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A SOLID SUBSTRATE IMMUNOLOGICAL ASSAY FOR MONITORING ORGANIC ENVIRONMENTAL CONTAMINANTS

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This project assesses the use of an immunoassay technique (antibody-antigen responses) as a possible warning system for exposure to a toxicant. If successful, it could ultimately develop into a system for monitoring human exposure to toxicants as film badges now monitor exposure to radiation which would be a novel breakthrough in an old problem that would have application in agriculture as well as occupational health.

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ABSTRACT

A solid substrate "film-badge" type monitor has been developed that is capable of detecting 2-aminobenzimidazole (2-ABZI) at less than one part per million in water in less than 10 minutes. The monitor makes use of the reaction which takes place between 2-ABZI in the sample and a monolayer of its antibody that has been deposited on a thin film of indium on a glass substrate.

A second approach in which the antibody is mounted on polystyrene and reaction of its antigen-binding sites with a fluorescein-labeled antigen are subject to competition with nonlabeled antigen in the sample, has been demonstrated in principle. Improvements in this alternate approach are proposed.

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SECTION 1

INTRODUCTION

BACKGROUND

Increasing concern for monitoring the natural environment and workplace can be better understood when it is noted that over one-hundred-billion pounds of organic chemicals, and in particular over one-billion pounds of pesticides, are manufactured annually in the United States alone (Ref. 1). Many sophisticated and sensitive assays have been developed for the detection and monitoring of environmental contaminants, but all suffer from one or more limitations when considered from the viewpoint of sensitivity, speed, low cost, simplicity, and specificity. In addition, movement of an individual throughout a particular industrial facility or agricultural area could well expose him to differing concentrations of a contaminant for differing periods of time, thus requiring that all possible sites of exposure be monitored.

This requirement logically leads to the consideration of workers carrying on their person exposure monitors, in the same manner that film badges are worn to monitor exposure to ionizing radiation. The present work has explored the feasibility of such badges based upon the immunochemical specificity and sensitivity of antibody for its causative agent, which in this case would be the contaminant of environmental concern.

Two approaches have been investigated. In one approach the antibody was bound to a very thin metal film deposited on a glass substrate. Subsequent reaction of the antibody with the contaminant was detected by changes in optical transmittance of the glass, film, protein complex. In the second approach, antibody was affixed to a polymeric surface, and reaction with the contaminant was observed by noting the inhibition of the reaction with fluorescein-labeled contaminant.

It has long been known that immunological techniques provide a highly specific and sensitive assay for many compounds of high molecular weights such as, for example, proteins. Recent work in which low molecular weight compounds have been coupled to carriers with resulting immunospecificity has extended the range of compounds which can be detected using such techniques. A specific example of such an assay has been recently demonstrated by IRT Corporation under the sponsorship of the Environmental Protection Agency. An assay was developed which allowed the detection of 2-aminobenzimidazole (2-ABZI, molecular weight 133), using a fluorescence polarization technique with a detection limit in the picograms/mℓ range (Ref. 2). Such an assay requires relatively sophisticated equipment, and is limited to the detection of the contaminant in aqueous solutions. It does, however, demonstrate that antibody, which is an essential part of the assay, can be produced to low molecular weight compounds with a high degree of sensitivity and specificity.

In a recent extension of conventional immunological techniques, Giaever (Ref. 3) has developed a visual method of detecting an antibody-antigen reaction. His method demonstrates that the protein, bovine serum albumin (BSA), can be adsorbed onto a metal film, and that the antibody to BSA will subsequently react with the BSA. In the initial step, BSA is bound to the metal film, and then reacted with anti-BSA, after which a change in the optical density of the film occurs and visual evidence of the reaction is obtained. In Giaever's experiments he was unable to carry out the procedure in the reverse order (Giaever, private communication), i.e., the anti-BSA being bound to the metal film with subsequent binding of BSA to the anti-BSA. This defined a major area in our research task since use of metal film as a substrate for a film badge system would not be suitable for detecting an antigen unless the antibody could be deposited on the metal film without destroying its specificity and sensitivity.

Inasmuch as investigation of the metal-film substrate system was a major objective of this work, the antibody deposition technique received considerable attention. The use of a polymeric substrate was analogous to the use of the metal film. However, the properties of film on polymer were not suitable for detection by optical transmission. Therefore, the use of a fluorescein-labeled compound was required.

2-ABZI was chosen as the contaminant of interest since an assay for this compound had been developed under a previous EPA contract (Ref. 2), and consequently results exist which will allow a comparative assessment of the sensitivity of the presently proposed assay to be made. However, limited quantities of the antibody to 2-ABZI exist, and, therefore, it was determined that in the initial stages of the program, where techniques were being developed and refined, it would be appropriate to use a more readily available antigen-antibody system, and consequently BSA and anti-BSA were used for this purpose.

OBJECTIVES

The objective of this program was to develop a solid substrate immunological assay for the detection of organic contaminants of environmental concern in a manner similar to the well-known radiological "film badge" type of monitor. Specifically, the following eight tasks were undertaken and successfully completed.

1. Metal-coated glass slides were prepared to serve as a solid support for the antibody matrix.

2. Antibody specific to BSA was deposited as a monolayer on the metal glass slide, and tested for reactivity to BSA.

3. Antibody specific to 2-ABZI was deposited as a monolayer on the metal-coated glass slide.

4. Antibody product was deposited on an organic polymer substrate as indicator slides.

5. Organic polymer-antibody preparation was examined for sensitivity, reproducibility, and specificity to BSA.

6. The glass indicator slides were evaluated for sensitivity and selectivity for 2-ABZI as a function of contaminant concentration.

7. The selectivity and sensitivity of the glass indicator slides were compared to results obtained by a fluorescence polarization immunoassay technique.

8. Applicability of the slides prepared in Task 3 to monitor the concentration of 2-ABZI in water was evaluated, and recommended configurations and monitoring techniques are herein proposed.

SECTION 2

CONCLUSIONS

A solid substrate "film-badge" type monitor has been developed that is capable of qualitatively detecting 2-aminobenzimidazole (2-ABZI) at less than one part per million (ppm) in water in less than 10 minutes. The badge is prepared by vacuum vapor deposition of a thin ($\sim$100 Å) film of indium (In) on a glass substrate, and attaching a monolayer of 2-ABZI antibody to the indium. The latter step is carried out under conditions that leave free the immunospecific positions of a significant number of antibody molecules. After deposition of the antibody on the indium, glass-substrate, the badge is air dried, and its optical transmittance measured on a densitometer. It may then be used by either immersion in an aqueous sample or by placing an aliquot of the sample on the monitor. After a short incubation period in the presence of the unknown, the badge is rinsed, air dried, and its transmittance once again measured. A decrease in the transmittance from its preexposure value indicates the presence of 2-ABZI. If only a part of the film is exposed to the sample, a visible difference between pre- and postexposure optical properties may be noted with ease when the sample contains detectable amounts of 2-ABZI.

The use of an alternate approach using somewhat more simplified measuring equipment has been demonstrated in principle. It consists of a polystyrene substrate to which antibody has been attached by a chemical reaction, and which becomes fluorescent upon exposure to fluorescein-labeled antigen. This effect is inhibited by prior exposure to a sample containing a nonfluorescent form of the antigen and is generally referred to as a "competitive binding assay." The major disadvantage of this second approach in its present stage of development is the high level of background fluorescence from the polystyrene, which limits its sensitivity; however, this is correctable. The potential advantage of the polymer system is for quantitative measurements.

