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A FLUORESCENCE IMMUNOASSAY TECHNIQUE FOR DETECTING ORGANIC ENVIRONMENTAL CONTAMINANTS

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A FLUORESCENCE IMMUNOASSAY TECHNIQUE FOR DETECTING ORGANIC ENVIRONMENTAL CONTAMINANTS

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ABSTRACT

This report describes the development and successful demonstration of an immunological assay for the detection of low molecular weight organic contaminants of environmental concern.

The specific technique described is a fluorescence polarization immunoassay, the theory of which is presented.

The preparation of the two required reagents, namely a fluorescent conjugate of the contaminant of interest, together with an antibody to the contaminant, is described in detail.

The specific contaminant chosen for this study was 2-aminobenzimidazole (MW = 133), a metabolite of certain fungicide agents used in agriculture. The particular fluorescent moiety chosen to form the conjugate with 2-aminobenzimidazole was fluorescein.

A successful demonstration of the assay has been accomplished, and a detection sensitivity in the sub-nanogram/mℓ range obtained.

A high degree of specificity of the antibody for the hapten has been demonstrated, and a successful quantitative recovery from an unknown solution has been obtained.

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SECTION I

CONCLUSIONS

The fluorescence polarization immunoassay (FPI) technique has been demonstrated to be highly sensitive and specific for the detection of 2-aminobenzimidazole (2-ABZI) in aqueous solutions. As a result of this successful demonstration, the following major conclusions can be drawn.

- Immunological techniques can be applied to the detection of low molecular weight organic contaminants in aqueous solutions.
- A high degree of specificity of the contaminant antibody for the contaminant can be obtained, as evidenced by the difference in initial rate and equilibrium polarization values for 2-ABZI and benzimidazole.
- The detection sensitivity (see Section V) is in the sub-nanogram/mℓ range, which compares favorably with other available analytical techniques (Ref. 1).
- The assay can be conducted using initial rate data in one to two minutes by non-professional staff, which makes it particularly valuable for the economic screening of large numbers of samples.

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SECTION II

RECOMMENDATIONS

In order to broaden the usefulness of this method and bring it to the stage of a practical analytical technique, a number of additional studies and developments are recommended. The usefulness of the technique as presently applied to the detection of a single organic contaminant could be improved by:

- Development of a wide band fluorescence polarimeter capable of being operated in the field.
- Use of fluorescent conjugates with fluorescent wavelengths different from fluorescein (e.g., indocyanine green) to avoid natural competing fluorescences which occur in host samples of interest, e.g., blood serum and tissue.
- A study of the stability of the reagents, i.e., antibody and fluorescent conjugate, under normal operating environmental conditions, with recommendations for improving this stability where necessary.

The demonstration of the ability of immunological techniques to detect low molecular weight organic contaminants lays the groundwork for the development of a rapid, sensitive, and inexpensive technique for environmental monitoring, e.g., reentry monitoring. This involves the deposition of contaminant antibody on a solid substrate such as glass, plastic, or paper, and monitoring of the physical characteristic of the surface to detect the presence of the contaminant.

There are basically two techniques by which this can be accomplished, and it is recommended that both be studied to determine the optimum and most cost-effective system. First, deposition of a metallic surface (indium or nickel, for example) on a glass substrate, bonding of the antibody to the metallic surface, and observation of the change in reflectivity which occurs in the presence of the contaminant. Second, deposition of the antibody on a plastic or cellulose substrate, exposure of the monitor to

a fluorescent-labeled form of the contaminant, and then a study of the inhibition of the bonding of this fluorescent conjugate by a sample thought to contain the contaminant of interest.

Finally, the application of the technique to multi-residue analyses using contaminants labeled with different fluorescent wavelength conjugates, would reduce the time and effort involved in multi-residue, multi-sample analyses.

SECTION III

INTRODUCTION

BACKGROUND

Environmental pollution is currently creating extremely serious problems on a global scale. One facet of this pollution involves the ever-widening agricultural use of chemicals such as pesticides, fertilizers, and similar substances. In addition, the industrial use of organic compounds is increasing at an alarming rate. An understanding of the gravity of the situation can be obtained when it is noted that the annual production of pesticides in the United States alone exceeds one billion pounds per year (Ref. 2). Repeated consumption of foodstuffs containing small quantities of these substances can pose the threat of direct accumulative toxicity. They can also seriously affect the ecological balance and produce insidious disruptions of the food cycle. This results from the tendency of organisms in a food chain to sequentially concentrate many of the nonbiodegradable chemicals in tissue as they feed on organisms containing only minute concentrations of them. This phenomenon is called "biological magnification" (Refs. 3,4).

Further, the spreading nature of this problem is indicated by the fact that species which exist far from civilization also appear to be contaminated with such pesticide residues. For example, eggs of the Bermuda Petrel, which spends most of its life ranging the open sea at great distances from applications of DDT, have been found to contain up to 5.1 ppm of DDE residues (Ref. 4). Such accumulations of these nonbiodegradable toxic chemicals could also readily involve man as he consumes foods below him in the eco-system which are so tainted.

The impact of the problems associated with the use of synthetic organic compounds is one which affects all members of society on a world-wide basis irrespective of geographic, socio-economic, or ethnic background.

Individuals come into direct contact with such compounds in industrial processes involving, for example, plastics, waxes, optical brighteners, and agricultural chemicals. Indirect contact evolves through absorption of these contaminants into the human food chain. To determine the impact of these contaminants on the environment, it is necessary to determine their final distribution and concentration. It is clear, then, that sensitive and precise detection and measuring systems are required to monitor the levels of these contaminants in the environment initially — for example, in ocean waters, reservoirs, and streams, and finally in the tissues of various living organisms.

The classical methods used in monitoring have been previously summarized (Ref. 1), and it is beyond the scope of this paper to discuss them. Of all methods, gas chromatography-mass spectrometry has achieved the most notable success in the detection of pesticides at the residue level. In an effort to improve upon the sensitivity and provide a considerably simpler technique capable of field operation, the use of an immunochemical technique has been investigated. The use of such a technique has been prompted by the extraordinary successes that have been recently achieved in the measurement of biological substances by specific immunological reagents and techniques (Ref. 5). For example, as little as 10^{-9} mg/mℓ of several pituitary hormones can be directly detected by such approaches. This level is equivalent to 0.001 ppb. Available evidence indicates that specific antibodies can be obtained against many organic compounds (Ref. 6). In particular, antibodies have been obtained against DDT and malathion (Refs. 7,8), and other pesticide residues (Ref. 1). The availability of a specific antibody against the structure or molecule to be detected immediately opens up the possibility of using immunochemical methods as a highly specific and sensitive (Ref. 5) detection technique.

Any means of applying an immunochemical reaction to a detection problem ultimately relies upon a reaction occurring between a substance (antigen or hapten) and its specific antibody. Perhaps the most general means by which this interaction can be employed in measurement and detection

has come to be known as "competitive binding assay". In principle, this method requires two essential reagents. These are a labeled form of the substance to be detected or measured, and an antibody or receptor specifically directed against the substance. The principle of the assay involves a preliminary measurement of the binding of the labeled antigen (substance being detected) with its antibody and then, a determination of the extent of the inhibition of this binding by known quantities of the unlabeled antigen, which corresponds to the unknown. From these data, a standard curve can be constructed which shows the degree of binding by the labeled antigen under certain specified conditions as a function of concentration of the unlabeled antigen or unknown added.

FUNDAMENTALS OF THE IMMUNOASSAY TECHNIQUE

Immunology as a subject is logically concerned with the immunity of living organisms to harmful agents, regardless of their origin, and includes resistance to disease, hypersensitive reactions as exhibited by allergic persons, and tolerance and rejection of foreign tissue such as those encountered in organ transplants. In this case, however, a more restricted view of the definition has been taken and has been applied to the mechanisms and techniques involved in the detection and measurement of organic contaminants of environmental interest.

A substance, which when injected into an animal stimulates the animal to produce antisera capable of reacting with it in a highly specific manner, is referred to as an antigen, and the specific protein produced is referred to as an antibody. These antibodies belong to a group of serum proteins known as immunoglobulins. The production of these antibodies as a result of the injection of the antigen takes place over a period of many weeks, and depends upon the immunization schedule. In general, "good" antigens are usually of large molecular size (>40,000) partially digestible by enzymes and are recognized as being foreign by the antibody-producing animal. It is immediately obvious, of course, that many compounds of environmental concern do not have a large molecular weight and would, therefore, appear to be incapable of stimulating antibody formation.

