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Inventions Leading to the Development of the Diagnostic Test Kit Industry – From the Modern Pregnancy Test to the Sandwich Assays

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ABSTRACT

The universities are encouraged by the government nowadays to stimulate innovations and also to provide the proper machinery for assisting the protection and commercialisation of innovations. A better understanding of the innovation process may help to create an atmosphere suitable for inventions at the university. Examples can be taken from successful innovations previously made at the university.

During the 1960's I made a series of inventions, which ultimately led to the development of the diagnostic test kit industry. The first, which I made as an undergraduate, was a simple and reliable test kit for diagnosis of pregnancy. This was followed by the solid phase radioimmunoassay and a solid phase assay for vitamin B12; next, the dual specific non-competitive sandwich assay and the in-vitro test for diagnosis of allergy, called RAST (Radioallergosorbent test). Organon in Holland with the pregnancy test kit, and Pharmacia in Sweden with test kits for radioimmunoassay, became pioneers among the diagnostic test kit industries. Pharmacia Diagnostics later became one of the leading diagnostic test kit companies in the world and has continued to be so in the field of allergy diagnosis.

Each one of these inventions started with a few unique observations leading to a technical development. The pregnancy test as well as the allergy test emerged from the development of assay methods with unique qualities with the subsequent search for appropriate applications. The foreseeing of a commercial value on a future market was a very important step. This was followed by the search for a suitable industry interested to exploit the invention with its new business opportunity i.e. apply for a patent, produce and market the products, which in my case consisted of the necessary reagents and equipments for particular diagnostic tests. Finally, an agreement had to be settled between the entrepreneur and the inventors. This report describes these inventions and particularly discusses some crucial steps of the innovation processes.

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INTRODUCTION

During the 1960's I made a series of inventions which ultimately led to the development of the diagnostic test kit industry. The first was a simple and reliable test kit for diagnosis of pregnancy. This was followed by the solid phase radioimmunoassay and a solid phase binding assay for vitamin B12; next, the dual specific non-competitive sandwich assays and the in-vitro test for diagnosis of allergy. At that time the university showed no real interest to encourage innovations leading to commercial products. Actually, I even experienced critical comments from several university colleagues. This attitude has completely changed during the last 20 years. Today, the universities are encouraged by the government to stimulate innovations and also to provide the proper machinery for assisting the protection and commercialisation of innovations. A better understanding about the innovation process may help to create an atmosphere suitable for inventions at the university. Examples can be taken from some of those innovations previously created at the university and having led to successful commercial products. The different steps on the road to a successful innovation are seldom described in the scientific literature.

The innovation process

There is no universal or single innovation process and it may vary considerably for different successful products. However, each one of the inventions that I made in the 1960's started with a few unique observations leading to a technical development. The pregnancy test as well as the allergy test emerged from the development of assay methods with unique qualities with the subsequent search for appropriate applications. The foreseeing of a commercial value on a future market was the next very important step. This was followed by the search for a suitable industry interested to exploit the invention with the new business opportunity i.e. apply for a patent, start a production and market the products, which in my case consisted of the necessary reagents and equipments for particular diagnostic tests. Finally, an agreement had to be settled between the entrepreneur and the inventors. This report will describe these inventions and particularly discuss some crucial steps of the innovation process.

THE PREGNANCY TEST

The Boyden technique

The idea to develop an immunological pregnancy test emerged from some crucial experiments that I did with Boyden's passive haemagglutination inhibition method aiming to measure human growth hormone (hGH) in blood. Boyden had found that protein antigens could be adsorbed to the surface of tannic acid treated sheep erythrocytes (1). He then used the Salk pattern technique to detect antibodies to the protein (2). When the protein coated cells sedimented in a test tube with a hemi-

spherical bottom in the presence of antibodies they formed a mat pattern, while otherwise the pattern was a ring or dot in the centre. He also found that addition of free antigen neutralized the antibodies and inhibited the mat pattern formation. This formed the basis for a method to detect the antigen in a solution.

A unique observation

As a student, I met Dr Carl Gemzell during the course in Obstetrics and Gynaecology at the Karolinska Institute and he showed me how he determined hGH in blood with a bioassay using hypophysectomized rats. In the autumn of 1959 I told Gemzell that I was interested to start some research in parallel with my medical studies. Gemzell, who himself had no experience in immunology, asked me if I was interested to learn some immunology and try to measure hGH in blood with an immunoassay in his laboratory at the King Gustav V:s Research Institute at the Karolinska Hospital. There was a recent short communication (3) on immunoassay of pituitary hGH using the Boyden technique and the same method had been used previously for the assay of insulin in buffer solutions. I developed this method for hGH but found that the instability of the hGH-coated tannic acid treated sheep erythrocytes was a great problem in the immunoassay. I solved this by formalin treatment of the cells prior to the treatment with tannic acid and the hormone. Formalin treatment of erythrocytes had been used since the 1920's in different agglutination reactions, but not for the assay of hormones (reviewed in 4).

The method worked well with pituitary hGH added to a buffer solution, but not to blood serum or plasma. As the erythrocytes had been stabilized to particles after formalin treatment they were not sensitive to changes in salt concentration and pH like the intact red blood cells. After the failure to develop a method for hGH in blood I tried to measure the hormone in plain urine. The most perfect Salk patterns were achieved, but hGH could be detected only after addition of pituitary hGH to the urine. The concentration of endogenous hGH in normal urine was too low. It seems that no one had ever tried to measure any compound in plain urine with the Boyden technique. This trivial experiment was the first crucial step in the innovation process of the pregnancy test. I had a perfect method for urine assays and looked for a suitable application: a hormone known to be present in large amounts in urine.

The immunological pregnancy test

Urine samples were sent to the hospital laboratory to test whether a woman was pregnant or not. Animals were then used in the laboratory to detect a hormone, chorionic gonadotrophin (hCG), excreted in urine in large amounts early in pregnancy. Could the immunoassay be used to measure hCG in plain urine? In February 1960 I decided to abandon the hGH project and started to immunize two rabbits with hCG to develop a pregnancy test and an assay method for hCG in urine. Gemzell was in USA when I got the idea to switch to hCG and few weeks later when he returned I had, to his surprise, the first results with the hCG test. Gemzell

immediately organized that urine samples were obtained from his ward. Only urine from pregnant women gave a positive reaction in the test. The first immunological pregnancy test with a high accuracy had been developed.

Freeze-dried reagents

I had a strong desire that my discovery would be widely used and generally replace the animal tests for pregnancy diagnosis. I realized that only a few larger laboratories could prepare the pregnancy test reagents for their own use. To perform the tests in smaller laboratories, out-patient clinics and pharmacies it was necessary to buy the reagents from some manufacturer. It would then be an advantage if the reagents could be freeze-dried. Therefore, I made some experiments which were crucial for the innovation. First, I investigated the possibility of freeze-drying the hCG-coated particles and the antiserum in two different bottles for about 20 tests. These reagents gave accurate results and could be stored at different temperatures. Then, I obtained ampoules with a hemispherical bottom and freeze-dried the two reagents together in these for single tests. The pregnancy test was now so simple that only urine and buffer had to be added to the ampoule. The ampoules were sealed and could be stored for future use and seemed suitable as 'test-kits' for a doctor's office or a pharmacy.