SECTION 3

RECOMMENDATIONS

Both the metal film and polymer substrates deserve further development. The indium film technique has been developed to the point where it is very close to fieldable and is able to give qualitative answers. The polymer system has been proven in principle, and its development, which would be straightforward, could lead to a system that gives quantitative results. Development of the metal film system should now be limited to work with indium films, and should focus on optimum conditions for antibody deposition, packaging, and stability. The problem of the nonspecific binding of protein must also be addressed. For example, the study of substrates to which antibody molecules might be chemically coupled without destruction of the antigen binding function would be a possible approach to this question of nonspecificity. The goal would be to maximize the number of free binding sites of antibody and the stability of the antibody surface, so that the monitor has optimum sensitivity and a reproducible performance both in absolute terms and in rate of reaction. Study of packaging and storage of the monitor should be undertaken with a view to developing long shelf life, and at the same time be compatible with ease of use.

Further development of the polymer substrate system would serve as a backup to the indium film system. In contrast to the indium film system, the polymer system is not suitable for visual observation; rather, it would require a simple fieldable electronic instrument to detect the inhibition of the fluorescence of the labeled contaminant in question by an unknown sample. It is recommended that one focus of further development be the acquisition of a suitable nonfluorescent polymer, i.e., a polymer that does not fluoresce at the same wavelength as the labeled reagent. Alternatively, the use of radiolabeled compounds in conjunction with a photographic plate

as a simplified counting system might be considered which may also solve the nonspecific fluorescence problem. In addition, the reagents relevant to the measurement of a contaminant of environmental concern should now be used, the sensitivity defined, and a calibration curve obtained.

SECTION 4

METHODS AND MATERIALS

PREPARATION OF METAL-COATED SUBSTRATE

The substrate chosen for these experiments was 22 x 22 mm microscope slide-cover glasses of No. 1 thickness. Metal was deposited on these cover glasses by means of a commercial vacuum coater system, which was equipped with a standard 18-inch-diameter bell jar, multiple electrical feedthroughs, pressure monitoring gauges, film thickness monitoring, auxiliary power supplies for substrate heating, and linear motion feedthroughs. The system was operated in the 10^{-5} torr range, and is capable of handling a wide range of resistance heated boats, coils, and crucibles for tungsten filament evaporation. The unit provides evaporation filament power from a 2 kVA multitap power transformer. It also includes a 6 kW electron-beam gun for electron-beam evaporation.

Indium films were prepared by tungsten filament evaporation out of a tantalum boat. Nickel films were prepared by electron-beam evaporation. The majority of films prepared were of indium, since system success was encountered early with indium, and its further development was the most efficient use of the available experimental time.

The procedure for preparing the metal-coated glass slide film, was first to clean the glass thoroughly by sequential treatment with mild detergent, distilled water, reagent grade acetone, and absolute alcohol, followed by drying in a warm airstream. The slide was then placed in the vacuum chamber and cleaned with argon plasma for 15 minutes.

Metal was placed in a quartz or tantalum boat, the pressure reduced to less than 10^{-5} mm Hg, and then heated to melting with a shutter in position above the boat to allow removal of possible contaminants which would result in

deposition of contaminants on the slide. When the metal surface on the shutter appeared clean it was removed without breaking the vacuum, and metal was deposited on the glass. The thickness of the deposit was measured with a Sloan thickness monitor.

Initially, metal films of approximately 1000 Å were prepared under various conditions of substrate temperature, rate of deposition, and purity of metal. Table 1 lists typical combinations of parameters for the indium. Subsequently, indium films approximately 100 Å thick were prepared. It is important that the metal is not deposited as a continuous film, but rather agglomerates into small droplets as described in Appendix A. These are illustrated in Figure 1, and optimally they measure 1000 Å across and are in the foam of an oblate spheroid.

COATING THE METAL FILM

The deposition of protein, such as BSA or immunoglobulin, was carried out by contacting a defined area of the metal film with a saline solution of the protein (0.15M NaCℓ), either by partial immersion of the film or by placing drops of solution on the film. After the deposition of protein, the films were thoroughly rinsed with distilled water and allowed to dry in air.

Testing the initial protein film for reactivity with another compound was carried out by contacting the film with an aqueous solution of the compound by either of the two methods previously outlined, followed by rinsing and drying.

The principal technique by which the initial deposition of a protein (or other compound) and/or subsequent reaction with another compound was detected, was by measurement of the optical transmittance of the films with a McBeth-Ansco Optical Transmission Densitometer. Operation of the densitometer requires that the sensor system be placed on the film so that there is minimal loss of light and maximum reproducibility of measurement. In order to obviate disturbance of the films, for example, by scratching of the surface by the densitometer, they were covered with a clean microscope slide-cover slip prior to the transmission measurements. The transmittance of the added cover slip was slightly less than 100%, so care was taken to use the same cover slip throughout a given experiment to take into account this additional absorption.

TABLE 1. INDIUM SLIDES

Slide No.	Indium Purity	Substrate Temperature (°C)	Thickness (Å)	Rate of Deposition (Å/sec)
1	0.9999	21	1006	7
2	0.9999	100	1007	7
3	0.9999	21	1000	50
4	0.9999	21	1000	7
5	0.99999	21	1000	7
6	0.99999	21	1000	50
7	0.99999	21	1300	7

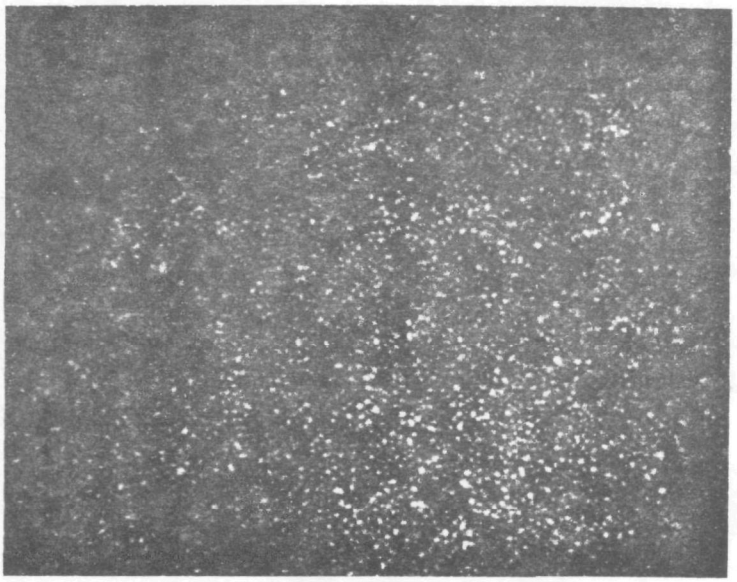

Figure 1. Agglomeration of indium particles on glass substrate (Reichart metalograph polaroid photograph)

Also, the densitometer was checked with respect to its "zero" and "calibration" readings prior to each measurement to guard against transient perturbations, and adjustments were made where necessary.

Changes in reflectance and in transmittance were also examined by the naked eye, since they were qualitatively observed with ease. This was aided by the fact that experimental treatments were addressed to less than a whole film, and a change in film thickness over the treated area resulted in an optical contrast with the untreated portion.

