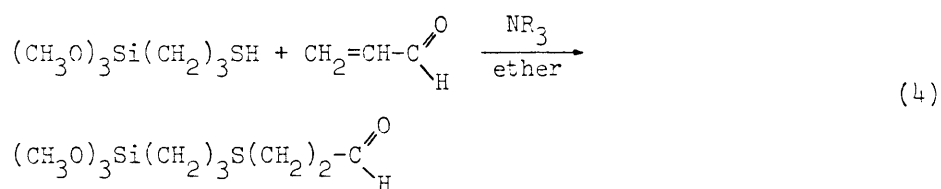


FIG. 3. Standard curve for  $T_3$  RIA, using hydrophilic aldehyde group containing coated tubes. Antiserum dilution: 1:150 000, 300  $\mu$ L antiserum; buffer: citrate, pH 2.5; incubation conditions for coating: 20 min (rotating) at room temperature;  $B_0/T = 35\%$ ;  $D = 70\%$ ; 50% Intercept = 120 ng/100 mL; NSB (non-specific binding) = 0.4%.

TABLE II. Accuracy and recovery data  
T3 RIA on hydrophilic aldehyde group  
containing tubes (values in ng/100 mL)

<u>Control sera</u>			
	normal	hyper	hypo
expected value	129 ± 35	275 ± 60	19 ± 10
value found	114 ± 18	240 ± 19	20 ± 2
CV	7.8% (n = 20)	3.9% (n = 6)	6.3% (n = 6)
<u>Recovery</u> (n = 10)			
expected value	found	CV %	% recovery
44	42	7.7	96.0
88	86	7.3	98.6
175	163	3.9	93.1
350	331	5.9	94.6
700	686	7.4	98.0

In order to avoid the chemical reaction steps due to the amino and anilino group containing surfaces, directly coupling surfaces containing aldehyde groups were developed. Silanes containing aldehyde groups were synthesized according to (4):



Antibodies were bound to the resulting hydrophilic surfaces at pH 2-3 in citrate buffer. Using the rotating principle it was possible to reduce the amount of antibody to 300 µL (dilution 1:150 000) per tube. The immobilized antibodies were stabilized by a treatment with a 0.1% (w/v) solution of bovine serum albumine.

Fig.3 shows a typical standard curve of a T3 RIA using these hydrophilic tubes. Assay characteristics are given in table II.



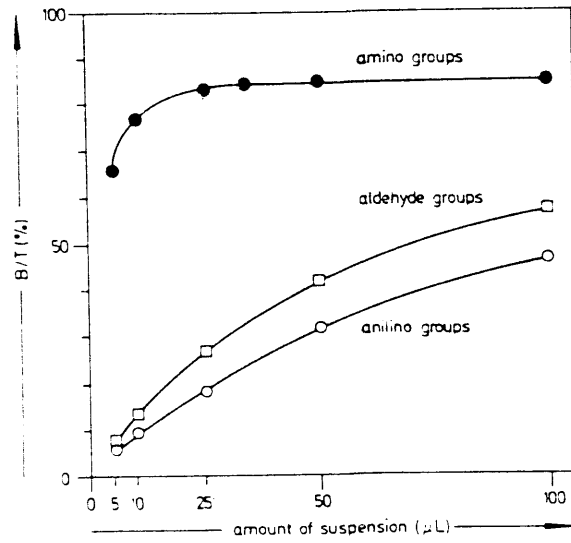


FIG. 4.  $T_4$  uptake with amino, anilino and aldehyde group containing particles.

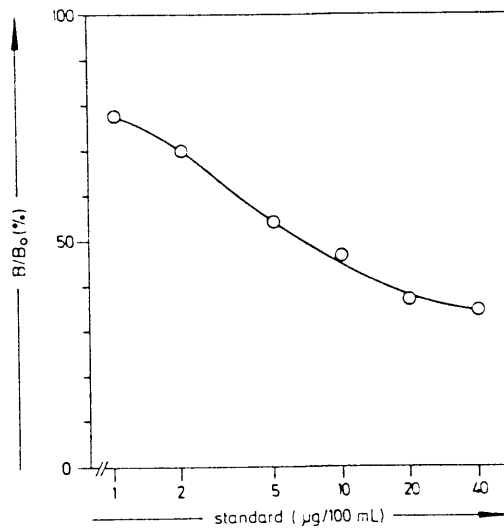


FIG. 5. Standard curve of the  $T_4$  assay with aldehyde groups.