Test-kit for a doctor's office

The principle of the immunological pregnancy test developed to a 'kit' for a doctor's office is shown in Fig. 1. One drop of a woman's urine plus 0.5 ml buffer solution is added to an ampoule containing freeze-dried reagents. The freeze-dried

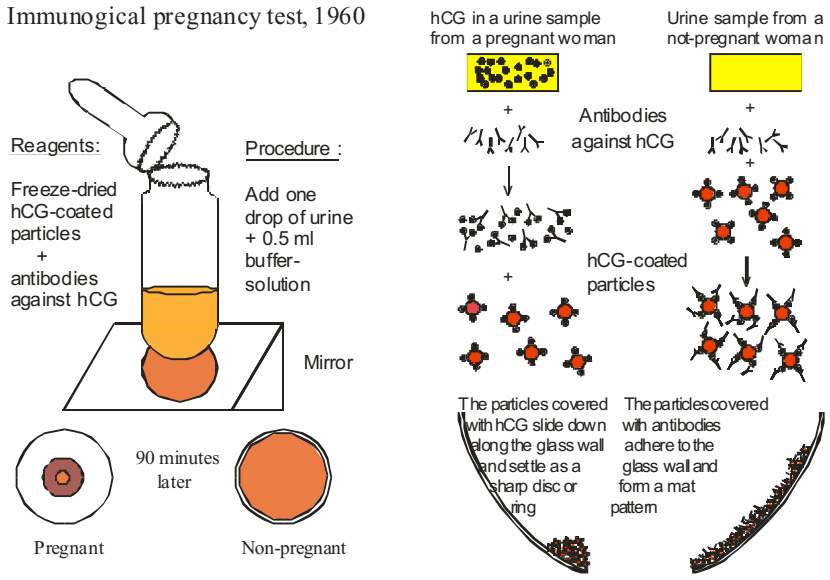

Figure 1. A test kit for diagnosis of pregnancy based upon immunoassay of hCG in urine. hCG in urine from a pregnant woman binds the antibodies and prohibits the hCG-coated particles to form a mat pattern. The hCG-coated particles were sheep red blood cells treated with formalin and tannic acid and finally coated with hCG.

reagents consist of antibodies to hCG and hCG-particles. The hCG-particles are sheep red blood cells pre-treated with formalin and tannic acid and finally coated with hCG. After 90 minutes the test is read by looking in a mirror underneath the test tube at the pattern formed by the particles on the hemispherical bottom of the ampoule. If the woman is pregnant her urine contains the hormone hCG which binds the antibodies, and the hCG-particles slide along the glass wall and settle as a sharp ring or disc. Urine from a not-pregnant woman contains no detectable hCG and the antibodies react with and cover the hCG-coated particles. When these antibody-hCG-coated particles sediment they adhere to the glass wall and form a mat pattern.

Ready to approach a manufacturer

In May 1960 I felt that we were ready to approach a possible manufacturer of the reagents for the pregnancy test. The method was remarkably reliable. Tests on over 300 morning urine samples had not given any incorrect result. The hCG-coated particles could be made in batches for 20,000 reactions and they were stable for months. The antiserum from one of the rabbits could be used for over one million reactions. The results from quantitative determinations of hCG in urine of 110 women in early pregnancy indicated that such determinations would be of clinical value. I made a draft of a publication with the title: 'An Immunological Pregnancy Test' with Leif Wide and Carl Gemzell as authors (5). My experience with the freeze-dried reagents was so exciting, that it seemed to be the right time to contact a suitable industry, before finishing the manuscript.

Organon, a suitable pharmaceutical company

Gemzell had earlier obtained financial support from N.V. Organon in Holland and he suggested that he should write a letter to explore their interest. On 1st of June 1960, Dr Marius Tausk, managing director of N.V. Organon in Holland, received a letter from Dr Carl Gemzell at the Karolinska Hospital, Stockholm, the first paragraph of which read as follows:

'A young doctor in my laboratory, L. Wide, has developed a new pregnancy test which seems to have many advantages. It is an immunological method, takes only one to two hours, gives a quantitative answer and can be performed in every laboratory or even in a doctor's office. It is also very inexpensive. A pre-requisite condition is that some material can be prepared and ,,,furnished preferably through some pharmaceutical company. This material, as we have found, can be stored and used at convenience….' (The letter published by M Tausk in ref 6.)

Pregnosticon

Dr Tausk found the letter exciting and appeared in Stockholm shortly thereafter. I demonstrated and discussed the pregnancy test with him and two weeks later with Mr Polderman, who became responsible for marketing the test by Organon under the name of Pregnosticon. I also showed that the antiserum and the hCG-particles

could be freeze-dried separately in bottles for e.g. 20 or 100 tests. These were suitable for laboratories performing larger series of pregnancy tests using test tubes with a hemispherical bottom. By testing a serial dilution of the urine, the concentration of hCG in urine could be estimated and expressed in IU/L.

Dr Tausk was impressed and Organon decided to bring it up immediately, and an agreement was proposed which was accepted by us. Organon then needed a few weeks for patent application, and after that we could send the manuscript for publication in Acta Endocrinologica. Organon put in large resources on this project and hoped to launch the first kit in 6 months. However, the freeze-drying of the reagents that had gone without any problem for me in laboratory scale turned out to be the main technical difficulty for Organon to solve, and the first Pregnosticon kit was not launched until spring 1962.

Pregnancy diagnosis from ancient day to1960

There is no diagnostic problem that has created more ideas for tests than finding out whether a woman is pregnant or not. The oldest recorded pregnancy test is found in Egyptian papyri about 3,350 years ago and was based upon the germination of seeds induced by a factor in the pregnant woman's urine. Several hundreds of methods have been described from antiquity to the present day (reviewed in 7, 8, 9,10). There was no simple test for diagnosis of pregnancy in 1960. They were all based upon injecting animals with plain urine or extracts of urine to detect a biological effect in the animal caused by the possible presence of hCG in urine. Two rabbits were commonly used for a test and 8 ml of the woman's urine was injected into their ear veins. Two days later the rabbits were killed and their ovaries were inspected for presence of a bleeding. The rabbits often died due to toxic effects of the urine and the test had to be repeated with an extract of the urine. Other laboratories used a mouse test with five mice which were injected twice daily for three days and then killed the following day for inspection of the ovaries. Toads and frogs, where either the laying of eggs or the expulsion of spermatozoa was observed, were used in many clinical chemical laboratories and by pharmacists. These animals could be used several times.

The idea to develop an immunological pregnancy test was not new in 1960. Numerous attempts were made from 1902 to the 1950's using antisera from animals immunized with extracts of human placenta or pregnancy urine, and around 1950, with purified preparations of hCG (references in 4). It can be noted that all the information needed to prepare the reagents for this pregnancy test were available nine years before I made the invention. In 1951 the Boyden technique (1), the formalin treatment of erythrocytes (11), purified hCG preparations (12) and specific antisera to hCG (13) had been described in the scientific literature. A scientist who was very close to develop an immunological pregnancy test was Helen Strausser making a PhD thesis in 1958 at Rutgers University, N.J., USA on some methodological studies with a modified Boyden technique (14). However, she analysed for the presence of hCG only in kaolin concentrates of 100 ml of pregnancy urine and

not in plain urine. Its potential use as a pregnancy test is not mentioned in the thesis, and the study was never published.

From bioassays to household kits for pregnancy diagnosis

The pregnancy tests made in rabbits were replaced by the immunological test at the University Hospital in Uppsala in January 1961, when I moved to Uppsala, where Gemzell had become the professor of Obstetrics and Gynaecology. Soon thereafter several other hospital laboratories in Sweden started with the immunological pregnancy test assisted by the detailed prescriptions that I finally presented in my PhD thesis the following year. I reported in this thesis, defended at Uppsala University, that the pregnancy test was positive about 21-23 days after estimated day of ovulation and that the test had an accuracy of 99.8 per cent on 2,230 urine specimens (4).

Pregnosticon was a success for Organon and became the most sensitive and accurate pregnancy test during a 20 years period. The variant with both reagents freeze-dried in the ampoule (see Fig. 1) was marketed as Pregnosticon-all-in. A 'household kit' of Pregnosticon was sold to the public under the name of Predictor. The women could from now on make the pregnancy tests at home.

Pregnancy tests performed on a glass plate, with the results given after only 2 minutes, were soon on the market both by Ortho in USA and by Organon in Holland. However, these tests were less sensitive and less accurate. It was not until the 1980's that pregnancy tests based upon the sandwich technique (see below), some using monoclonal antibodies, replaced the pregnancy tests developed 20 years earlier.