CULLULOSE SUBSTRATE SYSTEMS

Both Whatman No. 42 filter paper and cellulose thin-layer chromatography (TLC) sheet were cut into strips, and the strips were then immersed in a saline solution containing 12.5 mg of carbodiimide* for two hours, after which the strips were rinsed with water and immersed in a saline solution of anti-BSA reagent for three hours. The strips were then rinsed with saline and used as described in the Experimental and Results Section. Also, several cellulose TLC strips were reacted with normal rabbit immunoglobulin via the carbodiimide procedure.

POLYSTYRENE

Polystyrene strips were coated with antibody protein by direct immersion in a saline solution of the protein, and, after rinsing, reacted with protein solutions, including fluorescinated BSA (BSA-F).

Polystyrene discs were treated by the method of Filippusson and Hornby (Ref. 5) to form surface polyaminostyrene. The method involves initial nitration by immersion in an equimolar mixture of nitric and sulfuric acids for 20 minutes at 0°C, followed by rinsing with distilled water. The surface NO_3 groups are then reduced to amino groups by immersion in 6% (w/v) Na_2SO_4 in 2M KOH at 70°C for two hours, with stirring, followed by rinsing with dilute

*1-cyclohexyll-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate, from Aldrich Chemical Company.

HCℓ, and then water. One disc was set aside at this point as a blank. The amino groups of two discs were diazotized by reacting with fresh 1.5% (w/v) $NaNO_2$ in 0.6M HCℓ in an ice bath for 20 minutes with stirring. The discs were rinsed with 016M HCℓ and then with 0.001M HCℓ, and immediately contacted with a saline phosphate solution of anti-BSA reagent for two hours while cooling in an ice bath.

Anti-BSA and BSA solutions were dialyzed against saline solution to remove low molecular weight impurities, and then prepared at 300 ppm in saline. Also, normal rabbit IgG (which contained no antibody to BSA) was prepared at 300 ppm in saline by dialysis.

Fluorescein-labeled BSA was prepared by reacting 68.7 mg of BSA and 2 mg of fluorescein isothiocyanate (mole ratio of 5 FNCS to 1 BSA) in saline of pH 9 for two days at 4°C. The product, BSA-F, was purified by passing through a Sephadex G-25 column using saline as the eluent.

A viewing box with an ultraviolet lamp was used for visual observation of fluorescein-labeled antigen, and a fluorescence photon counter was used for quantitative measurement of the labeled antigen. The latter was equipped with a 5000 Å cutoff filter so that only photons with wavelengths greater than 5000 Å were counted, thereby reducing interfering fluorescence.

SECTION 5

RESULTS

METAL FILM SYSTEMS

Indium Films, BSA Experiments

It was found that the changes in transmittance of indium films in the 1000 Å range were relatively small subsequent to exposure to protein. For example, the films of Table 1 were subjected to the following sequence of experiments.

- The transmittance, T_0, of each quadrant of the indium-coated slide was measured prior to treatment.
- About 0.1 mℓ of anti-BSA solution (containing 290 μg of rabbit immunoglobulin directed against BSA per mℓ of saline solution) was placed in each of two quadrants, and allowed to incubate at room temperature for a measured length of time, t_1. The film was then gently and repeatedly rinsed with distilled water and allowed to air dry.
- The transmittance, T_1, of the once-treated quadrants was measured.
- About 0.1 mℓ of BSA solution (containing 290 μg of BSA per mℓ of saline solution) was placed on one of the quadrants previously treated with anti-BSA, and allowed to incubate for a measured length of time, t_2. The film was then gently and repeatedly rinsed with distilled water and allowed to air dry.
- The transmittance, T_2, of the twice-treated quadrant was measured.

The results are given in Table 2. It can be seen that while there is considerable change in transmittance effected by the first treatment, the second treatment produces a relatively small change, and in both cases the variation is quite large. Accordingly, subsequent experiments were directed toward thinner indium films, with the objective of improving the sensitivity.

TABLE 2. CHANGE IN TRANSMITTANCE WITH ANTIBODY AND ANTIGEN-ANTIBODY LAYERS ON 1000 Å INDIUM FILMS

Slide No.	T_0	t_1 (minutes)	T_1	t_2 (minutes)	T_2
1	0.164	31	0.161	22	0.161
2	0.242	30	0.205	21	0.202
3		PHYSICAL DAMAGE TO FILM (SCORING)			
4	0.173	34	0.153	26	0.141
5	0.092	30	0.084	20	0.080
6	0.154	32	0.144	15	0.144
7	0.161	30	0.090	19	0.090

Indium films of 50, 100, and 200 Å thickness were separately treated for 30 minutes with saline solutions containing 2.8, 28, and 280 ppm of BSA, and with a saline solution containing 294 ppm of anti-BSA. The results of these experiments are given in Table 3. It can be seen that the deposition of protein on indium film, whether BSA or its antibody (IgG), is easily accomplished and measured, and that relatively greater sensitivities are obtained with thicknesses in the range 100 to 200 Å.

The indium films that were coated with anti-BSA were then exposed to 294 ppm BSA solution. In no case was the transmittance significantly changed by exposure to the BSA solution, as shown in Table 4.

In order to check the possibility that the negative results of Table 4 might have been due to a characteristic of the experimental procedures, a 100 Å film was reacted first with 294 ppm BSA solution, and (after rinsing, drying, and measuring transmittance) then reacted with 294 ppm anti-BSA solution. The results were as follows.

Condition of Film	Transmittance
Before the experiment	0.385
After contact with BSA	0.31
After contact with anti-BSA	0.27

Thus, it was clear that we were able to duplicate the effect reported by Giaever (Ref. 3), but as he found, the reverse order of deposition resulted in greatly reduced sensitivity (Giaever, private communication).

The most likely explanation for these results is that the immunospecific end of the antibody (IgG) reacts with indium with much greater frequency than the opposite end, the so-called Fc end, of the molecule (see Appendix B). Under such a circumstance there would be relatively few immunospecific ends available for binding to the BSA; hence, there could not be a significant change in the film thickness, and consequently no significant change in the transmittance.

TABLE 3. REACTIONS OF PROTEIN SOLUTIONS WITH INDIUM FILM*

Solution	Transmittance for Various Thicknesses of Indium Before and After Exposure					
	50 Å		100 Å		200 Å	
	Before	After	Before	After	Before	After
BSA, 2.8 ppm	0.69	0.685	0.365	0.325	0.083	0.071
BSA, 28 ppm	0.725	0.70	0.36	0.305	0.093	0.078
BSA, 294 ppm	0.74	0.70	0.385	0.315	0.106	0.086
Anti-BSA, 294 ppm	0.67	0.66	0.385	0.285	0.098	0.068

*No exposures to more than one solution or more than one time were used in these experiments.

TABLE 4. INITIAL ATTEMPT TO REACT BSA WITH INDIUM COATED WITH ANTI-BSA

Thickness of Indium Film (Å)	Transmittance Before Exposure to BSA	Transmittance After Exposure to BSA (294 ppm)
200	0.068	0.069
100	0.285	0.280
50	0.66	0.66

The primary reactive groups of protein are free amino groups and free carboxyl groups. In order for there to be a marked difference between the two ends of the IgG molecule with respect to reactivity with indium, there must necessarily be a significant difference in the relative numbers of these reactive groups at each end of the molecule. Therefore, it appeared that manipulation of chemical factors could result in modification of the reaction such that the Fc end of IgG would predominantly bind to the indium, leaving specific reactive groups free.