Fortunately this is not the case, and so-called partial antigens or haptens can be produced and are capable of reacting with specific antibody. Haptens or partial antigens are defined as antigens which alone cannot induce antibody formation, but in conjugation with a suitable carrier can produce antibody against themselves, as well as against the carrier-hapten complex. Examples of such carriers include ovalbumin, bovine serum albumin, fibrinogen, and many others. In summary, the hapten once conjugated with a suitable carrier can stimulate antibody production. The remarkable thing about this antibody stimulation is that some antibody will be produced which is highly specific in its reaction with the hapten alone. It is this phenomenon which allows the use of immunological techniques in the detection and quantitation of organic contaminants of relatively low molecular weight, and under a variety of practical circumstances.

REVIEW OF IMMUNOASSAY TECHNIQUES

The usual method of labeling the antigen to be identified in an immunoassay requires the introduction of a radioactive label. When such a radiolabel is used, an essential and crucial step in the radio-immunoassay (RIA) is to separate physically that portion of the labeled antigen which is bound to the antibody from that which is unbound or free. Only in this way is it possible, by radioactive counting, to determine what fraction of the radiolabel remains bound, or is being bound, in the presence of the unknown.

Alternatively, a direct way of implementing competitive binding principles in an immunoassay is to employ a fluorescent label which allows the assay to be carried out in principle, either by fluorescence polarization measurements, or in some cases by fluorescence intensity measurements. Unlike RIA, no separation of the bound and free forms of the labeled antigen is necessary, since a simple, rapid optical measurement gives the essential information without physical separation.

Examples of other radioassays include radioreceptor assay, which employs a partially purified tissue receptor as the active reagent, and immunoradiometric assay, which uses radioactive labeled antibody. In the latter case,

separation of bound and unbound antibodies is accomplished by attachment to the antigen which is deposited on a solid substrate. Radioenzymatic assays involve enzymes as the reactive agent. Once again, a separation procedure is required. In this case, separation of the radioactive pools requires separation of two compounds, one formed from the other by the enzyme catalyzed reaction. These techniques, however, tend to be inferior to RIA and FPI in terms of sensitivity and specificity (Ref. 1).

APPLICATION OF IMMUNOASSAY TECHNIQUES IN PESTICIDE RESIDUE METHODOLOGY

The application of immunological techniques to the analysis of pesticide residue levels appears to be of relatively recent origin, and Ercegovitch (Ref. 1) in 1971 gave an excellent review of work in this area. This work appears to be limited to research carried out on the herbicide aminotriazole and parathion with sensitivities in the microgram range, and by Centeno (Ref. 7) and Haas and Guardia (Ref. 8) using a tanned cell hemiglutination inhibition test with detection limits of 0.1 μg and 1.0 μg for DDT and malathion, respectively.

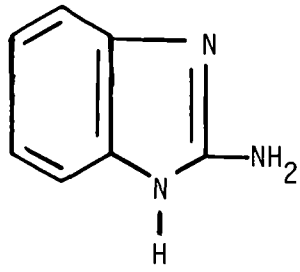
APPLICATION OF FLUORESCENCE POLARIZATION TO IMMUNOASSAY TECHNIQUES (FPI)

The essential feature of applying this phenomenon to an immunoassay consists in first labeling the antigen molecule with a fluorescent moiety and then observing the degree of polarization of the fluorescent light, or in certain cases, the intensity of fluorescent light measured, when standard quantities of the labeled antigen and antibody together with the unknown are allowed to interact. The dependence of polarization (and also occasionally, the fluorescent intensity) upon the extent of reaction between the antigen and antibody forms the basis for the quantitation and immunoassay. Reaction between the antigen and antibody results in an increase in size of the kinetic unit and in a retardation of the rotary brownian motion, which in turn is manifested by an increase in the polarization of fluorescence. In the presence of unlabeled antigen in the sample, a smaller percentage of the labeled antigen is bound to the antibody and in this

circumstance the polarization observed will be lower. Hence, the standard immunoassay curve, which can be constructed from this type of data, would show the polarization of fluorescence for certain standard chosen experimental conditions plotted as a function of the amount of unlabeled antigen.

OBJECTIVE

The objective of this program was to demonstrate the feasibility of using the technique of fluorescence polarization immunoassay for the detection of low molecular weight contaminants. The structure of 2-aminobenzimidazole (2-ABZI), chosen as a model compound for this demonstration, is shown below.



Molecular weight ∿133

While this compound alone does not find extensive use in either agriculture or industry, it may be found as the result of the use of a number of agricultural chemicals. For example, the degradation of certain fungicides results in 2-ABZI. Consequently, the demonstration of a successful fluorescence polarization immunoassay for this compound would serve a twofold purpose, namely the capability of immunological techniques to assay for low molecular weight compounds, and the sensitivity and specificity of the assay technique to a compound of environmental interest. The scope of work for the program was as follows.

TASK 1: From a list of available pesticides or metabolic derivatives selected by the Contracting Officer, choose by analysis or experiment, a single product that can be labeled with a fluorescent dye and can be used to demonstrate the feasibility of a fluorescence polarization immunoassay.

TASK 2: Prepare a fluorescent sample of one selected pesticide or metabolic derivative using chemical and/or free radical induced labeling techniques.

TASK 3: Prepare immunogens from the selected pesticide or metabolic derivative and immunize animals with the immunogen to obtain antibody.

TASK 4: The binding affinity of the fluorescent labeled compound developed under Task 2 for the antibodies developed under Task 3 will be determined, and a standard immunoassay curve for the pesticide or derivative will be constructed.

TASK 5: Recovery experiments will be performed on known quantities of the selected compound, added to natural water and one other medium specified by the Contracting Officer.

SECTION IV

METHODS

PREPARATION OF REAGENTS REQUIRED FOR THE ASSAY OF ENVIRONMENTAL CONTAMINANTS

Of the techniques available for the synthesis of the fluorescent derivatives, two have been considered. First, classical organic syntheses, and second, free radical labeling. The organic synthesis adopted clearly depends on the structure of the contaminant itself, and will vary widely. Alternately, a mixture of the substance to be labeled, together with the fluorescent dye, can be irradiated. The multiplicity of free radicals formed during the irradiation then affords a mixture of compounds, some of which will generally be fluorescent-labeled derivatives (Ref. 9).

Preparation of an antibody against a contaminant begins by coupling it to a highly immunogenic molecule such as ovalbumin, and introducing the complex into an animal, for example rabbits, by means of intradermal injections. The initial immunization yields "primary response" antibodies, which are usually of fairly low specificity. Booster immunizations can be given at eight-week intervals and secondary response antibody collected ten days after the booster. This is generally more specific and of higher titer.

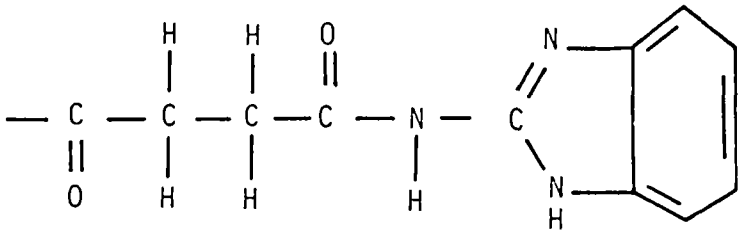
PREPARATION OF FLUORESCENT DERIVATIVE OF 2-ABZI

Several organic syntheses were carried out in an effort to prepare a fluorescent derivative of 2-ABZI. Although several of the syntheses proved to be unsuccessful, they will be described for the purposes of completeness.

CLASSICAL ORGANIC SYNTHESES

The initial synthesis effort was to prepare the conjugate Fluorescein-NH-H_1, where H_1 is a hapten derived from 2-ABZI via reaction at the 2-position of 2-ABZI. Fluorescein amine was used in the preparation of this conjugate.

H_1 is prepared by reacting 2-ABZI with succinic anhydride. The cyclized product opens up on reacting with amines. H_1 is then of the form:



, and when conjugated

forms Fluorescein-NH-H_1.

It was determined that this 2-position derivative of 2-ABZI was the succinate salt compound $(2\text{-ABZIH}^+)_2\text{-}C_2H_4O_4^+$, and consequently, alternate syntheses were initiated.

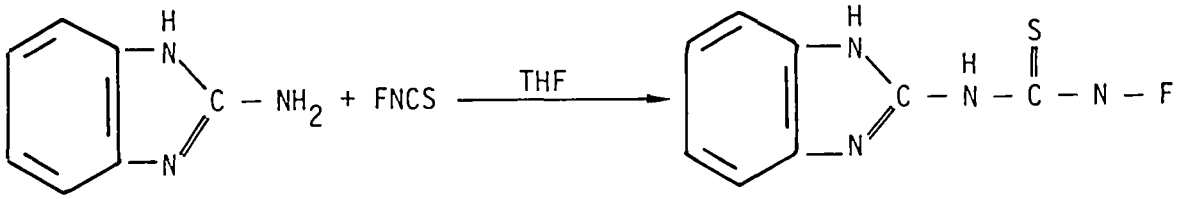
Based upon the consideration that conjugation at the number two position of 2-ABZI may result in a weaker immunogen than might be the case for conjugation at alternative positions, separate attempts were made to conjugate at the one-position and five-position of 2-ABZI. In each case the effort was to prepare a butyric acid derivative, which could then be coupled to either ovalbumin or fluorescein as desired.