Clinical significance of quantitative immunoassays of hCG

In my PhD thesis (4) I described the value of a quantitative immunoassays of hCG in urine in various clinical conditions based upon more than 10,000 assays of the hCG concentration in urine. Information about the hCG level in urine was shown to be of importance for diagnosis of ectopic pregnancy, threatened abortion, intra-uterine foetal death, hydatidiform mole and choriocarcinoma. The immunoassays replaced the bioassays for hCG in urine which had been used for many years in particular for the diagnosis of choriocarcinoma (15). Quantitative immunoassays of hCG in urine could be made with the Pregnosticon kit from Organon, which became the first test-kit on the market for a quantitative assay of a hormone.

SOLID PHASE COMPETITIVE BINDING ASSAY

An experiment giving rise to new inventions

Two of the other inventions, the solid phase competitive and the non-competitive binding assays, emerged from one particular experiment with the hCG-coated particles used in the pregnancy test. The first crucial observation was that the particle pattern could be transformed from a mat to a ring and then back to a mat and then again to a ring by adding antiserum, hormone, antiserum and hormone in a sequence. This was true even when I washed the particles between the additions of

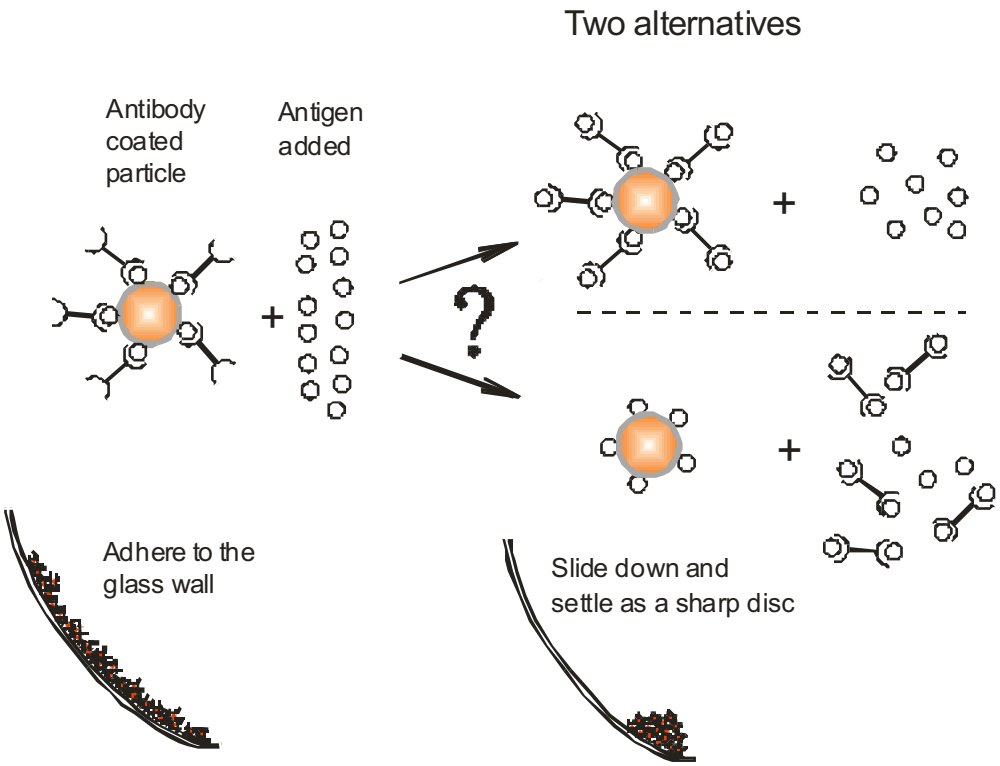

Figure 2. Alternative reactions after the addition of excess amounts of antigen (hCG) to the antibody-coated particles used in the pregnancy test.

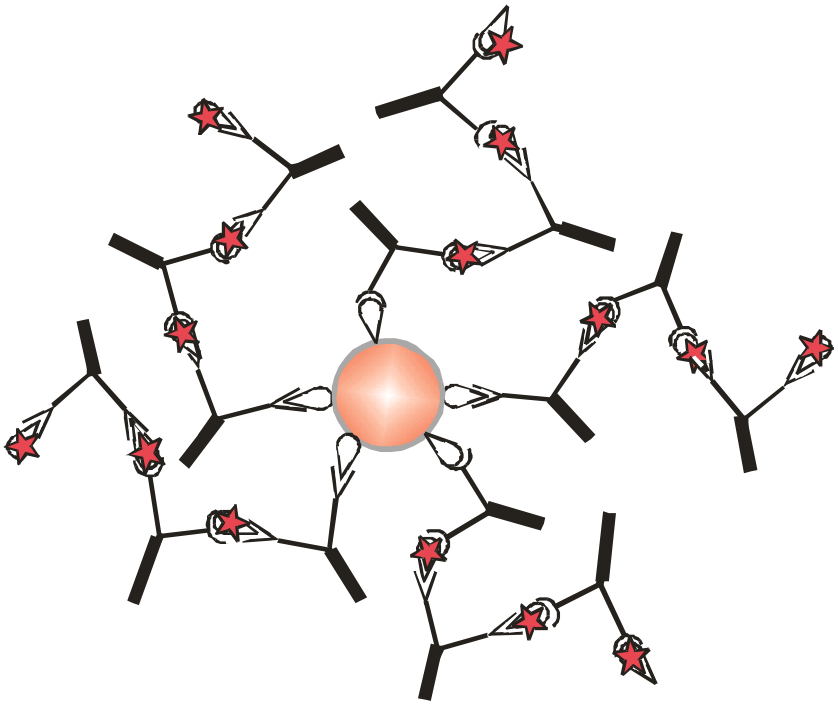

Figure 3. The effect of alternately adding polyclonal antibodies against hCG and ^{125}I-labelled hCG to hCG-coated particles. Formation of several layers of antibodies and labelled hormone.

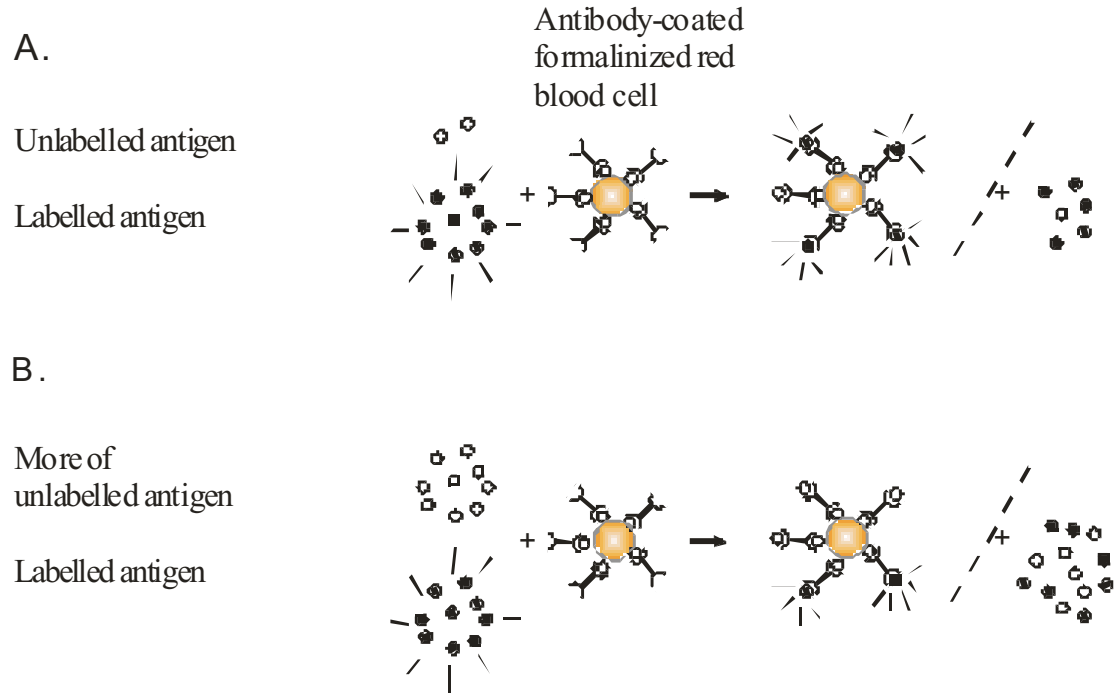

Figure 4. Unlabelled antigen inhibits the binding of labelled antigen to the antibody-coated particles. Effects of a smaller (A) and a larger (B) amount of unlabelled antigen.