Inasmuch as the two groups are opposite with respect to acid-base properties, it was anticipated that the desired effect might be obtained by simple adjustment of pH. In particular, one could possibly increase the reactivity of the carboxyl groups (which are more prevalent at the Fc end) by increasing the pH of the antibody solution.

Consequently, an experiment was carried out in which an aliquot of 294 ppm anti-BSA solution, approximately 0.03M in KOH, was contacted with a 100 Å indium film, and then (after rinsing, drying, and reading transmittance) the coated film was exposed to a 294 ppm BSA solution. The results were as follows.

Condition of Film	Transmittance
Before the experiment	0.43
After contact with anti-BSA	0.36
After contact with BSA	0.31

Significant changes, of approximately 15% in transmittance were apparent under these conditions, and suggested that the optimum concentration of KOH be determined. Subsequent experiments conducted with 200 Å films are summarized in Table 5.

As can be seen, the desired sequence of protein deposition was achieved with a small improvement in the sensitivity over a wide range of alkalinities.

TABLE 5. EFFECT OF KOH ON THE TRANSMITTANCE OF ANTIBODY MOUNTED ON 200 Å INDIUM FILMS

Condition of Film	Anti-BSA Solutions, Concentration of KOH		
	0.03M	0.004M	0.0008M
Before the experiment	0.079	0.076	0.078
After contact with anti-BSA	0.063	0.071	0.072
After contact with BSA	0.055	0.060	0.060

Since the speed with which an unknown sample can be analyzed is of considerable importance in a practical situation, it is of interest to determine the rate of reaction of protein with its antibody. Consequently, transmittance was used to follow the BSA, anti-BSA reaction as a function of time with 100 Å films only, since, as previously demonstrated, this thickness gave a near-optimum sensitivity.

The abbreviations below are used in the presentation of the results.

AB-film: An anti-BSA monolayer on an indium substrate film.

AG-AB film: The triple-layer BSA attached to the AB-film.

T_∞: Transmittance of the AG-AB-film after lengthy reaction of BSA solution with AB-film.

T_n: $(T - T_\infty) \times 1000$.

T_{no}: (T of the AB-film less T_∞) x 1000.

In the first series of experiments, an anti-BSA solution was made alkaline and used within one hour to prepare AB-film; the AB-film was then reacted with 10 mℓ of BSA solution within 24 hours. The extent of the resulting reaction as a function of time for BSA solutions of 0.4, 1.1, and 7 ppm concentrations is given in Table 6.

TABLE 6. REACTION OF BSA SOLUTIONS WITH FRESHLY PREPARED AB-FILM

0.40 ppm BSA[a]		1.1 ppm BSA[b]		7 ppm BSA[c]	
Time (minutes)	T_n	Time (minutes)	T_n	Time (minutes)	T_n
0	30	0	73	0	86
2	20	2	30	1.1	63
5	12	5	27	2.2	40
15	3	9.5	8	3.3	28
30	0	15	2	5.3	10
		21	0	6.5	25
				8.5	10
				16	0

[a]The regression line is given by $\log T_n = -0.065t + 1.443$.
[b]The regression line is given by $\log T_n = -0.093t + 1.806$.
[c]The regression line is given by $\log T_n = -0.106t + 1.862$.

A similar experiment, but with AB-film, prepared and stored one week in the open at room temperature, gave the results shown in Table 7.

TABLE 7. REACTION OF BSA SOLUTION WITH WEEK OLD AB-FILM

2.8 ppm BSA*	
Time (minutes)	T_n
0	30
1	27
3	23
8	15
18	0

*The regression line is given by $\log T_n = -0.037t + 1.473$.

Identical experiments, but with AB-film freshly prepared from anti-BSA solutions that had been alkaline for approximately one week, gave the results shown in Table 8.

TABLE 8. REACTION OF BSA SOLUTION WITH AB-FILM FRESHLY PREPARED FROM OLD, ALKALINE ANTI-BSA SOLUTIONS

0.4 ppm BSA[a]		2.8 ppm BSA[b]		28 ppm BSA[c]	
Time (minutes)	T_n	Time (minutes)	T_n	Time (minutes)	T_n
0	17	0	45	0	42
1	15	1	36	1	30
4	10	2.5	25	2	22
8	9	5	18	3.5	18
16	4	8	13	5.5	13
24	0	13	6	8.5	7
		19	0	21.0	0

[a]Regression line: $\log T_n = -0.037t + 1.210$.
[b]Regression line: $\log T_n = -0.065t + 1.610$.
[c]Regression line: $\log T_n = -0.086t + 1.572$.

The anti-BSA solution had been alkaline for five days prior to preparation of the slide used versus the 2.8 ppm BSA, and it was eight days old prior to preparation of the slide used versus the 0.4 and 28 ppm BSA solutions.

The behavior of anti-2-aminobenzimidazole (anti-2-ABZI) deposited on indium was next investigated. As in the case of anti-BSA, solutions of the antibody in saline were made slightly alkaline prior to deposition on the indium.

Several attempts to deposit 2-ABZI on the anti-2-ABZI films failed. At least such effects were not evidenced by any changes in transmittance of the film. Furthermore, after such attempts, films were exposed again to anti-2-ABZI (which would have adhered to 2-ABZI, but not to anti-2-ABZI) without affecting the transmittance.

One hypothesis for the failure of 2-ABZI to react with its antibody was that the alkaline treatment that inhibited a reaction of the "antibody-end" of the IgG molecule with the indium film also inhibited its reaction with its hapten. This would be true if one of the amino groups of the hapten (2-ABZI has one each primary, secondary, and tertiary amino groups) was necessary for the reaction with its antibody. The alkaline pH suppresses the reactivity of the amino groups. Therefore, a solution of 2-ABZI, which was on the alkaline side of neutrality, was neutralized with HCℓ, and a fresh attempt to react the hapten with the antibody films was carried out. This attempt was successful, as shown by the fact that the value of transmittance dropped from 0.355 to 0.330 upon contact with the 2-ABZI solution for 30 minutes.

Sensitivity

In order to test the sensitivity of the system, an anti-2-ABZI film on 126 Å indium was contacted with a 2.5 ppm solution of 2-ABZI in three points on a single slide with the following results.

Condition	Point A	Point B	Point C	Average T
Before contact with 2-ABZI	0.345	0.335	0.330	0.337
After contact with 2.5 ppm 2-ABZI for 30 minutes	0.320	0.315	0.320	0.318

The average difference of 0.019 in transmittance between the pretreatment and posttreatment (with 2-ABZI) films is significant at better than the 5% limit of confidence by Student's t-test of differences between means.

A similar test against 0.25 ppm 2-ABZI gave the following results.

Condition	T, Individual Measurements			Average T
Before contact with 2-ABZI	0.335	0.325	0.345	0.333
After contact with 0.25 ppm 2-ABZI for 30 minutes	0.325	0.325	0.310	0.320

The average difference of 0.013 in T between the pre- and posttreatment films is significant at better than the 20% limit of confidence by the t-test. An important aspect of this experiment was that the exposure to 0.25 ppm 2-ABZI resulted in a difference in optical density that was readily noticeable to the unaided eye.

In a similar test, 0.025 ppm 2-ABZI failed to produce any change in transmittance within 30 minutes which was detectable visually or by available instrumentation. Increasing the exposure to 24 hours resulted in the metal substrate being physically degraded by the experimental 0.025 ppm 2-ABZI solution.