Treatment of 2-ABZI with 4-chlorobutyric acid in a direct attempt to prepare a one-position derivative resulted in a viscous, oily product that was isolated by chromatography, but would not crystallize. An indirect attempt to prepare the compound by first preparing the ethylester of the butyric acid side chain was made. This intermediate, ethyl-γ-[1-2 aminobenzimidazol] butyrate, was crystalized (m.p. 95 to 97°). The proton magnetic resonance spectrum of the compound is consistent with the desired 1-position derivative of 2-ABZI.

Preparation of a 5-position derivative was started with γ-phenyl-butyric acid, which was nitrated at the para-position. The nitro group was reduced to the amino-group with zinc, and then acetylated with acetic anhydride.

The meta-position was then nitrated, and the p-amino m-nitro phenyl butyric acid intermediate product was purified prior to reducing the nitro group and cyclization across the resulting adjacent amino groups to obtain the desired product 2-ABZI with a butyric acid size chain at the 5-position.

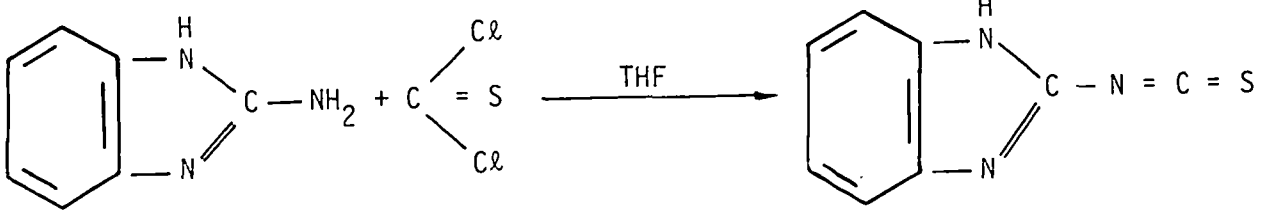
In addition, a further attempt to product a conjugate at the 2-position was made as follows. 2-ABZI and fluorescein isothiocyanate (FNCS) were reacted to produce a conjugate with fluorescein coupled to the 2-position of 2-ABZI, by the following reaction.



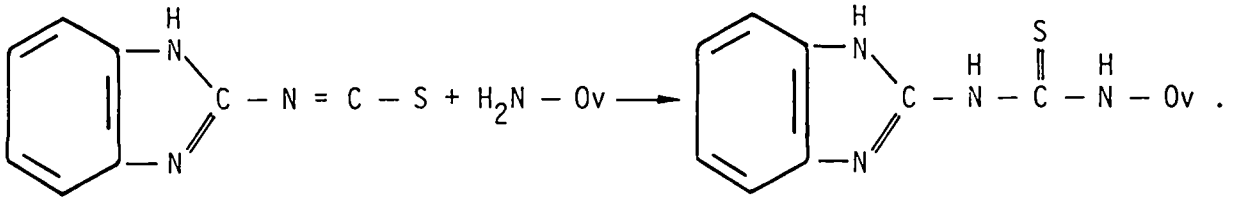
The 2-FNCS-ABZI mixture was subjected to separation by a two-step, thin-layer chromatography procedure. Firstly, on silica gel with 15% methanol in ethyl acetate until the faster moving unreacted dye was separated from the labeled product. The latter was removed from the silica gel with methanol and separated on cellulose TLC using borate buffer (pH 8.8). The fluorescent zone adjacent to the leading point contained the desired product, which was removed with methanol. Since reactivity was successfully attained with this conjugate at the 2-position, no further work was undertaken on the 1- and 5-position syntheses.

PREPARATION OF 2-ABZI PROTEIN CONJUGATE

Although numerous protein carriers can be used in the preparation of a successful immunogen, in this case the studies have been limited to ovalbumin, since this carrier has been used successfully in much of our earlier work. The preparation of immunogen was carried out by first reacting tetrahydrofuran solutions of 2-ABZI and thiophosgene to produce the isothiocyanate of 2-ABZI, which precipitated:



The precipitate was filtered, washed, and air dried. An 18-mg aliquot was dissolved in 1 mℓ of dimethylformamide and slowly added to an 8 mℓ, 0.15 M saline solution of 500 mg of ovalbumin. The solution was stirred overnight, dialyzed, and freeze dried to give a yellow-tan product. Spectral analysis (U.V.) indicated the product was a conjugate of 2-ABZI and ovalbumin in the mole ratio of ∿2:1. This indicated that the reaction of 2-FNCS-ABZI with amino groups of the ovalbumin led to a successful conjugation:



This will be designated 2-Ov-ABZI.

The ovalbumin conjugate was mixed 1:1 with Freund's complete adjuvant and 2-mg portions were injected into two sites of each of three rabbits (female New Zealand Whites). After eight weeks, the rabbits were boosted and then bled 10 days later (15 mℓ per rabbit); the blood was allowed to clot, and the serum separated by centrifugation. The globulins were separated from the serum by precipitation with $(NH_4)_2SO_4$ (at 0.38 saturated ammonium sulfate), followed by centrifugation. The globulin fraction was solubilized to its original serum concentration in 0.15 M saline solution for testing by FPI.

RADIATION SYNTHESIS

Mixtures of 2-ABZI and fluorescein solutions, and mixtures of 2-ABZI and ovalbumin solutions have been subjected to 17 megarads of bremsstrahlung (E_{max} = 10 MeV) from an electron linear accelerator, to examine the use of ionizing radiation chemistry in synthesizing both the immunogen with 2-ABZI hapten and the fluorescein conjugate with 2-ABZI.

A dose of 17 megarads is equivalent to the deposition of 1.1×10^{21} eV/mℓ of solution. Assuming that about one molecule of desired product is formed for every 10^3 eV deposited (G-product ≈ 0.1), this would produce 1.1×10^{18} molecules, or ~ 1.8 micromoles of product per mℓ of solution. This would amount to about 1 mg of 2-ABZI-fluorescein, and about 91 mg of 2-ABZI-ovalbumin per mℓ.

Since the radiation chemistry process gives mistures of all possible products, the method seldom permits direct synthesis of a pure product. The 2-ABZI and fluorescein mixtures contained much more than 91 mg of product (visible as highly colored precipitate, for example), and some separation of the desired product was necessary.

These irradiated solutions proved to be too heavily irradiated (17 megarads resulted in the denaturation of the ovalbumin). Accordingly, new solutions of 2-ABZI and ovalbumin, and 2-ABZI and fluorescein, were prepared and irradiated. These solutions were given 3.3 megarads of electron (10-MeV) irradiation. However, since success was obtained with the chemical syntheses, the workup of these irradiated conjugates was discontinued in order to concentrate on the development of the immunoassay. The practicality of the radiation synthesis approach, however, has been demonstrated on a parallel program (Ref. 10).

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SECTION V

RESULTS

DETECTION SENSITIVITY

With the successful production of antibody to 2-ABZI, and also a fluorescent conjugate, a series of preliminary experiments were conducted to estimate the detection sensitivity. In these experiments, varying concentrations of 2-ABZI were incubated with the globulin aliquot (which contains the antibody), labeled 2-ABZI was added, and the polarization recorded as a function of time. The results of these experiments are shown in Figures 1 and 2. The initial rate of polarization was diminished in proportion to the logarithm of the 2-ABZI concentration, i.e., effectively the kinetics are first order, which is demonstrated by the following data points, which are shown in graphical form in Figure 3.

Log (picograms of 2-ABZI per mℓ)	Initial Polarization Rate[a] $(dp/dt)_o$ (units of polarization/min)
----	67.0[b]
3.64	32.4
4.12	19.0
4.64	12.6
5.12	3.9

[a] Initial rate x 10^4.

[b] Basic reaction of 1 μg 2-FNCS-ABZI with 200 μg of globulins in 3 mℓ of solution.