antiserum and hormone. There were two possible explanations to this phenomenon as shown in Fig. 2. I got a chance to explore this with ^{125}I-labelled hCG in 1965 at the Department of Clinical Chemistry, University Hospital, Uppsala. I found, as is shown in Fig. 3, that antibodies and hormone could be added in several layers on the hCG coated particles. Next crucial observation was that the amount of labelled hCG that bound to the particles was directly proportional to the amount of antibody added in the previous step. This observation led to a new assay principle: the non-competitive 'sandwich technique' (see below). Another observation was that unlabelled hCG competitively inhibited the binding of labelled hCG to the antibody coated particles. The effect of two different amounts of unlabelled hCG is shown in Fig. 4. The particles were separated from the solution by centrifugation and then carefully washed several times. This was followed by determination of the radioactivity bound to the particles which decreased in relation to the amount of unlabelled hCG. This competitive binding assay, using antibody coated formalinized sheep erythrocytes and ^{125}I-labelled hormone, was evaluated in our department in 1965 for determination of hCG, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and insulin in human serum. The highly purified human pituitary gonadotrophin preparations used in the assay were isolated by Dr Paul Roos, Department of Biochemistry, Uppsala University.

The principle of the competitive binding assays

The principle of the competitive binding assay with a graded response, shown in Fig. 5, was first developed by Arquilla and Stavitsky in 1956 for immunoassay of human insulin (16). In their method the signal was the antibody triggered release of haemoglobin from intact erythrocytes attached to insulin. A considerable improvement of the competitive binding assay was made a few years later by Roger Ekins (17) in London, UK, and Rosalyn Yalow and Salomon Berson (18, 19) in New York, USA. They independently developed considerably more sensitive competitive binding assays by introducing radioactively labelled competing analytes used for the assay of thyroxin and insulin, respectively.

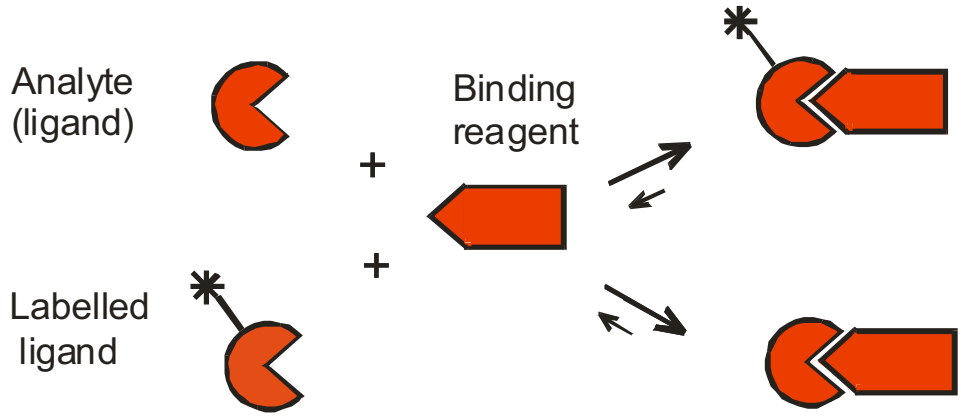

Figure 5. The principle of competitive binding assays using a labelled ligand, described 1956-60 (16-19). The analyte and a fixed amount of labelled ligand compete for a limited amount of binding reagent. The amount of labelled ligand bound is inverse to the amount of analyte.

From formalized erythrocytes to Sephadex particles

The lack of information about the levels of the protein hormones in blood and urine of the patients had been a frustrating experience for me when I worked as a gynaecologist at the Uppsala University Hospital. In spite of its great potentials to measure these hormones, the radioimmunoassay described by Yalow and Berson had not become used in the clinical routine in the hospital chemical laboratories. I assumed that this was most likely due to the cumbersome and laborious chromatoelectrophoretic method they used to separate free and bound labelled hormone (19). The technique I had developed using antibody coated particles facilitated this separation procedure to a simple washing step. However, even if the performance of the radioimmunoassay now was simple, very few hospital laboratories would have the facilities to prepare the reagents needed for the assay. I saw a large potential market for test kits containing the necessary materials for radioimmunoassays. It then seemed desirable to replace the formalized sheep erythrocytes, used as a solid matrix, with some insoluble polymer like cellulose or cross-linked dextran (Sephadex).

Dr Jerker Porath, at the Department of Biochemistry in Uppsala, had developed a method to couple enzymes to Sephadex using an isothiocyanate-derivative (20). I contacted him and obtained activated Sephadex to which we coupled antibodies against hormones. These antibody coated particles had a high specific and low non-specific binding of the radioactively labelled antigen but the precision was so poor, that the first preparations could not be used in the immunoassay. Eventually I solved this problem by ultrasonic disintegration of the Sephadex particles. Dr Rolf Axén, working in Porath's group, suggested that we should investigate CNBr-activated Sephadex. Already after the first experiment with coupled antibodies could I report to him, that

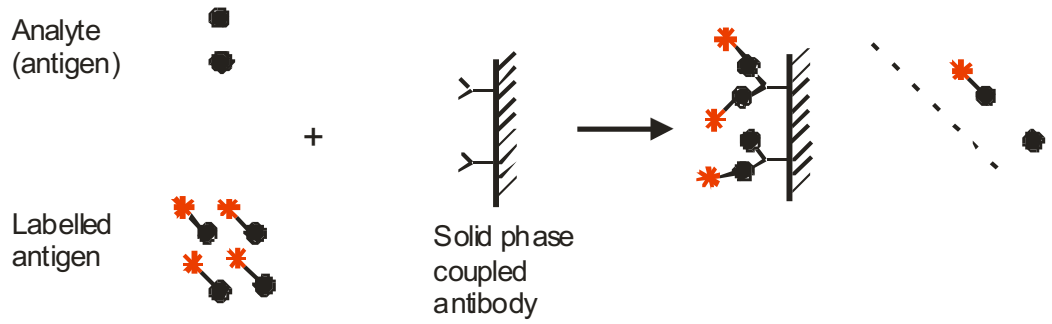

Figure 6. Solid phase radioimmunoassay. A competitive immunoassay using solid phase coupled antibody and a labelled antigen. The use of a solid phase facilitates the separation procedure of antibody bound and free labelled antigen to a simple washing step.

this immunosorbent was perfect for use in the immunoassay. The CNBr-method (21) was simple to perform and I could immediately introduce it into my own laboratory. I started to investigate Sephadex and cellulose particles of different sizes for use in the radioimmunosorbent (RIST) assay.

To Pharmacia with inventions and a new business idea

In March 1966, I contacted Pharmacia in Uppsala to present the invention and convince them to enter into a new business area: marketing reagent kits to hospital laboratories for radioimmunoassay of hormones and many other compounds in blood. There were no radioimmunoassay kits on the market at that time. I suggested that they should start with a patent application for the solid phase competitive radioimmunoassay and include the use of CNBr-activated Sephadex particles. I represented the inventors and negotiated with Gösta Virding, Managing Director of Pharmacia. After about six weeks of considerations at Pharmacia an agreement was settled between Pharmacia and the inventors: Wide, Axén and Porath. The patent application got a priority date of 2 of June 1966, after which we could publish our results (22, 23). In the same month, after Pharmacia's priority date, Kevin Catt et al in Australia submitted a manuscript for publication describing a similar solid phase radioimmunoassay (24). The general principle is shown in Fig. 6.