Specificity

A number of experiments were carried out to test the specificity of the system for measuring 2-ABZI, which are described below.

An indium film (126 Å) was coated with a layer of BSA and then dipped in a 2.5 ppm solution of 2-ABZI for 30 minutes, and gave the following measured values of transmittance.

Condition	Average Transmittance
With BSA layer	0.402 ± 0.009
After contact with 2-ABZI solution for 30 minutes	0.398 ± 0.003

It can be seen that the transmittance was not significantly altered by placing the BSA-coated indium film in contact with a 2.5 ppm solution of 2-ABZI.

A second 126 Å indium film was coated with anti-2-ABZI, and then contacted for 30 minutes at different points with solutions of 2-ABZI, benzimidazole, and 2-amino 5-chlorobenzimidazole. These imidazole compounds effected the following changes in transmittance.

Condition	Point Contacted With benzimidazole	Point Contacted With 2-amino 5-chlorobenzimidazole	Point Contacted With 2-ABZI
Prior to contact	0.315	0.295	0.345
After contact	0.310	0.290	0.320

These experiments indicate that 2-ABZI does not react with a random protein (BSA), but does react with its antibody (anti-2-ABZI), as shown in column 3, and that it has greater affinity for its antibody than do analogous imidazoles (benzimidazole and 2-amino 5-chlorobenzimidazole), as shown in columns 1 and 2.

Similar experiments, in which an attempt was made to react diethylstilbesterol (DES) with anti-2-ABZI, proved negative, and confirmed the high degree of specificity of the antibody for its hapten, as shown in the following results.

Condition	Average Transmittance
Prior to contact with DES solution	0.354 ± 0.005
After contact with DES solution	0.352 ± 0.006

In the course of conducting these experiments, it was determined that 2-ABZI would also coat the bare indium-coated glass slides (slides that did not have an anti-2-ABZI coating), and that it was possible to subsequently react both anti-2-ABZI and BSA with the 2-ABZI, as shown by the following results.

Condition	Slide No. 1 Average T	Slide No. 2 Average T
Bare indium film	0.408	0.465
After 30-minute contact with 0.25 ppm 2-ABZI	0.370	0.422
Subsequent to 30-minute contact with anti-2-ABZI	0.318	
Subsequent to 30-minute contact with BSA		0.380

Thus, although the reaction of 2-ABZI with a protein film is specific to its antibody, the reaction of proteins to a film of 2-ABZI on indium film appears not to be specific.

It was found, in addition, that the 2-ABZI analogs, benzimidazole and 2-amino 5-chlorobenzimidazole, also coat the indium film. However, DES did not coat the indium film, so it appears that it is not coatable with all organic compounds.

Nickel Films

Experiments were conducted to investigate the possible advantages of using nickel films instead of indium. Two thicknesses of nickel were used, namely 100 Å and 160 Å. The nickel films were immersed in both neutral and alkaline saline solutions containing 294 ppm of anti-BSA for 30 minutes. The films were then rinsed and dried. The transmittance of the films were

then measured and immersed in neutral saline solutions containing 7 ppm of BSA for 30 minutes, and again rinsed and dried. Measurements of transmittance showed no change after the addition of both anti-BSA and BSA. Additional experiments, in which an attempt was made to reverse the process, i.e., first lay down BSA on the nickel and then anti-BSA, also showed no change in transmittance.

POLYMERIC SUBSTRATES

Experiments were conducted to investigate the feasibility of affixing antibody to a strip of polymer in such a manner that the strip could be used to detect antigen. Unlike the indium-on-glass technique, the reaction of antigen with the antibody was not expected to give a visible effect. However, the reaction would use up binding sites in proportion to antigen concentration, so that a subsequent reaction with fluorescein-labeled antigen would be proportionally inhibited. Thus, the method would work by first exposing the strip to the sample suspected of containing the antigen, and then determining the extent of suppression of the reaction between labeled antigen and the strip.

Whatman No. 42 Filter Paper

One of the strips that had been reacted with anti-BSA via the carbodiimide reaction was immersed in the fluorescein-labeled BSA (BSA-F) for 40 minutes, and then rinsed with saline.

A second strip was immersed in the BSA reagent for 40 minutes, rinsed, and then reacted with BSA-F reagent and rinsed again. Both strips were examined under ultraviolet light, and it was found, as was expected, that the strip that had been reacted with BSA prior to BSA-F was less fluorescent than the strip that had not been exposed to BSA. However, the fluorescence of both strips was irregular, which indicated nonuniformity of the substrate or the treatment.

Cellulose TLC Strips

Two cellulose TLC strips, reacted with anti-BSA via the carbodiimide reaction, and two strips that were reacted with normal rabbit IgG reagent via carbodiimide, were exposed to BSA-F and rinsed. It was found by observing ultraviolet fluorescence that the BSA-F had not reacted with the strips containing normal IgG to the same degree that it had reacted with the other strips, which indicated a specificity of reaction. However, again the fluorescence was unevenly distributed, and it was concluded that another support medium might be more appropriate.

Polystyrene Culture Tubes

To each of three polystyrene culture tubes (a, b, and c) was added 0.8 mℓ of anti-BSA reagent, and to a fourth tube (d) was added 0.8 mℓ of normal rabbit IgG reagent. Each tube was incubated three hours at room temperature, and then rinsed three times with saline solution.

To tubes a and b were added 1 mℓ of BSA reagent, and they were then incubated for 18 minutes, followed by a triple rinse with saline. Then, 0.5 mℓ of BSA-F reagent was added to each of the four tubes, and they were then incubated for 18 minutes, followed by a triple rinse with saline. A fifth tube (e, untreated) was used as a blank. All five tubes were examined for equal periods of time with a fluorescence photon counter. The results obtained were as follows.

Tube	Gross Counts (millions)	Treatment
e	0.95	None
d	1.20	Normal IgG, then BSA-F
a	1.73	Anti-BSA, BSA, then BSA-F
b	1.47	Anti-BSA, BSA, then BSA-F
c	2.48	Anti-BSA, then BSA-F

These results indicate the following: (1) IgG attached to the polystyrene; (2) the reaction of BSA-F with anti-BSA (shown in tube c) was specific, since it did not react with the normal IgG in tube d; and (3) the reaction of BSA-F with anti-BSA was inhibited by prior exposure of the anti-BSA to BSA in tubes a and b.

High-Impact Polystyrene

White, high-impact polystyrene strips were found to have an excessively high blank fluorescence level, which made them unsuitable for further experiments.

Black Polystyrene

Black, buna-loaded polystyrene sheet was examined as a candidate substrate material. One tab was taken as a blank, and another was simply exposed to the anti-BSA reagent for 30 minutes, rinsed, exposed to the BSA-F reagent for 10 minutes, and rinsed. The strips were then counted with the fluorescence photon counter. The blank gave 360,000 counts per minute, and the treated strip gave 820,000 counts per minute. Thus, the ratio of sample fluorescence to background fluorescence was greater for this material than for culture tubes, and the material is a candidate substrate for the purpose at hand.

Polystyrene Discs

One of the discs with an anti-BSA coating prepared as described in Section 4 was reacted with BSA-F reagent. Its fluorescence intensity, in counts per minute, were observed with the fluorescence photon counter and compared with those of the blank disc and a disc having an unreacted anti-BSA coating. The results were as follows.