Least squares fitting of these data points gives the regression line:

$$Y = 98.79 - 18.68X.$$

The observed data points show a standard deviation of 16%, relative, about the regression line. The value of $(dp/dt)_o$ in the absence of 2-ABZI

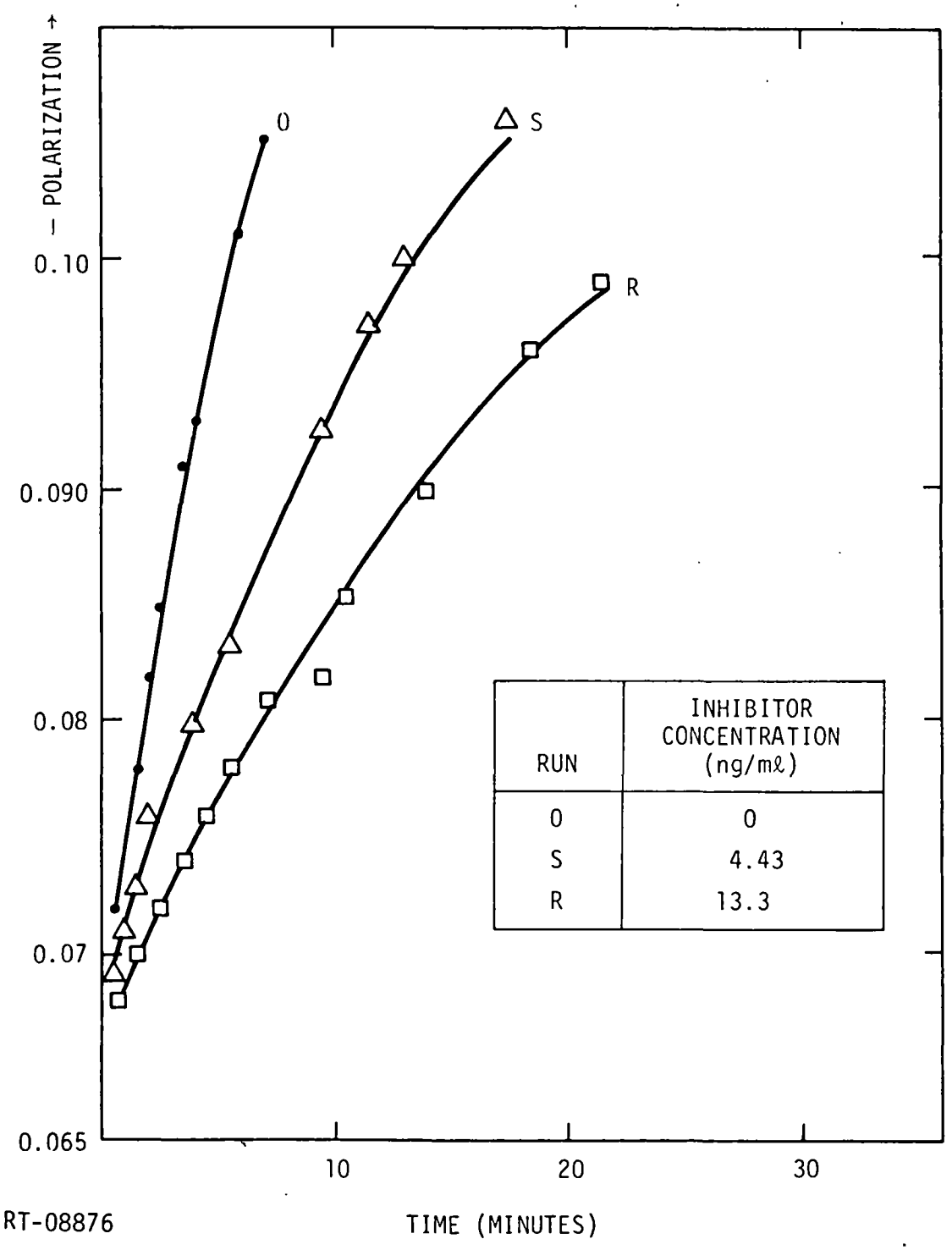


Figure 1. Rate of Change of Polarization as a Function of Inhibitor Concentration

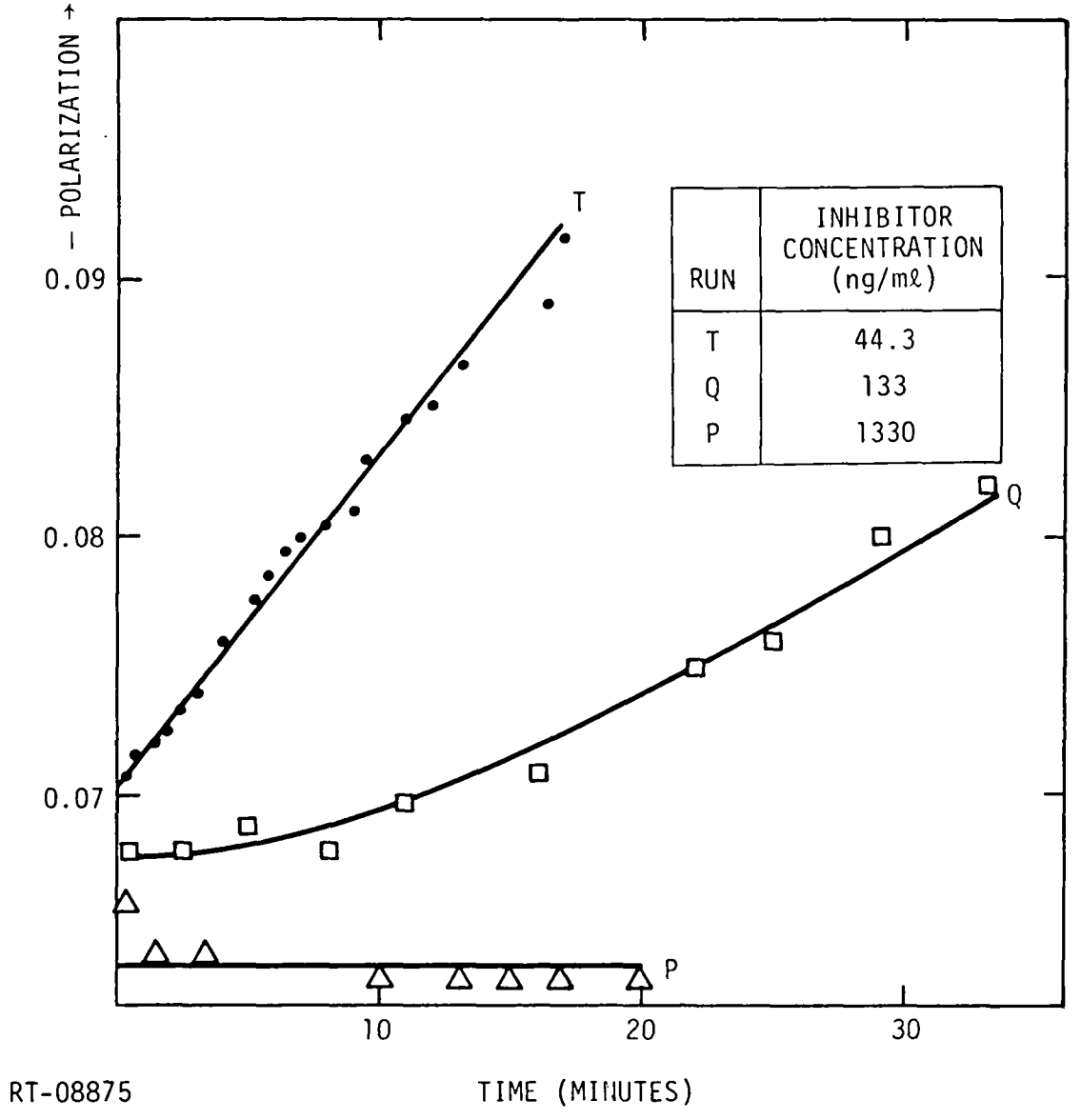


Figure 2. Rate of Change of Polarization as a Function of Inhibitor Concentration