During the negotiations with Pharmacia I stressed that the solid phase competitive assay was not restricted to immunoassays and I wanted the agreement to cover non-immunological systems as well. They asked me to confirm this with an example. A few months later I presented Pharmacia with a method for the assay of vitamin B12 in blood. It was a solid phase competitive binding assay using Co^{57}-labelled vitamin B12 as a tracer and intrinsic factor coupled to CNBr-activated

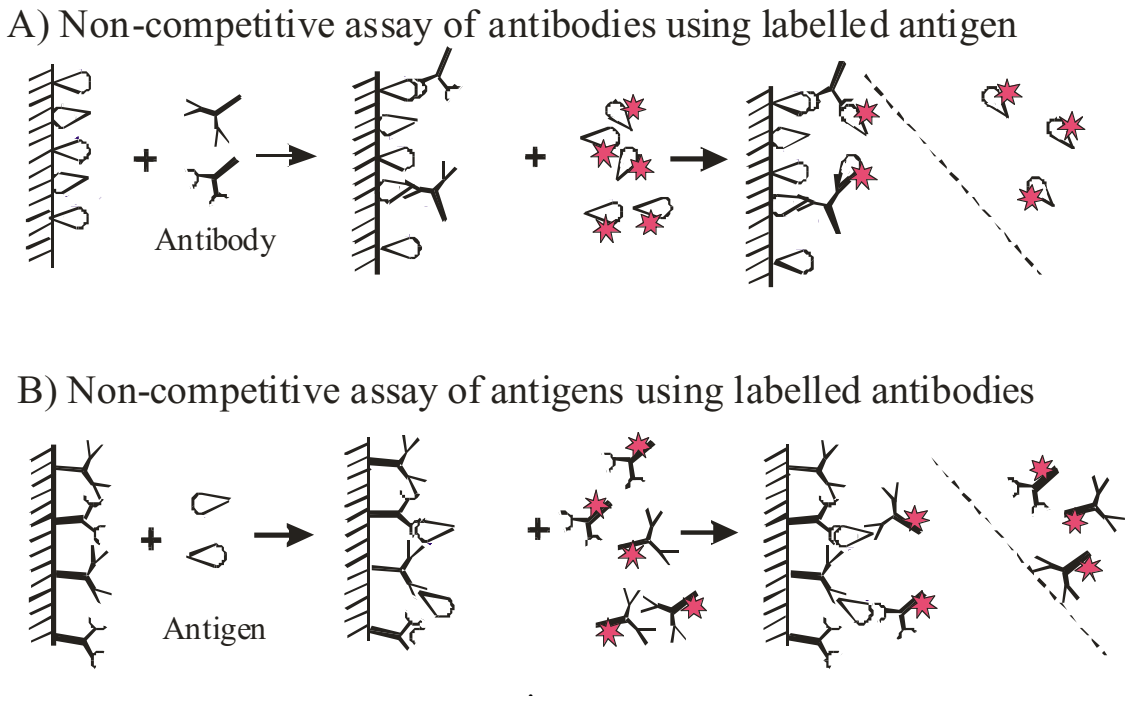

Figure 7. A) Solid phase coupled antigen in excess binds the polyclonal antibodies. Labelled antigen in excess binds to the antibodies. Unbound labelled antigen is removed by a washing step. B) Solid phase coupled polyclonal antibodies in excess bind the antigen. Labelled polyclonal antibodies in excess bind to the antigen. Unbound labelled antibodies are removed by a washing step.

Sephadex as a binding reagent (25). An agreement was settled between the inventors and the company, and in October 1966 Pharmacia applied for a patent on the solid phase vitamin B12-assay.

FROM SANDWICH ASSAY TO ALLERGY TEST

A new assay principle – a non-competitive 'sandwich technique'

As mentioned above, the experiment with hCG-coated formalinized erythrocytes, anti-hCG and ^{125}I-labelled hCG gave rise to the solid phase competitive binding assays as well as to the non-competitive sandwich assays. The non-competitive method was first used as a very simple method to detect antibodies using a labelled antigen (Fig 7a). I then examined a non-competitive assay of antigens using the

combination of solid phase coupled antibodies and labelled antibodies (Fig 7b). The antigen had to have at least two binding sites. In this experiment the antiserum was polyvalent with antibodies to different epitopes on the hCG molecule. The IgG fraction of the antiserum was purified and used both for coupling to the solid phase and for labelling. The difference of the dose response curves of competitive and non-competitive immunoassays, using a labelled antigen and a labelled antibody, respec-

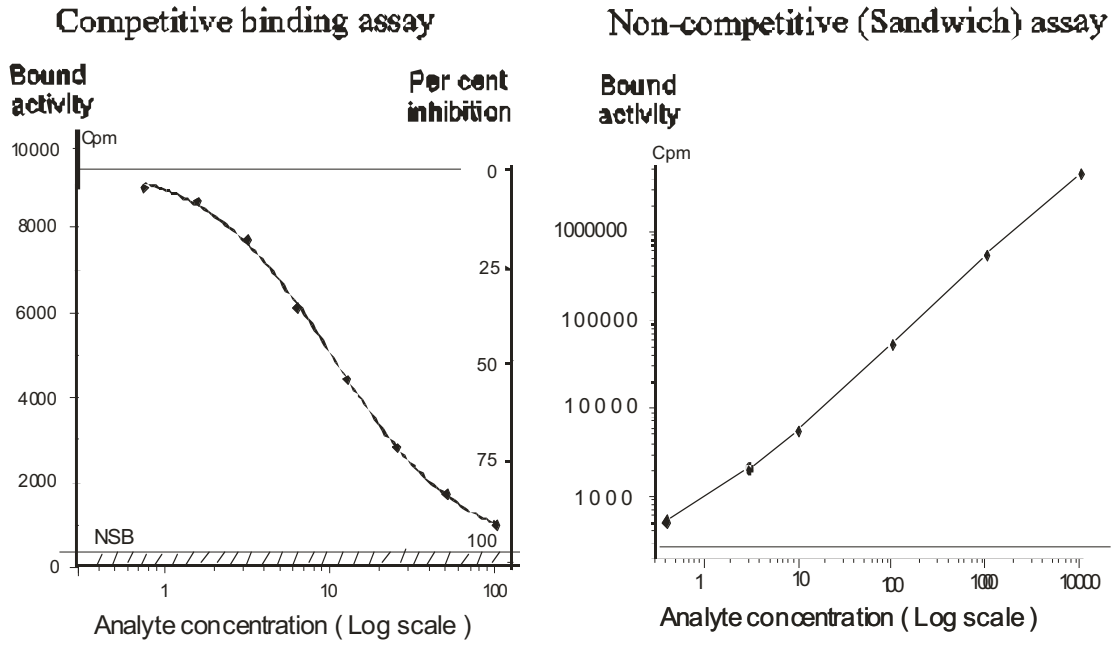

Figure 8. Comparison of dose response curves of competitive and non-competitive (sandwich) binding assays. The bound fraction of labelled ligand and labelled binding reagent, respectively, is shown on the Y-axis.

tively, is shown in Fig 8. In both cases the bound fraction of the labelled reagent is indicated on the Y-axis.

The design of a dual specific allergy test

The non-competitive assay using labelled antibodies had not been described in the literature. I had observed that the method had a higher sensitivity and a better precision than the competitive immunoassay. It also had a shorter reaction time, as the reagents were added in excess. If the labelled and the solid phase coupled binding reagents were directed to different sites on the analyte, a dual specific assay was obtained. Excited over the wide potential of this assay method with its unique qualities, I was looking for an application with a novelty and clinical significance similar to the successful pregnancy test.