Disc	Count Rate
Blank	430,000
Treated with anti-BSA only	230,000
Treated with anti-BSA, then BSA-F	1,590,000

It can be seen that these samples gave the best signal-to-noise ratio of those examined to this point. The anti-BSA apparently diminishes the incident and/or fluorescent light.

SECTION 6

DISCUSSION OF RESULTS

In comparing the two metal films, indium and nickel, the quality of indium for the purpose at hand was quickly demonstrated, while it soon became apparent that a relatively greater amount of effort would be required to define the usefulness of nickel films. Since, in these studies we are only concerned with demonstrating the feasibility of the technique, indium was used exclusively in rate and sensitivity experiments.

The hypothetical basis for experiments that led to the deposition of antibody in such a manner that its specific reactivity was partially retained was useful in that pragmatic context. However, the experimental success cannot be taken as proof of the validity of the hypothesis, nor can the follow-up hypothesis regarding unblocking of sites specific for 2-ABZI be considered as proven by experimental success. It is recognized that the model depicting IgG as consisting of linear and parallel chains is idealized, and that the molecule is highly convoluted (Ref. 6). It would be as reasonable to suppose that the changes in pH effected the molecule's chemical configuration with respect to exposed groups. Obviously, however, the initial hypotheses were useful in that they served to guide the experimental efforts in a direction which resulted in a practical solution to the problem of interest.

As expected, BSA and anti-BSA system served as an adequate model in the developmental work on the indium film system, in the sense that the procedures developed needed only slight modification for application to 2-ABZI and its antibody. It is interesting that the sensitivities of the indium substrate system in its present form are comparable for both BSA and 2-ABZI.

For example, 2-ABZI was easily detected at 0.25 ppm, but not detected at 0.025 ppm. By comparison, the BSA data in Table 6 vary about the regression lines as follows:

0.4 ppm BSA:	±65%
1.1 ppm BSA:	±23%
7 ppm BSA:	±33%

Clearly, the 0.4 ppm BSA left an observable change in the optical density of the film (as did 0.25 ppm of 2-ABZI); the large variance associated with the measurements, however, indicates that 0.4 ppm BSA was near the limit of detection. Also, it may be noted from the values of T_n at time zero in Table 6 that the net change in optical transmittance was much less for 0.4 ppm than for 1.1 ppm or 7 ppm BSA.

The regression lines of Table 6 are plotted in Figure 2, and the regression lines of Tables 7 and 8 are plotted in Figure 3.

Although the curves of Figures 2 and 3 have a progression of slopes that suggest a relation between rate of transmittance change and BSA concentration, it is premature to conclude that such is the case. For one thing, the standard deviations of two of the curves (1.1 and 7 ppm) of Figure 2 are such that there is no significant difference between their slopes. Also, the conditions relevant to Figure 3 were strictly comparable in only two of the four curves. More importantly, it is clear that the change in transmittance follows an apparent first-order kinetics over the range of concentrations used. This suggests a primacy of the following relationship, since attachment of BSA to a binding site on the film will decrease the transmittance in a linearly proportional manner:

$$-dS/dt = kS \qquad (1)$$

where

S = number of binding sites/cm^2 to which BSA may attach

t = time

k = rate constant*

*Equation 1 integrates to the expression: $\ell n(S/So) = -kT$, which gives straight lines when plotted on semilogarithm paper, as in Figures 2 and 3.

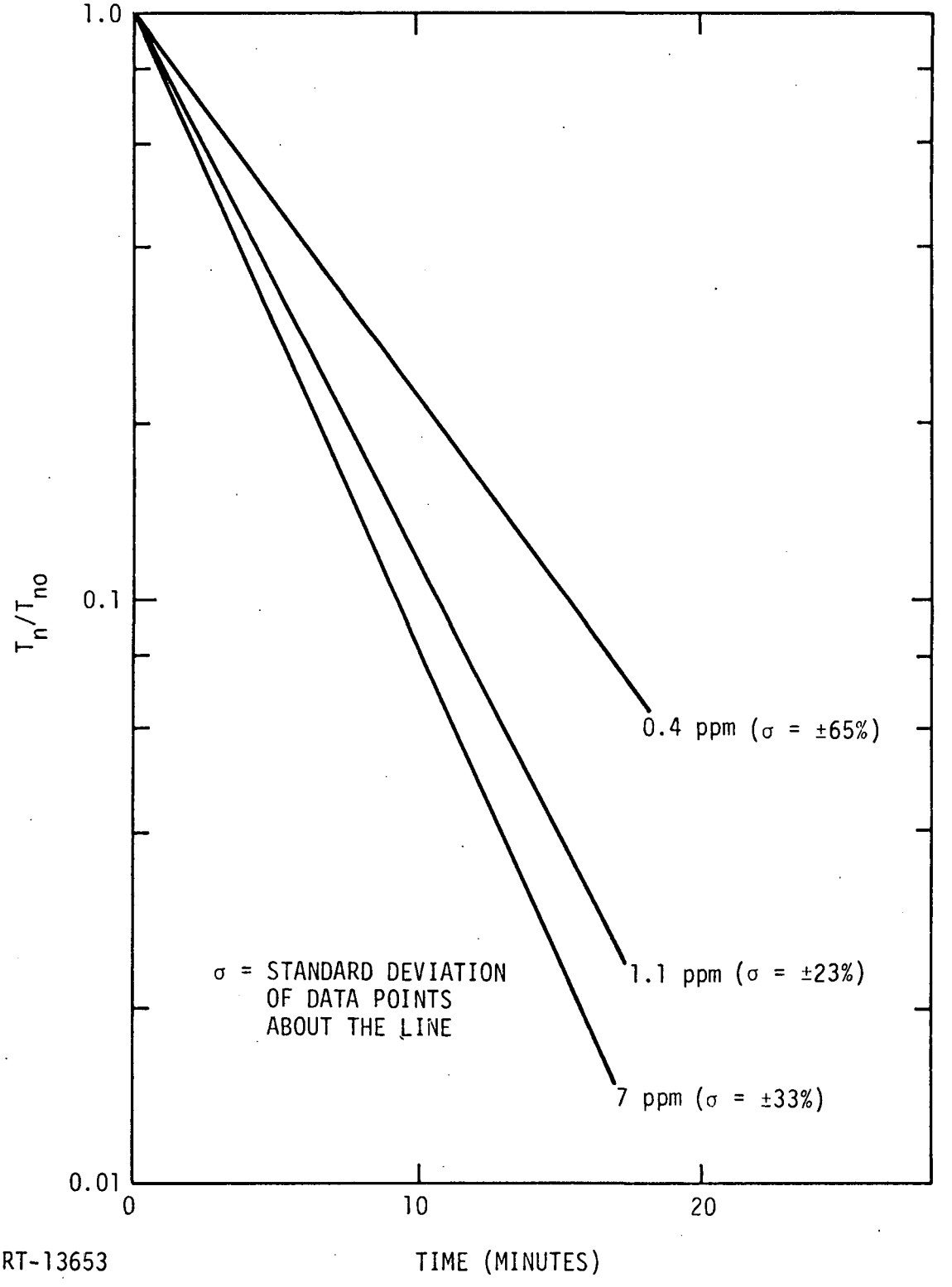

Figure 2. T_n/T_{no} of fresh AB-film versus BSA solution as a function of time and BSA concentration