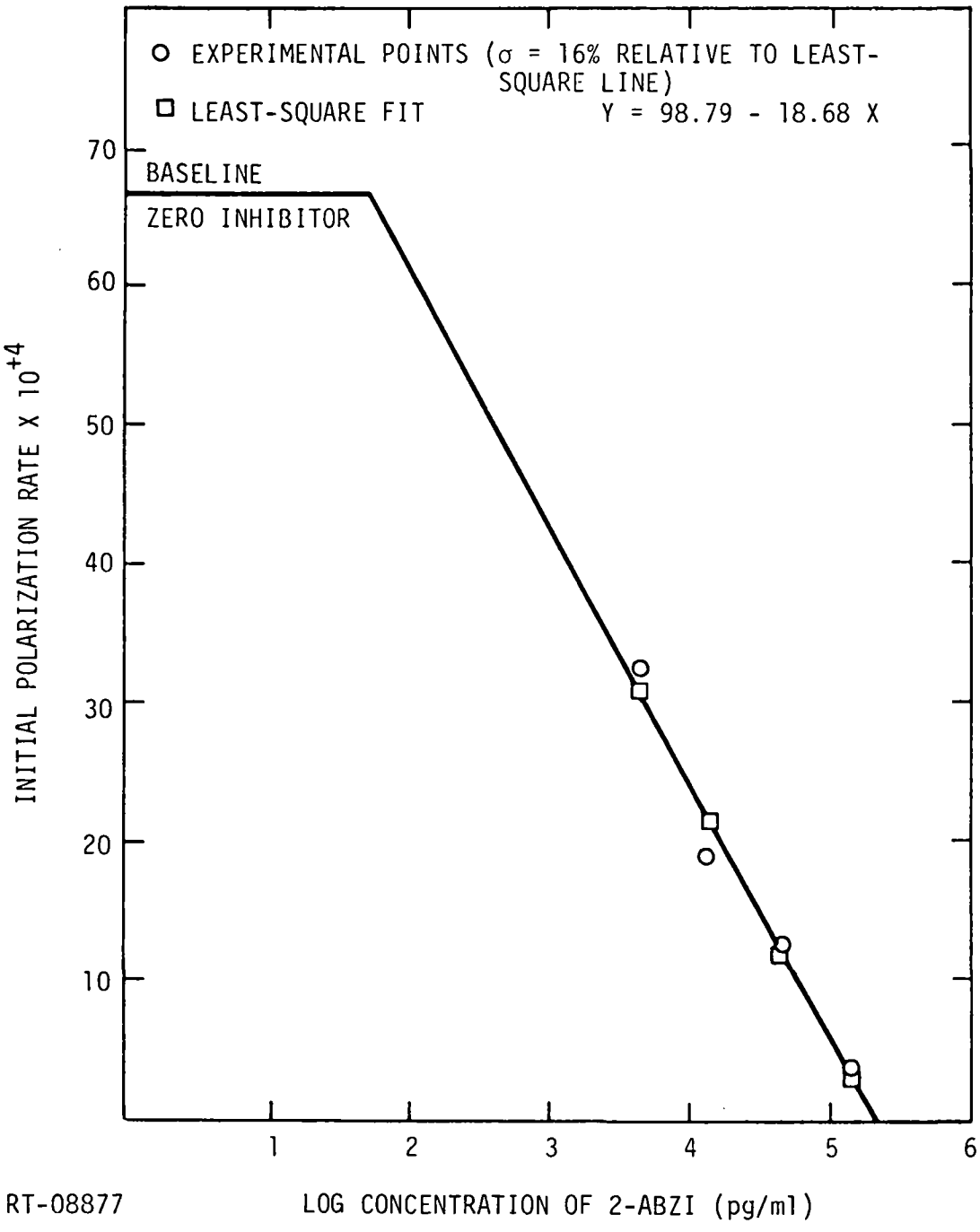


Figure 3. Standard Inhibition Curve

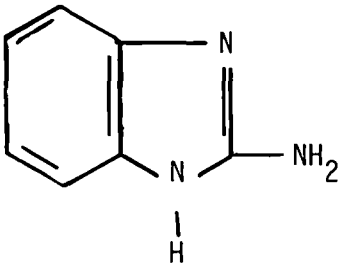
(67×10^{-4} in the foregoing tabulation) is intercepted by the regression line at X = 1.7; i.e., when the concentration of 2-ABZI is 50 picograms per mℓ. This level cannot be detected under the described conditions, since this concentration cannot be differentiated from zero inhibitor concentration, i.e., this concentration corresponds to the "noise" of the system. However, changes in the initial rate of polarization of 3×10^{-4} units are readily observable. Thus, a rate of change of 64×10^{-4} units can be differentiated from the noise level. From Figure 3 this can be seen to give a log concentration of 2-ABZI (pg/mℓ) of approximately 2, which corresponds to a minimum detection sensitivity for 2-ABZI of 100 pg/mℓ.

SPECIFICITY

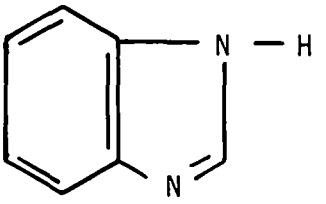
In devising an immunoassay, the degree of specificity attained is a prime factor in determining the ultimate usefulness of the test. No one parameter has ever been universally adopted as a measure of immunological specificity, but one of the best would seem to be the binding affinity between antibody and the antigen or hapten. The binding affinity is a reasonable measure of specificity, since cross-reacting antigens or haptens usually show lower affinities than that of the structure contained in the immunizing antigen. The physical factors contributing to the binding affinity finally reduce to the number and strengths of all the atomic interactions between the antigenic determinant group and the antibody combining site. The nature of these interactions includes hydrogen bonding, electrostatic attraction, hydrophobic bonding, and a variety of weaker dispersion forces. The better the match between groups on antigen and antibody, the greater will be the free energy of interaction when the two molecules combine, and the greater the "specificity".

To estimate the specificity of this assay, inhibition experiments involving 2-ABZI and benzimidazole were conducted. The structure of these two compounds and their respective molecular weights are as follows.

2-Aminobenzimidazole (MW = 133)



Benzimidazole (MW = 118)



In these experiments antibody (200 µL) was added to 3 mℓ of buffer, and then 3 µL of benzimidazole or 2-ABZI was added to achieve a 10^{-5} M solution of the respective benzimidazole compound. The fluorescence polarization was read at timed intervals after 50 µL of the 2-ABZI fluorescent conjugate was introduced.

The resulting data are shown in Table 1, and in graphical form in Figure 4. The results for the case in which no inhibitor has been added is also given.

The significant difference between both the initial rate of change of polarization and the equilibrium value of the polarization for the case of inhibition by 2-ABZI and benzimidazole demonstrates the high degree of specificity of the antibody for its hapten.

RECOVERY EXPERIMENT

The objective of this recovery experiment was to demonstrate the ability of the fluorescence polarization technique to determine on an absolute basis the presence of 2-ABZI in an unknown sample.

For these measurements, new reagents were prepared. Firstly, fresh tris buffer at pH 8.0 and 0.01M concentration was prepared and filtered through 0.22µ millipore filter to remove any microorganisms. Next, fluorescein-tagged, 2-aminobenzimidazole (FABZI) was prepared by reacting fluorescein isothiocyanate with 2-ABZI in tetrahydrofuran, and purified by sequential TLC procedures, as described in Section IV.

Table 1. RELATIVE INHIBITION PRODUCED BY BENZIMIDAZOLE AND 2-ABZI

2-FNCS-ABZI No Inhibitor			2-FNCS-ABZI vs 2-ABZI Inhibitor			2-FNCS-ABZI vs Benzimidazole Inhibitor		
Time (min)	$p^{(a)}$	$p-p_o$	Time (min)	$p^{(a)}$	$p-p_o$	Time (min)	$p^{(a)}$	$p-p_o$
(0)	(0.076)	0	(0)	(0.076)	0	(0)	(0.084)	0
0.28	0.0815	0.0055	0.30	0.076	0	0.30	0.086	0.002
0.38	0.086	0.010	0.55	0.076	0	0.48	0.088	0.004
0.75	0.0895	0.0135	1.0	0.074	-0.002	0.70	0.0895	0.0055
1.00	0.095	0.019	2.0	0.074	-0.002	1.0	0.0905	0.0065
1.58	0.100	0.024	3.0	0.074	-0.002	1.65	0.095	0.011
2.5	0.105	0.029	5.0	0.0775	0.0015	2.70	0.101	0.017
4.0	0.118	0.042	7.0	0.0775	0.0015	4.0	0.107	0.023
5.0	0.125	0.049	10.0	0.076	0	5.0	0.110	0.026

(a) p at time zero, p_o, obtained by extrapolation of observed data.