I got the idea to develop an allergy test early in the autumn 1966 while listening to a dermatologist, Lennart Juhlin, at a meeting of the Society of Experimental Biol-

ogy in Uppsala. This society, sponsored by Pharmacia and organized by the Department of Medical Chemistry, Uppsala University, was a forum to increase interdisciplinary collaboration and exchange of ideas and knowledge in Uppsala. Juhlin had just returned from a year in Philadelphia, USA and gave a lecture on urticaria, histamine and reaginic activity. I asked him during the lecture whether it had been shown that the reaginic activity in allergy was associated with an antibody. He told us that a group in Denver (lead by K Ishizaka) had recently shown that the reaginic

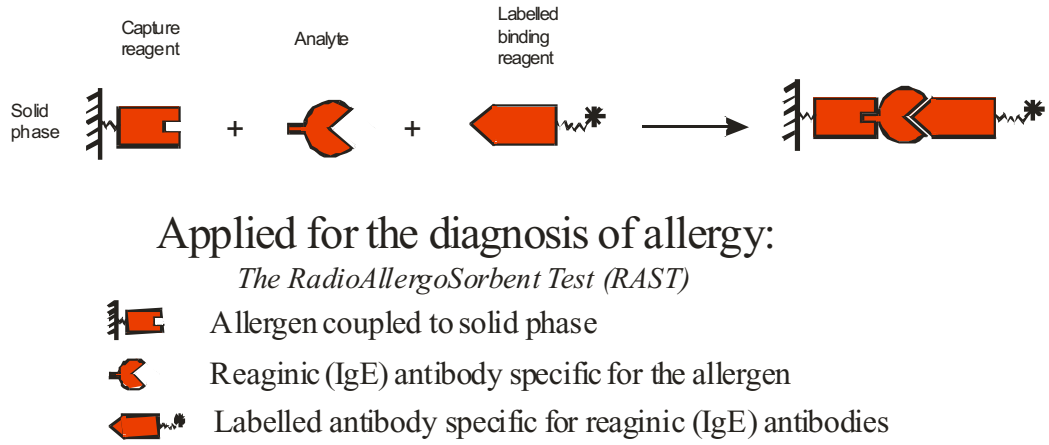

Figure 9. General principle of the dual specific non-competitive sandwich assay. The analyte binds to the capture reagent as well as to the labelled binding reagent. The capture reagent and the labelled binding reagent are added in excess. Unbound labelled binding reagent is removed by a washing step. A washing step may also be essential after the analyte is bound to the capture reagent. The application for diagnosis of allergy is described in the text.

activity was associated with antibodies. These antibodies did not belong to the known immunoglobulin classes γG, γM, γA or γD-globulin (IgA, IgM, IgA or IgD) but to a fifth unique immunoglobulin class called γE-globulin (26-28).

With this information, I designed during the lecture a dual specific sandwich test for reaginic antibodies, as shown in Fig 9. The reaginic antibody in a serum sample was first bound to the specific allergen attached to a solid phase. The allergen specific reaginic antibody was then detected with a labelled antibody specific against the γE-immunoglobulin (IgE) class. A prerequisite for the test was that the reaginic antibody could be bound simultaneously both to the allergen and to the labelled antibody. A signal (e.g. radioactivity, fluorescence) from the label bound to the solid phase indicated that the patient had reaginic (IgE) antibodies and was allergic against the particular allergen.

The merits of the dual specific sandwich technique for this application are illustrated in Fig. 10. It could be expected that the serum sample might contain antibodies of other immunoglobulin classes directed to the specific allergen. If the solid phase allergen was in a large excess, these antibodies of other immunoglobulin

classes would hardly interfere. They would not be detected by the labelled antibody. Also the labelled antibody should be added in excess, and the reaginic (IgE) antibodies directed to other allergens should then not interfere. However, as a precaution, the reaginic antibodies against other allergens could be removed in a washing step before the addition of the labelled antibody.

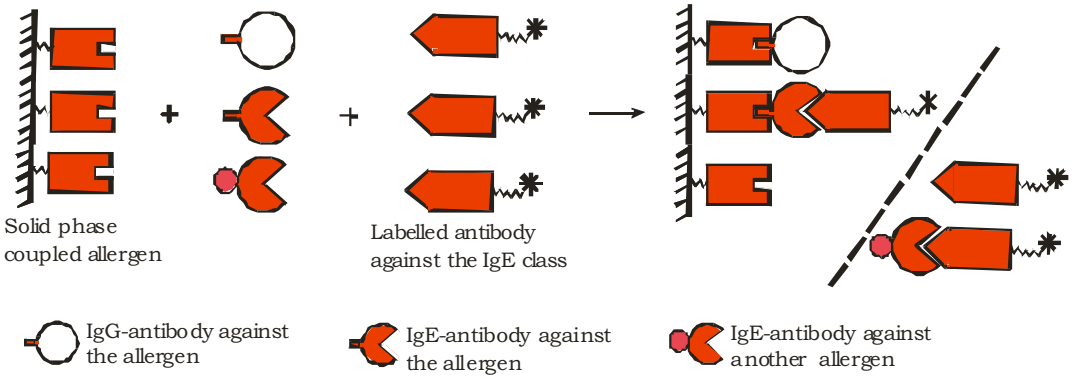

Figure 10. Selective detection of IgE-antibodies against a particular allergen in the presence of IgG-antibodies to the same allergen and IgE antibodies to another allergen.

Penicillin allergy

On the following day, I met Dr Juhlin and showed him my schematic drawings of possible methods to detect the reaginic antibodies. Juhlin had a particular interest in penicillin allergy and suggested the use of penicilloyl as allergen in the test. We agreed upon collaboration on penicillin allergy, which eventually resulted in several joint publications (29, 30). During the autumn 1966 Juhlin sent me blood samples from penicillin hypersensitive patients, and I obtained the penicilloyl which was supposed to be used in the assay.

A remarkable coincidence – a RIST assay of an atypical myeloma protein was developed

A remarkable coincidence then occurred. An atypical myeloma protein that did not belong to the known immunoglobulins A, D, G, and M had been detected and purified by two colleagues in Uppsala: Gunnar Johansson and Hans Bennich (31). They had been unable to find a normal counterpart to the myeloma protein (called myeloma-IgND) when investigating 300 sera from blood donors and patients with a single radial immunodiffusion (SRD) test.

When I heard about their efforts which were presented in a seminar, I informed that radioimmunoassays had a much higher sensitivity than the SRD test. The chromatoelectrophoresis, used as a separation step in the radioimmunoassay of Yalow and Berson (19), may not be applicable for the gammaglobulins. However, I had invented, but not yet published, a new principle which I termed radioimmunosorbent

(RIST) assay, which may be suitable in this case. I invited them to a collaboration in which they should give me the purified myeloma protein and a specific antiserum, and I should label the protein and couple the antibodies to Sephadex and develop a RIST assay for the myeloma protein.

With this RIST method, a protein was detected in normal sera and this protein had a position on electrophoresis, gelfiltration and DEAE-chromatography corresponding to the atypical myeloma protein. It was concluded that myeloma-IgND represented a new class of human immunoglobulin. The study was published in Immunology by Johansson, Bennich and Wide (32) with the title: A New Class of Immunoglobulin in Human Serum. In this study we found that one of 62 blood donors had an IgND level 15 times higher than the average.

The critical reagent for the allergy test was in my laboratory

This blood donor showed clinical signs of extrinsic asthma (32). It was a fair chance that the myeloma-IgND belonged to the immunoglobulin class γE (IgE) shown to be associated with the reaginic activity (26-28, 33, 34). When I, late February 1967, heard about the asthma diagnosis, I realized that the critical reagent for the allergy test may have been in my laboratory for several months. Within a few days I had obtained sera of allergic patients from the Allergy Outpatient Clinic in the hospital and common allergens from the pharmacy for coupling to solid phase: animal dandruff from horse, cat and dog; pollen from birch and grass; and extracts of shellfish and house dust. It was an immediate success for the design of the dual specific allergy test. The anti-IgND was used as an anti-IgE in the assay to detect allergen specific reaginic antibodies, and the results correlated excellently with those of provocation tests with the same allergens. This was a strong support for a similarity between the IgND and the γE-immunoglobulin (IgE).