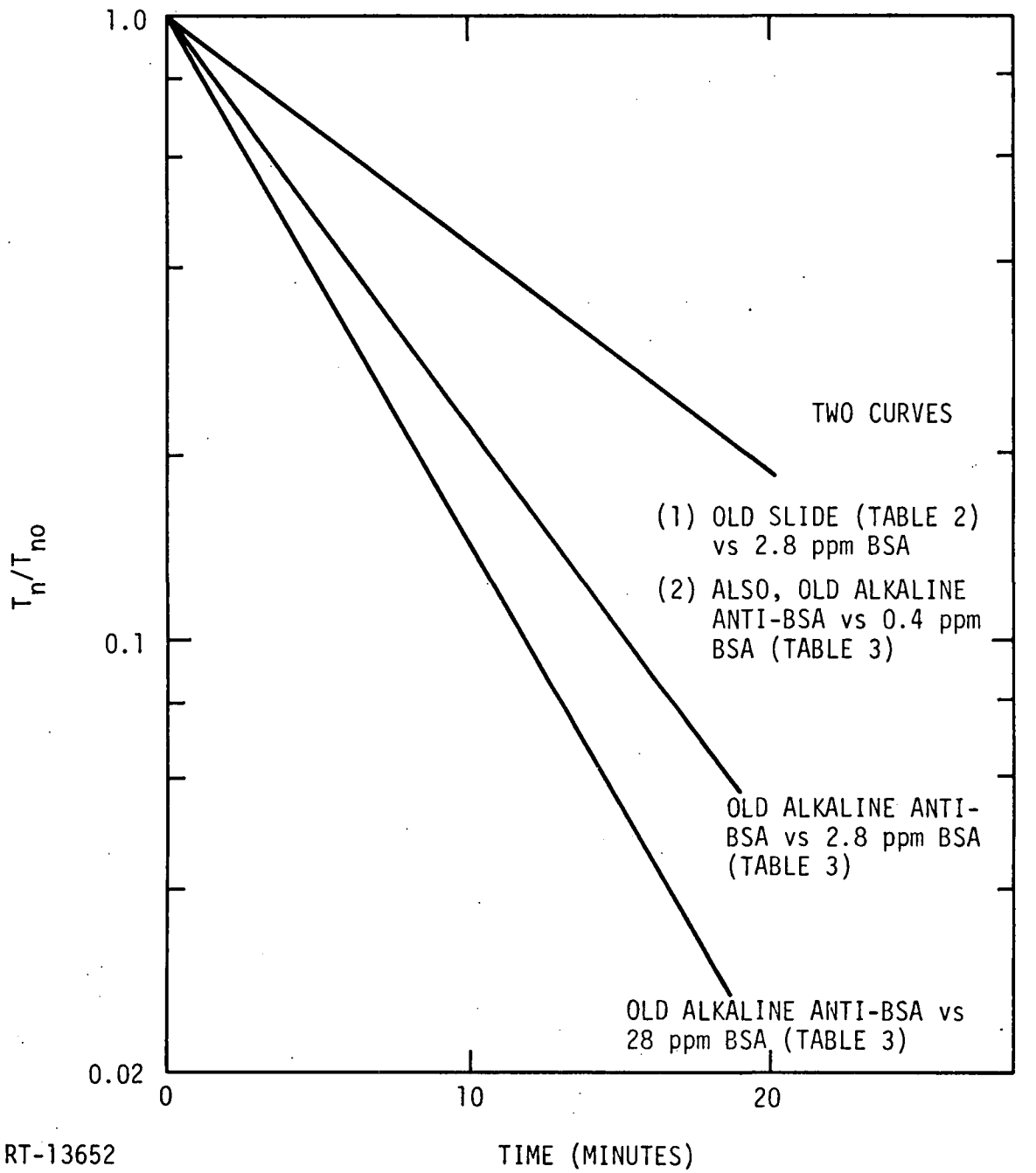

Figure 3. Influence of aging on the reaction between AB-film and BSA solutions

For the present purposes, a detailed understanding of the kinetics is not necessary. It is relatively more important that we now know a technique which is capable of detecting a substance at the sub-ppm level, and that the time required for the detection is short (under ten minutes). Also, the data indicate that it is best to prepare AB-films with fresh solution, and that such films tend to deteriorate if left exposed to air, light, and dust.

Although equivalent effort resulted in more progress with the polymeric system than the nickel system, it was less productive than was the case with the indium system. Nevertheless, the attachment of antibody to a diazotized polyaminostyrene surface has been demonstrated, and it appears to be a suitable method for preparation of a surface for subsequent reaction with antigen and labeled antigen. Unfortunately, the substrates tested to date are inherently fluorescent, and consequently a meaningful estimate of the true sensitivity of the system could not be made. The hope that pigmented polystyrene would be superior by virtue of elimination of all but surface fluorescence proved disappointing, apparently, by virtue of pigment fluorescence.

Inasmuch as styrene has a pale blue fluorescence, the fluorescence of pure polystyrene should have been blocked by the 5000 Å cutoff filter in the photon counter. The observed fluorescent background of the polystyrene discs, therefore, was probably due to impurities, which indicates that a pure polystyrene would be more suitable for the desired application.

Alternatively, since it was observed that a coating of anti-BSA reduced the measured fluorescence of a polystyrene disc (probably due to a decrease in transmittance) it is possible that polystyrene might serve as a substrate where a change in transmittance is measured as an indicator of hapten presence, as in the indium film experiments. The disadvantage of this system is that the change in transmittance is not easily discerned with the naked eye. The advantage would be that the polymer substrate is less fragile than the metal-on-glass substrate.

A major advantage of the polymer system operating in the fluorescence mode, and with a field instrument for measuring fluorescence, is that it is easily made quantitative via a calibration curve. The fluorescence intensity

is inversely proportional to the hapten in the sample, since the latter uses up antibody sites that would otherwise be available to the labeled hapten.

Based on the foregoing discussion, it appears that continued work on both systems would be profitable. The indium film system is almost fieldable in its present form, and can be implemented relatively quickly to give qualitative results. It will be desirable to develop a packaging system and define a storage system that will optimize stability and utility in the field. Once a suitable polymer substrate is located (or, if necessary, specially synthesized), the polymer system can be brought quickly to a point of providing quantitative analyses with a portable fluorometer.

With respect to the special synthesis of polymer, it is recommended that radiation polymerization be used, since it will obviate the need to use catalysts, which can enhance the fluorescence of the polymer.

It would also be of interest to attempt to increase the change in transmittance of the indium monitor on exposure to hapten. At the present time, hapten only effects a change of about 10%. Such an increase could be accomplished by optimizing deposition of antibody, whereby a greater fraction of molecules attach to the indium by their Fc ends rather than their Fab ends.

It is recognized that the failure to detect 25 ppb of 2-ABZI with the indium badge was probably due to the use of an unstirred solution where diffusion was rate limiting at such a low concentration. However, the indium badge approach was conceived of as being a simple, straightforward, inexpensive test that requires no special stirrers, instruments, or other field equipment; thus, the unstirred solution was more realistic in that context.

SECTION 7

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APPENDIX A

PHYSICAL BASIS FOR THE VISUAL OBSERVATION OF THE ANTIBODY-ANTIGEN REACTION

The ability of an antibody to bind selectively to its antigen with a high degree of specificity is well known. The ability of proteins, and in particular antibodies, to bind to metal surfaces is less well known, and of much more recent origin. Giaever (Ref. 3) has demonstrated that bovine serum albumin (BSA) can be made to bind to indium-plated glass slides, and that in turn anti-BSA can be made to bind to the BSA-coated slide.