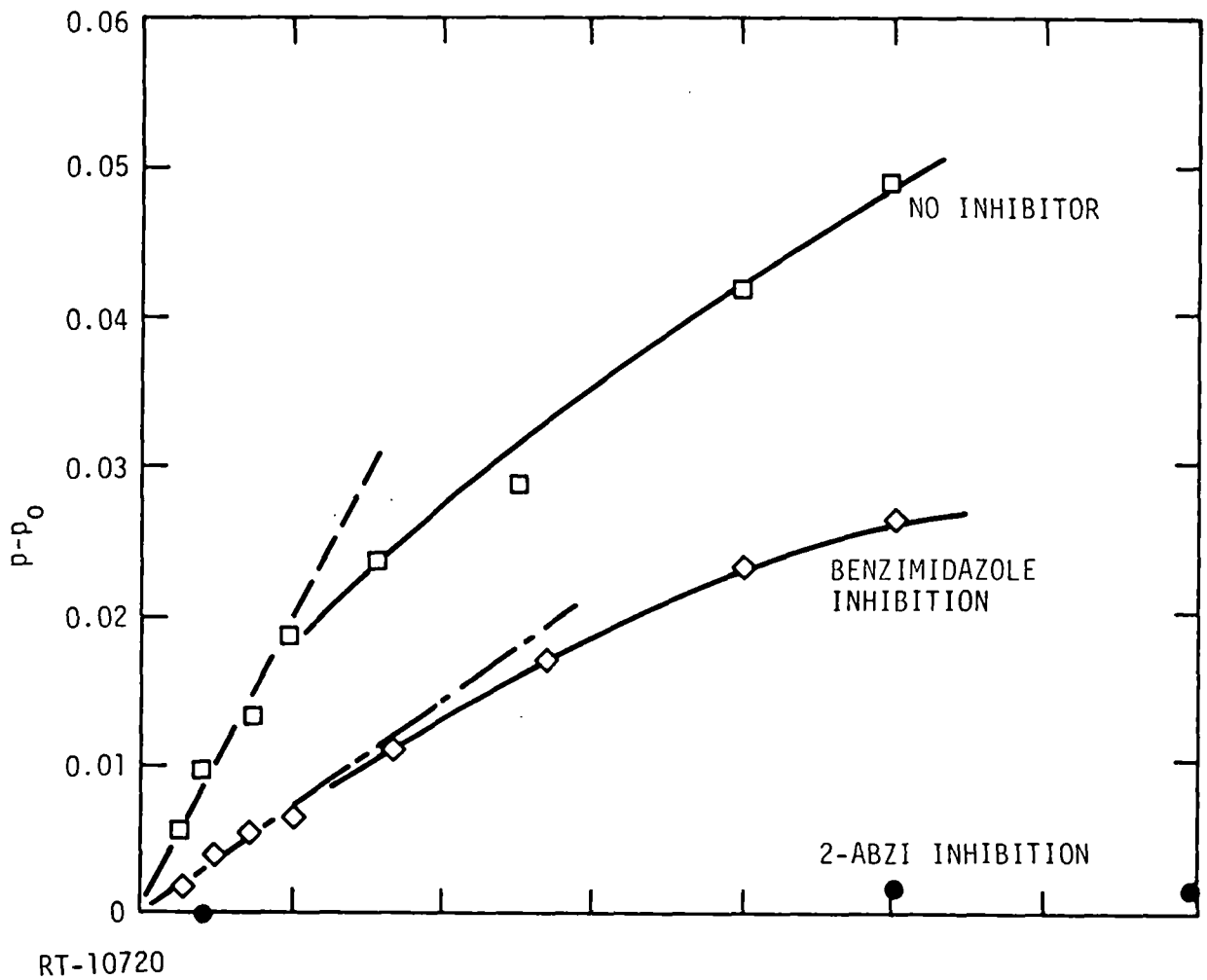


Figure 4. Comparative Inhibition: Comparison Between 2-Aminobenzimidazole and Benzimidazole

Previously unused immunoglobulin preparation, which had been frozen immediately after preparation, was then thawed. This preparation, obtained from rabbits innoculated and boosted, was at normal serum concentration in the thawed preparation.

A set of inhibition curves was then developed for varying concentrations of inhibitor. All polarization measurements were made after mixing the reactants in 3.0 mℓ of tris buffer. The resulting curves are shown in Figure 5. The initial rate of change of polarization for the four different levels of inhibitor concentration shown, were as follows.

Log (picograms of 2-ABZI per mℓ)	Initial Polarization Rate[a] $(dp/dt)_o$ (units of polarization/min)
3.60	227
3.12	433
2.60	617
2.12	842

[a] Initial rate x 10^4.

These data points are shown in graph form in Figure 6. Analyzing these four points by least squares gives the following linear relationship.

$$\text{Log Inhibitor Concentration (pg/m}\ell) = -0.00247\ dp/dt_o + 4.171.$$

Two solutions were then prepared containing known but different quantities of 2-ABZI.

The change in polarization as a function of time for these two unknowns, containing the antibody, tris buffer, and 1 μℓ of fluorescent-labeled 2-aminobenzimidazole was then recorded. The results are shown in graphical form in Figures 7 and 8.

The estimated initial rate of change from these two curves is 730 x 10^{-4} and 670 x 10^{-4}, which from Figure 6 corresponds to 272 pg/mℓ and 347 pg/mℓ, respectively. The quantities added to these unknowns corresponded to 270 pg/mℓ and 330 pg/mℓ.

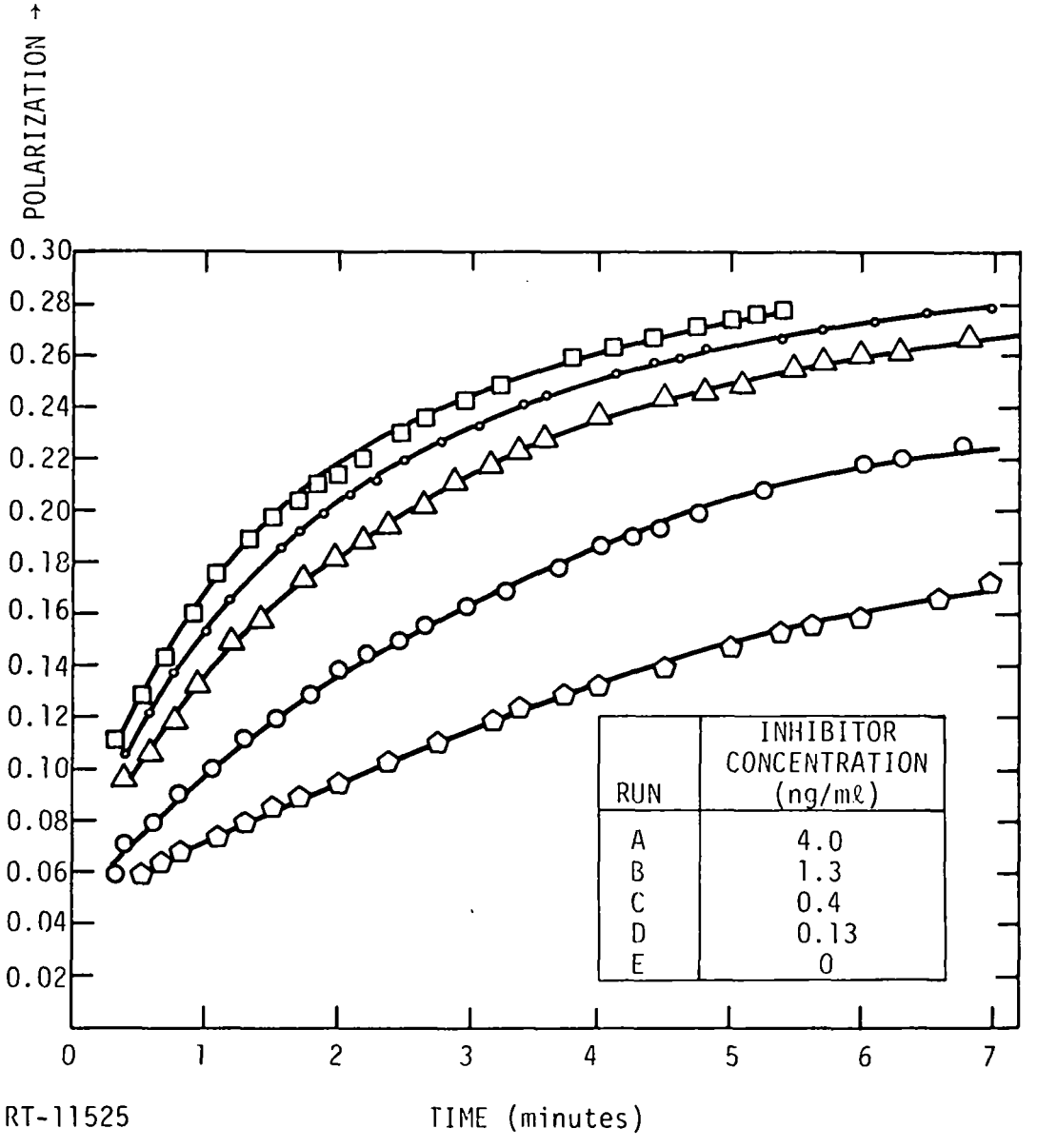


Figure 5. Rate of Change of Polarization as a Function of Inhibitor Concentration