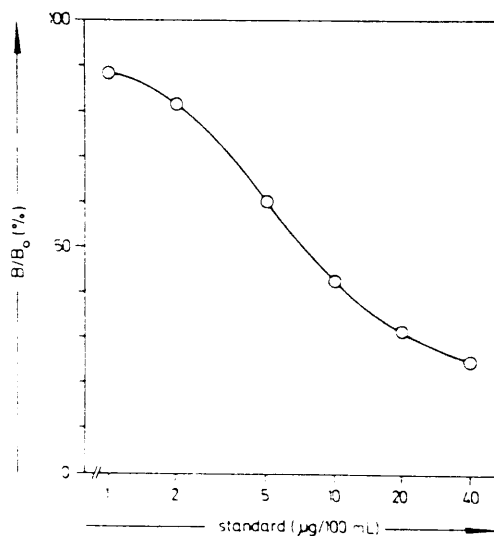


FIG. 6. Standard curve of the  $T_4$  assay with anilino groups (diazo coupling).

In preliminary experiments hydrophilic aldehyde group containing tubes were applied to further assays including testosterone and estriol, with reasonable results. So far these assays were not optimized.

## 2.2. Particles as separation medium

Therefore, we prepared the silane condensates in form of particles with a diameter of about  $1 \mu\text{m}$ . Special reaction conditions in (1) and (2) yield proper sizes. In case of amino, anilino and aldehyde group containing particles it was possible to define their size with variation of the catalyst concentration (HCl). Thus slowly precipitating granulates were obtained and applied in form of suspensions. Antibody coating was performed with essentially the same techniques as with tubes. Granulates containing different functional groups were used as solid phase separation system in a  $T_4$  RIA. Fig. 4 shows the  $T_4$  uptake.

Fig. 5 and 6 show the standard curves of the optimized  $T_4$  assay. From the amino groups no usable standard curve could be obtained. Due to the steeper slope of the standard curve we obtained better results with particles containing anilino groups. Table III gives the details.

TABLE III. T<sub>h</sub> assay data from particles

	B <sub>0</sub> /T (%)	D <sub>(1-40)</sub>	50% IC	CV (%)
Anilino	42	58	5.2	9.5
Aldehyde	41	43	6.8	28.6

In further tests we found that other geometric forms, including coated balls or coated capillaries, may be used for a T<sub>3</sub> RIA, too.

In summary, the results of the study show that a novel solid phase system has been developed that is easily prepared and gives highly reproducible assay results.

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#### DISCUSSION

In response to a question, Mr. Schmidt indicated that the studies with coatings containing amino groups had shown little difference in B<sub>0</sub>/T values between tubes treated with glutardialdehyde and tubes not so treated. The immobilization of Ab in the latter could involve either hydrophobic interaction between the coating and corresponding structures in the Ab or ionic or dipole-dipole interaction between the amino groups and corresponding structures in the Ab. Comparable results had been obtained in studies involving the immobilization of enzymes on supports

containing amino groups, although the slower decay of enzyme activity in glutaraldehyde-treated as compared with untreated tubes suggested that less stable binding occurred in the latter.

Mr. Schmidt described experiments (not mentioned in his paper) in which Ab-coated tubes had been regenerated after use by treatment with a 50% wt. vol. methanol/water mixture for 15 min. Such regeneration, which removed bound Ag but not Ab, could be repeated up to 20 times without loss of Ab-binding capacity. The primary aim of these experiments had been to investigate the possible use of coatings on capillaries in automatic analyser systems operating on flow principles. None the less, their implications with regard to prospects for the commercial production of coated tubes were recognized in discussion.