The unique aspect of this discovery is that as the BSA is bound to the indium-plated glass slide it changes the light-scattering properties of the indium, and the indium looks darker. As the anti-BSA is bound to the BSA, the scattering properties change once more and the slide appears darker still.

The sensitivity of this technique has been demonstrated to be comparable to existing radioimmunoassay techniques (i.e., nanogram to picogram/mℓ range) it is simple to perform, and should be relatively inexpensive.

The observation by Giaever that protein absorption on an indium-coated slide causes the slide to darken can be understood in terms of the theory of light scattering by small particles, and as he points out, this theory is based on Maxwell's equations.

As Giaever recognized, the unique nature of a thin coating of indium on glass is that instead of forming a continuous coating, it deposits in the form of small droplets, typically 1000 Å across. (Other materials also deposit in this form, but usually the individual spheres coalesce to form a continuous film at much smaller thicknesses.) These particles are smaller than a wavelength of visible light, and consequently, the appearance of these films will be described by the theory of scattering from small particles.

Scattering from the uncoated indium is determined by noting that the skin depth of the indium at optical frequencies is negligibly small compared to the particle size. Hence, the particle may be approximated as having infinite conductivity. In this approximation, the spatial dependence of the scattered light intensity at point (r,θ) from a small particle is given by:

$$I(r,\theta) = \frac{(2\pi)^4 a^4 I_0}{r^2 \lambda^4} \left[\frac{5}{8}(1 + \cos^2\theta) - \cos\theta\right] ,$$

where I_0 is the incident light intensity, λ is the wavelength, and a is the particle radius. In this formula, θ is measured from the direction of the incident light. Here, we see that light is scattered preferentially in the backward direction since $[5/8(1+\cos^2\theta)-\cos\theta]$ is a maximum when $\theta = \pi$. Although interaction of the light scattered from an array of particles will change the quantitative form of this equation, the qualitative conclusion remains: the array of particles will be highly reflective, i.e., the film will look "metallic".

Next, we consider the effect of coating these particles with a nonconductor. Scattering from such a structure, a nonconducting layer on a conducting sphere, has apparently not been treated in the literature. However, for our present purposes, we can consider the limiting case of scattering from a nonconducting particle and recognize that the observed scattering will approach this value as the coating becomes thicker. Under the same conditions as above, the scattered intensity from a dielectric particle is:

$$I(r,\theta) = \frac{2(2\pi)^4 a^6 I_0 |\tilde{n} - 1|^2}{9 r^2 \lambda^4} (1 + \cos^2\theta) \; G\left(\frac{4\pi a}{\lambda} \sin\frac{1}{2}\theta\right) ,$$

where

$$G(u) = \frac{9\pi^{\frac{1}{2}}}{2u^3} J_{3/2}(u)$$

$J_{3/2}$ is the Bessel function of the first kind, of order 3/2. From this expression, we can determine that the scattering is approximately isotropic for $a/\lambda << 1$, with the backscattered intensity slightly less than the forward intensity. For increased a/λ, the forward intensity grows at the expense of the backscattered intensity.

Consequently, it can be concluded that a dielectric coating on the indium spheres will reduce the intensity of the reflected light, i.e., the surface will darken. A second layer will further reduce the backscattered light, yielding additional darkening. In addition to these effects, due entirely to the angular dependence of the scattering, additional darkening would be produced if the dielectric layer was absorbing at optical frequencies. This would be manifest in the above formula through a reduction in $I(r,\theta)$ produced by a nonzero imaginary part of $\tilde{n}$.

APPENDIX B

THE ANTIBODY MOLECULE

Although there are several kinds of antibody molecules, the most prolific one is immunoglobulin G (IgG), which has a molecular weight of ∿140,000. It is commonly depicted as comprised of four parallel chains of protein, two of which are heavy chains (molecular weight of ∿50,000 each) joined together by a disulfide bond, and two of which are light chains (molecular weight of ∿20,000 each) joined one each to a heavy chain by disulfide bonds, as shown in Figure B-1. Also shown in Figure B-1 are the dominant end groups, COOH and NH_2, and the plane of cleavage with papain digestion that splits IgG into a crystalline (Fc) portion and an antigen-binding (Fab) portion.

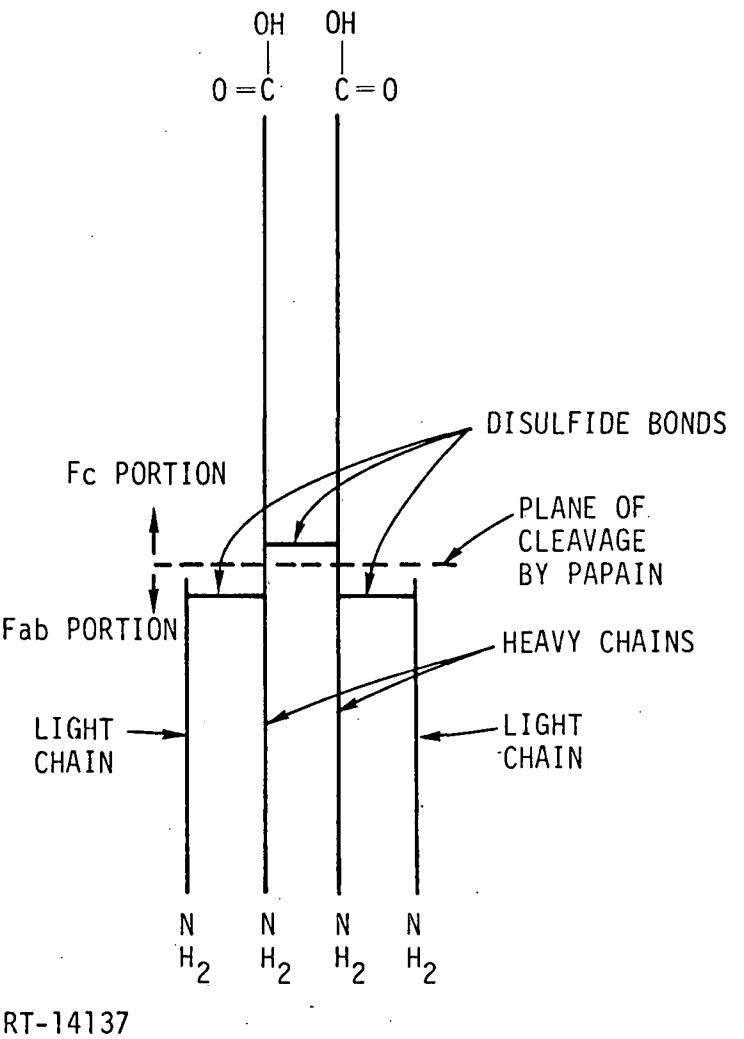

Figure B-1. Schematic of IgG structure

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16. ABSTRACT

A solid substrate "film-badge" type monitor has been developed that is capable of detecting 2-aminobenzimidazole (2-ABZI) at less than one part per million in water in less than 10 minutes. The monitor makes use of the reaction which takes place between 2-ABZI in the sample and a monolayer of its antibody that has been deposited on a thin film of indium on a glass substrate.

A second approach in which the antibody is mounted on polystyrene and reaction of its antigen-binding sites with a fluorescein-labeled antigen are subject to competition with nonlabeled antigen in the sample, has been demonstrated in principle. Improvements in this alternate approach are proposed.

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