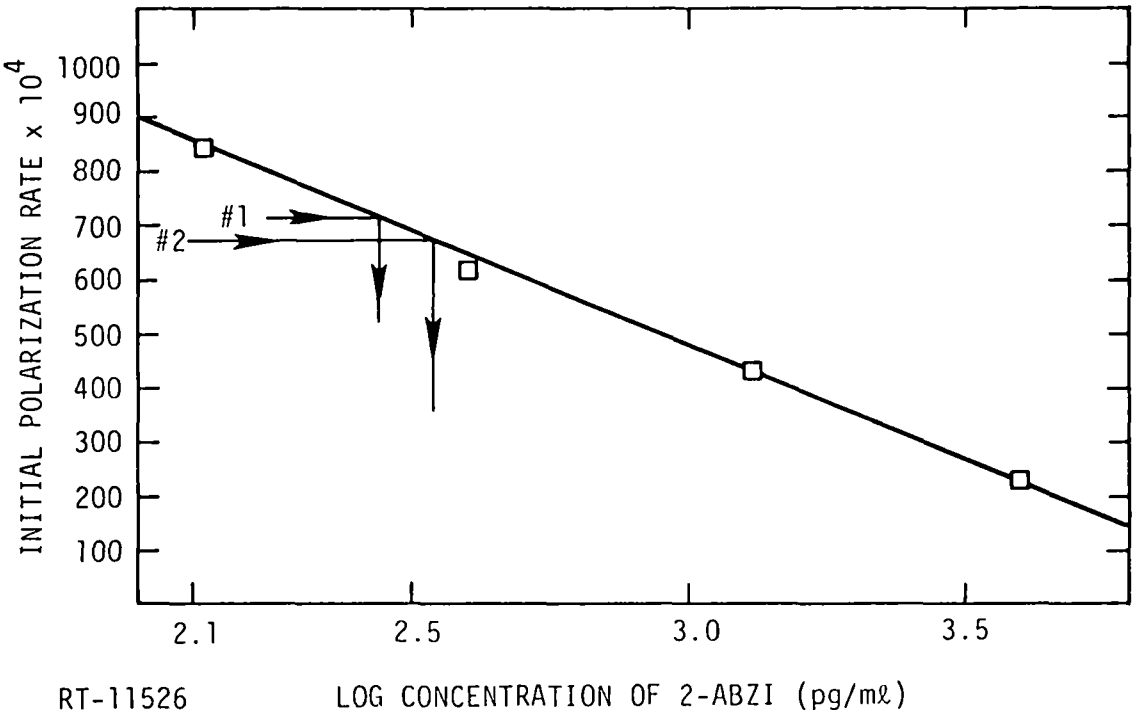


Figure 6. Standard Inhibition Curve

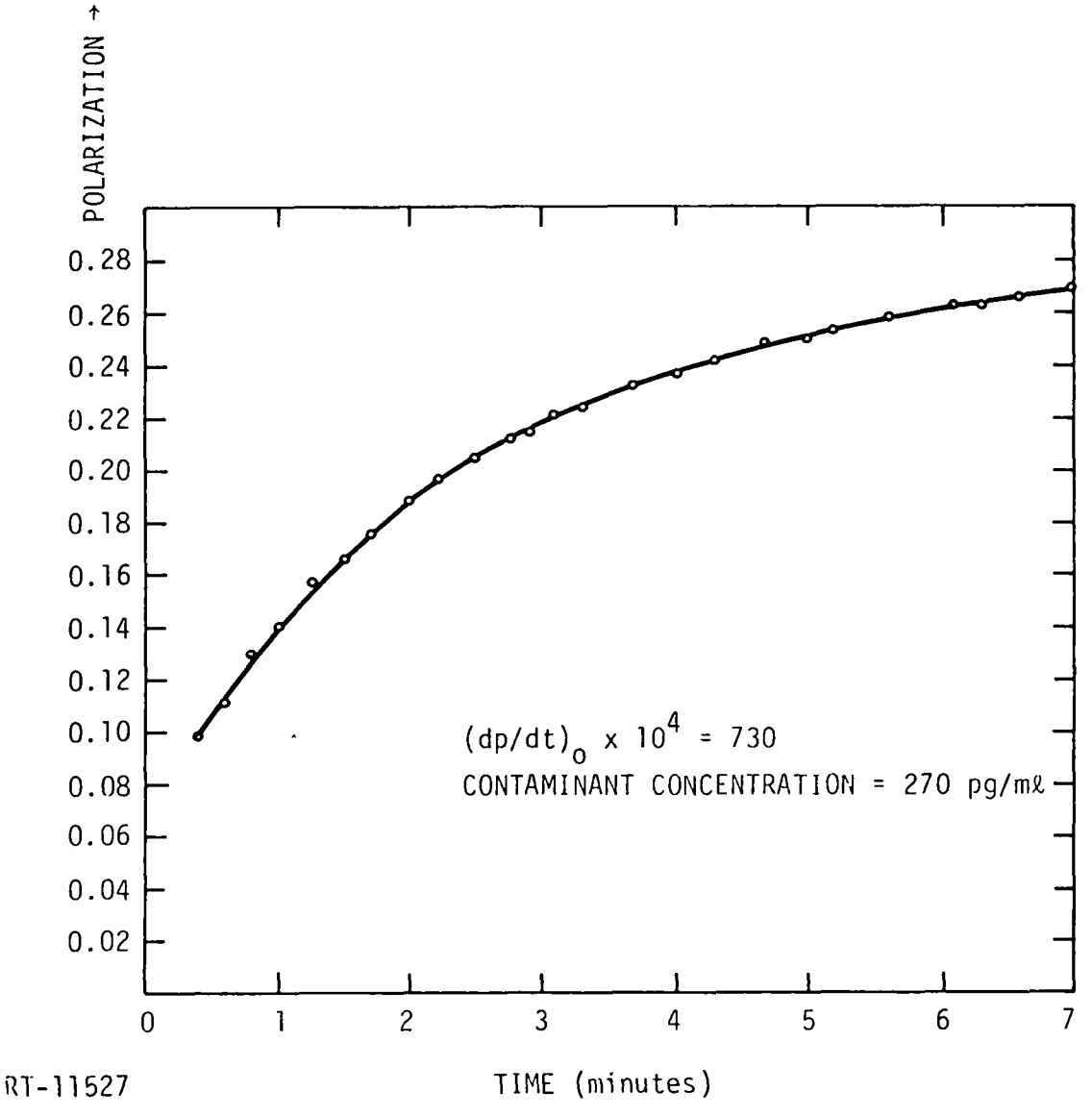


Figure 7. Polarization as a Function of Time for Unknown Sample No. 1

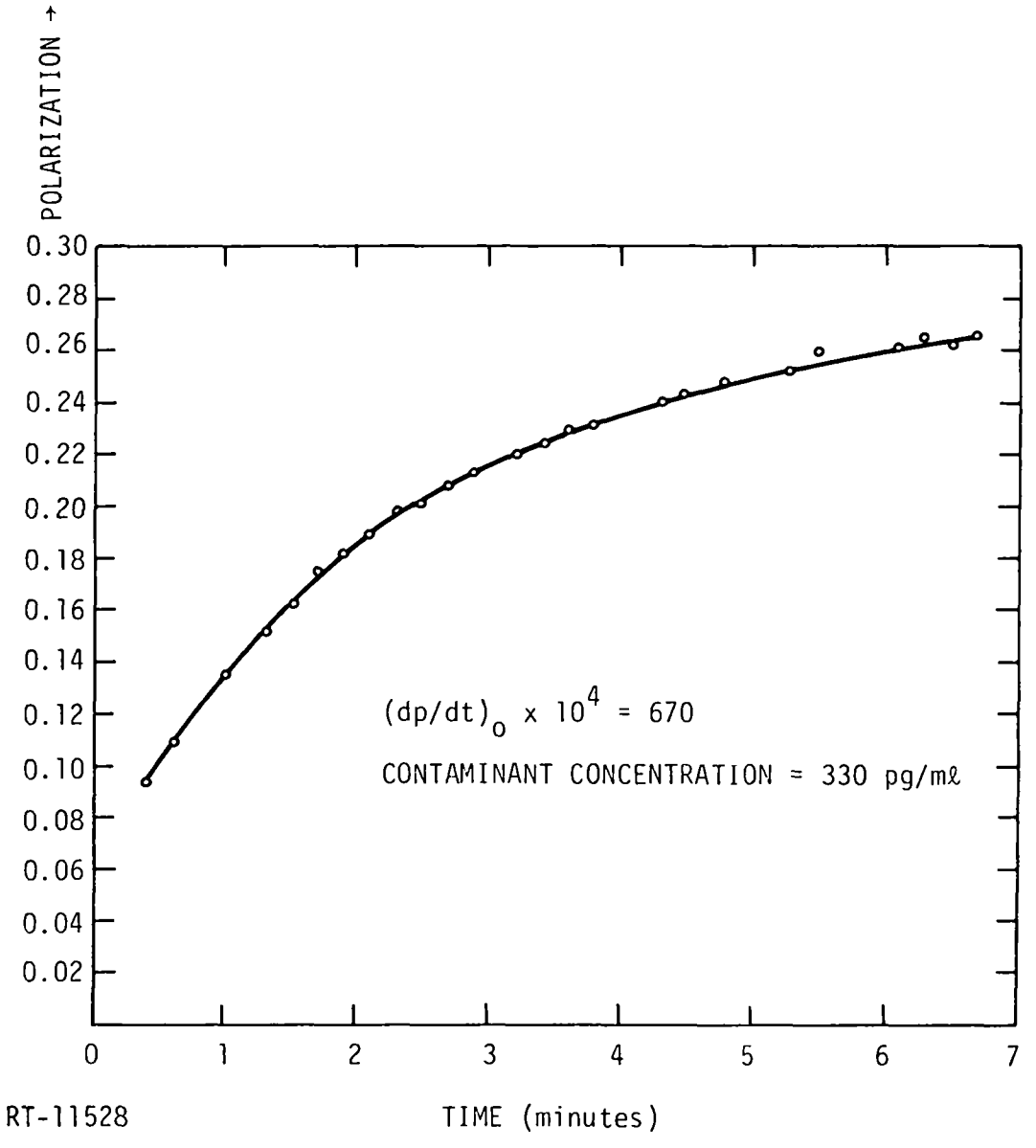


Figure 8. Polarization as a Function of Time for Unknown Sample No. 2

This demonstrates the ability of the technique to determine subnanogram quantities of 2-ABZI in an unknown sample to approximately 5% of the true value for the worst case analyzed in this experiment.