The following month specific antisera to myeloma-IgND and γE-immunoglobulin (IgE) were exchanged between the laboratories in Uppsala and Denver (K Ishizaka). The two proteins shared major antigenic determinants, and eventually it was reported that the myeloma-IgND and the IgND in normal serum were of the IgE-immunoglobulin class (35, 36).

To Pharmacia with the radioallergosorbent (RAST) test

I had kept the non-competitive sandwich assays, including the design of the allergy test, as a secret as I regarded it as a patentable invention. By the time I found out that the allergy test functioned with the anti-IgND, I disclosed the method for Hans Bennich and Gunnar Johansson. I suggested that Bennich, who had the purified IgND protein, should make a larger batch of immunosorbent purified anti-IgND. Then, after I had shown that this functioned well in the test, we could offer the allergy test to Pharmacia to apply for a patent and to produce and market a diagnostic test kit. An agreement had to be made between the three of us and Pharmacia and this should include, in addition to the invention, also the supply to Pharmacia of the necessary amounts of myeloma-IgND and anti-IgND to start the production. I suggested the name RadioAller-

goSorbent Test or RAST in analogy with RIST (see above). My negotiation with Gösta Virding started in June 1967 but was not finalized until the beginning of September.

A citation classic

As soon as the patent application was sent, I started to make a draft of a manuscript by L Wide, H Bennich and SGO Johansson with the title 'Diagnosis of Allergy by an In-vitro Test for Allergen Antibodies'. This was published in The Lancet in November 1967 (37) and became, in 1985, a Citation Classic in Current Contents (38) as it for the first time described the allergy test as well as the dual specific non-competitive sandwich assay using labelled antibodies.

Further applications of the sandwich technique

The use of a catching antibody and a labelled antibody to measure an antigen was published in 1969 and presented by me at a Karolinska Symposium on Immunoassay of Gonadotrophins (39) : 'The gonadotrophin in the unknown serum is first bound to

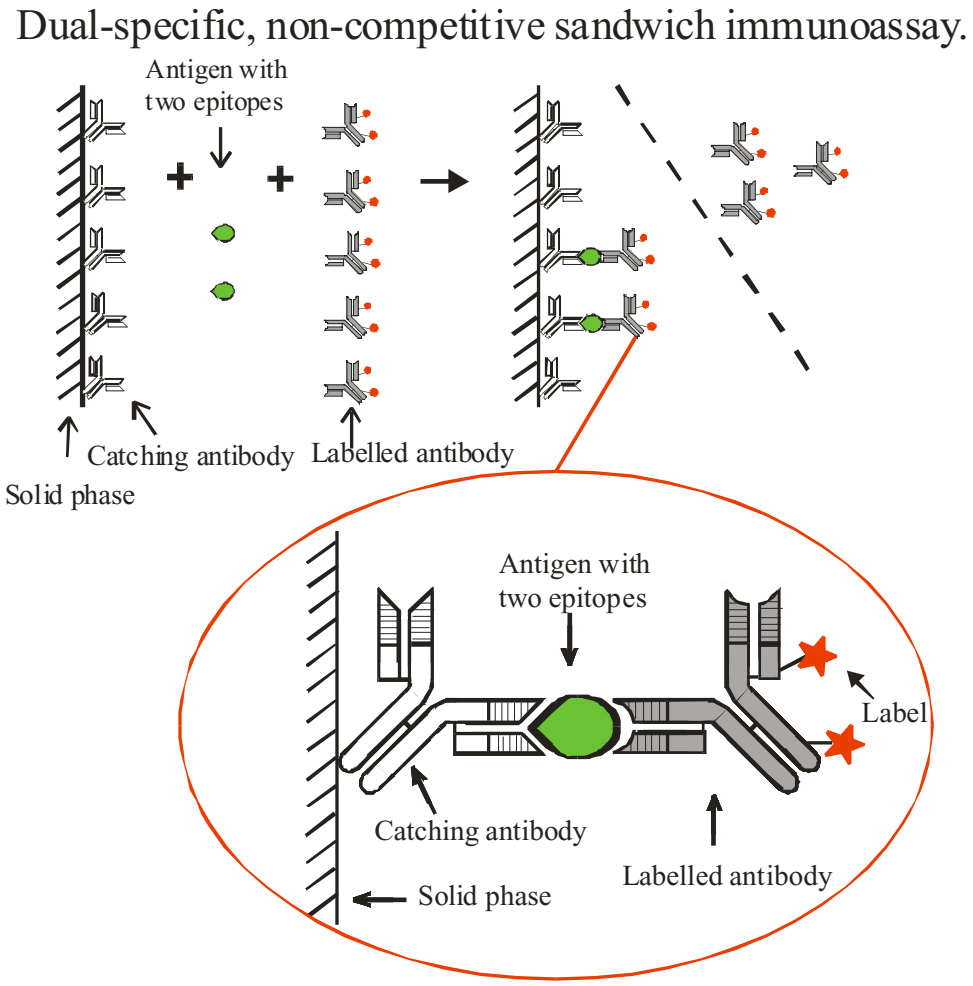

Figure 11. The principle of the dual specific sandwich assay using a catching antibody and a labelled antibody directed against different sites on the antigen.

polymer-coupled antibodies. After washing the solid phase, labelled antibodies are added. They bind to the gonadotrophin on the solid phase and the uptake of radioactivity is in relation to the amount of hormone present in the original serum.' In the same publication, I introduced the term 'sandwich technique' as a general term for the non-competitive assays. The principle of the dual specific sandwich assay using a catching antibody and a labelled antibody directed against different sites on the antigen is shown in Fig. 11.

The following year I described three different variants of the sandwich technique at a European Workshop in Edinburgh on Radioimmunoassay Methods. The sensitivity of the IgE assay was reported to be at least 10 times higher with the sandwich technique than with the competitive radioimmunoassay using the same antiserum (40).

Other names than the sandwich assays were proposed, such as 'Verknüpfungs Test'(41), 'two-site assay' (42) and 'immunoradiometric assay', the latter name originally used for a competitive immunoassay with labelled antibodies (43). Today the term sandwich assay is commonly used, and it covers also the non-immunological systems using the same basic principle, e.g. for DNA analyses.

GENERAL REMARKS

The inventors' choice of a suitable entrepreneur

A very important step in the innovation process is the choice of entrepreneur. It was Gemzell who choose Organon for the immunological pregnancy test, and I had no objection to that, or suggested any alternative. The amount of resources, both number of people and laboratory space that they immediately put into the project, was impressive. Organon had an excellent quality control and their product Pregnosticon was highly reliable. Organon was a possible choice also in 1966 for the new invention, the solid phase radioimmunoassay. However, I had lost the scientific contact with Organon and their reply was unfavourable when I asked for a research grant in 1965.

Pharmacia in Uppsala became an alternative. This company, on the other hand, was much smaller and without any experience within the diagnostic area or immunology. Pharmacia had the advantage of being close to us, had a tradition of good contacts with scientists at Uppsala University, and their knowledge about insoluble polysaccharides could be essential. It was also attractive to favour a Swedish industry, and a maximal profit of the invention was never my objective. My co-inventors, Axén and Porath, had no objection to let Pharmacia have the first option. It turned out to be a successful choice.

The innovations from the entrepreneurs' view

The entrepreneurs, in this case Organon and Pharmacia, had to estimate the potential market for the product, how the project could fit into their organization, their possibility to manufacture the product and the chances to obtain a patent protection.

The potential market of the pregnancy test must have been obvious for Organon in view of the well documented desire throughout ages of developing a simple and reliable test, which hitherto had failed.

It was much more difficult for Pharmacia to evaluate the potential market of test kits for radioimmunoassay. Over six years had elapsed since the first radioimmunoassay methods were described, and they were not used at the hospitals in the medical service. Pharmacia had limited experience in the field of immunology. Conventional market researches seemed meaningless to do, as there were no similar products on the market, and only a few people could see the future demand. A pioneer research and development in a new area had to be made. I had several long discussions with Gösta Virding, in which I convinced him about a large fruitful future market for these products, and his never failing support for the 'Wide-project' became important.

There were periods during the development of the projects, both at Organon and at Pharmacia, when strong opinions expressed that the projects should be abandoned, as resources had to be taken from other areas within the companies. It was then of vital importance for the projects that they were supported by the managing directors, Mauris Tausk and Gösta Virding, respectively.

The agreements between the inventors and the entrepreneurs

It is a very unequal situation when a young inventor from the university negotiates with professional businessmen and lawyers. The agreement with Organon in 1960 was too strictly limited to the particular technical performance of the method as we had described it. When in 1964, Organon heard of Strausser's unpublished PhD thesis from 1958 (14) (see above) they claimed that this anticipated the novelty of the invention and that they therefore reduced the royalty to half and limited the period for payment to ten years. On the other hand, they were willing to extend the agreement to include also developments and improvements of the test made by Organon as long as the basic principle of the original test was applied. We had of course taken the latter for granted when the agreement was settled in 1960.

The discussions with Organon were useful experiences when I later negotiated with Pharmacia. Pharmacia got the rights to decide the contents of the patent applications. In the agreement with the inventors they defined the invention as it was described in their patent application. I had objections against this way of limiting the definition of the invention to Pharmacia's patent application. I could foresee many products based upon the basic principle of the invention and not covered by the patent applications. One example, which I discussed when the first agreement was signed, was the use of a solid phase coupled second antibody, later called DASP (Double Antibody Solid Phase). Different variants of the sandwich technique were other important examples, as Pharmacia covered the technique with patents only in the allergy field. In1968, I made numerous attempts to persuade Pharmacia to apply for patents on the other different applications of the sandwich technique. When they hesitated to do this, I succeeded to get an informal agreement on the use

of the basic principle of the non-competitive 'Sandwich technique'. I realized that it was important for the future to describe and define in text and drawings the basic principles of all our inventions. In this work I had valuable support from Björn Ingelman, a successful inventor at Pharmacia.

During the negotiations for the RAST method I wanted to change a paragraph against which I had objections in the previous agreements. It was about the rights to sell patents, patent applications and licenses to a third part. After several meetings with Virding we decided that Pharmacia was not allowed to do this with any of the inventions without the permission of the inventors.

An independent inventor from the university has his particular view on the exploitation process. An industrial partner may look at the process and at the agreement in a somewhat different way. This difference may not have been clarified and penetrated before the agreements are signed. We experienced this with a shock when the first royalties were paid from Pharmacia. We inventors lived with the view and took it for granted that the royalties were based upon the sales to independent external customers. Pharmacia regarded their own foreign sales organizations, which were subsidiaries, as customers. The royalties were calculated on Pharmacia's internal price between these subsidiaries and the part of Pharmacia with whom we had signed the agreement. This considerably reduced the royalties that we had expected to get. It is advisable for b,oth parties to take the time necessary to agree on all important principles in order to reach a mutual understanding and create a win-win-situation. A lawyer with experience and a special interest in these particular problems may help to avoid future surprises.

It was not until the 80's, thus after 20 years of experience with negotiations that I felt that I could negotiate on more equal terms. All agreements were rewritten and became valid between the inventors and the Pharmacia group, including affiliated companies as Wallac OY and Electro-Nucleonics Inc. Pharmacia obtained the rights to grant patent licenses. We also agreed upon the definitions of the innovations and signed formal agreements on the DASP-principle and the 'sandwich technique'.

Some reflections

Even if the university showed no active interest in commercial innovations in the 1960's, I experienced there a stimulating atmosphere for creative research. The institutions at which I worked possessed some free research money that could be used for new ideas. The institutions could supply with technical support in the form of technicians as well as instruments, and they were equipped with a workshop where an instrument maker was employed. I experienced a strong support from the staff both at King Gustav V:s Research Institute in Stockholm and at the departments in Uppsala. All this facilitated a creative research work. Examples have been given above on the value of seminars and meetings in local research societies for the exchange of information and start of collaborations.

Innovations are seldom created on order. The pregnancy test as well as the allergy test started with the development of methods with unique qualities, and then fol-

lowed by the search for appropriate applications. This sequence of 'having found a solution and then look for its problem' is not unusual during an innovation procedure. It can, as exemplified above, be rather trivial observations that can lead to a deviation from the original research into new areas.

It can be difficult to formulate the claims in a patent application for an invention within a completely new area. The diagnostic test kit area was new for Pharmacia's patent group. They were chemists and came to focus on the chemistry in their patent applications. In 1968, I presented at Pharmacia several drafts for patent protections of the general principle of the sandwich technique, covering fields outside allergy. I stressed the high sensitivity, precision and specificity, and furthermore the short reaction time and the possibility to use other solid matrixes than particles, when compared with the competitive immunoassays. We discussed it with a patent bureau and preliminary drafts were made. However, the patent group at Pharmacia feared that such an application could interfere with their previous patent for the allergy test and they decided to stop these attempts. As a consequence, Pharmacia had no general patent protection when, in 1976, they launched the sandwich assay of hGH using a solid phase catching antibody together with a labelled antibody.

EPILOGUE – GLOBAL GROWTH OF DIAGNOSTIC TEST KIT INDUSTRY

From a project group to Pharmacia Diagnostics

The so called 'Wide-project' at Pharmacia developed slowly during the first year, but after that in an increasing pace. Under the successful leadership of Carl-Erik Sjöberg it grew to a diagnostic division within Pharmacia and later to the company Pharmacia Diagnostics. Pharmacia became one of the pioneers in the field of reagent-kits for radioimmunoassay in 1971, when marketing a RIST-kit for insulin. The following year they were first on the market with tests for total IgE and the solid phase assay for vitamin B12.

The use of paper discs instead of Sephadex particles was an improvement of the sandwich techniques that I presented for Pharmacia in 1968 (44). This eliminated the centrifugation step used in the original version. Pharmacia became a pioneer on the market with kits for assays based upon the non-competitive sandwich technique. In 1974 the company launched the test for allergen specific IgE (Phadebas RAST) and two years later they introduced the labelled antibody methods for the assays of hGH and total IgE. They were all based upon the use of paper discs as the solid phase. Pharmacia reached 'break even' for the diagnostic products ten years after the start of the project. About ten years later the number of employees had increased to 1,500. When the profits from the diagnostic products rapidly increased, Pharmacia showed its gratitude to Uppsala University with a generous donation for research. During a long period of time Pharmacia Diagnostics was one of the leading diagnostic test kit companies in the world and has continued to be so in the field of allergy diagnosis.

The diagnostic test kit industry grows fast on a global multi thousand million dollar market

The non-competitive sandwich technique combines an ultra-high sensitivity, a high specificity and a short reaction time, which are features of crucial importance particularly in many clinical applications. In 1975 Köhler and Milstein (45) described a method for in vitro synthesis of monoclonal antibodies. Such monoclonal antibodies are particularly useful in sandwich methods and offer advantages for the kit manufacturers by the means by which these antibodies are isolated and produced. Large amounts of antibodies can be synthesized against a single antigenic site.

A few immunoassay-kit manufacturers realized the advantages of the sandwich technique in the 70's, but during the following decade the number of such companies increased rapidly. Several companies applied for patents on the use of sandwich techniques in the 80's causing many bitter patent disputes (46). The diagnostic test kit market grew globally and was a multi thousand million dollar market in the 90's. The sandwich technique is now used in most of the immunoassays and in other fields such as the array-based DNA-analysis. The pregnancy tests in use today are also based upon the sandwich technique.

The radioisotopes that successfully had been used as labels in the binding assays were to a large extent replaced by enzyme, fluorescence, and chemiluminescent labels. Competitive binding techniques are still applied for assay of small size analytes, and the vast majority uses the solid phase separation system.

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