SECTION VI

DISCUSSION OF RESULTS

It is of interest to compare the FPI system for 2-ABZI to that of a typical case, as represented by dinitrophenol (DNP) (Ref. 11) and its antibody, and to the optimum system evaluated to date, i.e., fluorescein and its antibody (Refs. 12,13). The relevant parameters for these systems are tabulated below.

Parameter	DNP (Ref. 11)	2-ABZI	Fluorescein (Refs. 12,13)
Concentration of unbound binding sites[a] - R	10^{-5} M[b]	2.4×10^{-8} M[b]	?
Heterogenity Factor[c] - a	0.6	0.72	1.0
Average association constant - K_o	4.5×10^{7}	1.4×10^{10}	1×10^{11}
Second order rate constant - k	5×10^{7}/M-sec	2.5×10^{6}/M-sec	4×10^{8}/M-sec
Detection limit	10^{-7} M	10^{-9} M	10^{-11} M

[a] Antibody from boosted animals. In the case of fluorescein, very late antibody was used.

[b] At normal serum concentration.

[c] An inverse proportion is implied between a and the variety of binding sites.

Comparison of the three systems tabulated above shows that the FPI system for 2-ABZI performs better than average, but not as well as for the best test system evaluated to date, namely, fluorescein.

Despite the fact that fluorescein is a considerably larger and more complex molecule than 2-ABZI, there is reason to believe that the FPI system for the latter could be brought to perform nearly as well as the fluorescein system. In particular, it seems likely that affixing carrier protein to

one of the positions of the 6-membered ring of 2-ABZI would provide for greater participation of the unique triple amine constellation in antibody formation relative to the present case.

The present 2-ABZI-Ov conjugate presents the hapten with the 6-membered ring foremost in a manner that apparently minimizes the uniqueness of the molecule as it appears to the antibody-forming system. Both the small production of antibody, which suggests that less than the usual number of lymphocytes recognized the antigen as a foreign entity, and the comparatively small rate constant, k, which suggests a relatively large steric factor in the antibody-hapten reaction, are consistent with this view. It has been shown that the FPI method can be applied to the measurement of 2-ABZI with great sensitivity and specificity. The reaction between the hapten and its antibody, as prepared in the present program, has been defined in terms of order, equilibrium constant, antibody heterogeneity, and rate constant.

SECTION VII

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APPENDIX 1

THE PHYSICAL BASIS OF FLUORESCENCE POLARIZATION IMMUNOASSAY

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THE PHYSICAL BASIS OF FLUORESCENCE POLARIZATION IMMUNOASSAY

To adequately understand the basic principles of fluorescence polarization in an immunoassay, some basic discussion of the polarization phenomenon itself must be presented. In classical terms, the emission from a single molecule may be regarded as radiation from a single oscillating dipole; this radiation has an oscillating electric field parallel to the direction of oscillation of the dipole and is said to be polarized in the same direction. Now, if a randomly oriented assembly of molecules is excited by fully polarized light, their fluorescence is only "partially" polarized (partially polarized light may be thought of as being a mixture of polarized and unpolarized light), even if the molecules are prevented from rotary brownian motion in solution, as may be seen by the following consideration.

For simplicity, assume that the direction of the absorption and emission oscillators in a single molecule are the same and that they are rigidly fixed with respect to the geometric axis of the molecule. Furthermore, assume the molecule is to be rigidly fixed in position during the interval between absorption and emission (typically 10^{-8} second). The probability of absorption of light is proportional to the square of the magnitude of the component of the electric vector of the exciting light in the direction of the oscillator. This probability is proportional to $\cos^2\theta$ (see Figure A1-1), where θ is the angle between the incident field E which is parallel to the Z axis, and the direction of the absorption oscillator. Because the probability of absorption falls off as θ increases, molecules oriented so that θ is small are preferentially excited, while those with large θ have little chance of absorbing. Since the absorption and emission oscillators are parallel, the emitted light will be partially polarized with a degree of polarization P. This quantity is defined (Ref. A1-1) in terms of the intensities I polarized either parallel or perpendicular to the incident electric field, and is given by

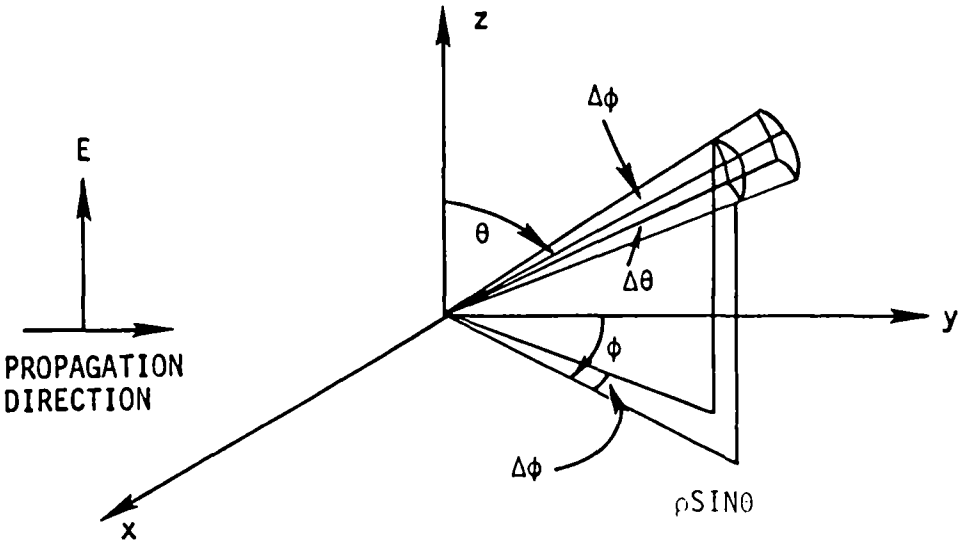

Figure A1-1. Polarization Coordinate System

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad .$$

In Figure A1-1 the area of one face of the elemental volume is equal to

$$(\rho \sin\theta \Delta\phi)\ (\rho \Delta\theta) \quad .$$

The elemental solid angle is then given by

$$\frac{\rho^2 \sin\theta d\phi d\theta}{\rho^2}$$

$$= \sin\theta d\theta d\phi \quad .$$

If the molecules in the medium are distributed randomly, the number of oscillators excited in a given elemental solid angle must be proportional to the solid angle and the probability of absorption of light by such an oscillator.

However, since the probability of absorption of light is proportional to the square of the magnitude of the component of the electric vector of the exciting light in the direction of the oscillator, i.e., $\propto \cos^2\theta$, then, the number of oscillators excited in a given elemental solid angle is

$$(\cos^2\theta \sin\theta\ d\theta\ d\phi) \quad .$$

If the radiating oscillators have the same direction as the absorbing oscillators, then the amplitudes of the electric vector, parallel to the excitation vector and observed from the XY plane, i.e., $\theta = 90°$, is:

$$(1)(\cos^2\theta \sin\theta\ d\theta\ d\phi) \cos\theta$$

and the intensity which is proportional to the square of the amplitude, is given by

$$\cos^2\theta \sin\theta \, d\theta \, d\phi \, (\cos\theta)^2 \, (1)^2$$

$$= \cos^4\theta \sin\theta \, d\theta \, d\phi \quad ,$$

integration over a sphere gives

$$I_{||} = \int_0^{2\pi} \int_0^{\pi/2} \cos^4\theta \sin\theta \, d\theta \, d\phi \quad .$$

The intensity of vibrations perpendicular to the excitation vector is computed similarly as follows. The number of oscillators excited in a given elemental solid angle is as before:

$$(\cos^2\theta \sin\theta \, d\theta \, d\phi) \quad .$$

The amplitude of the electric vector perpendicular to the excitation vector and observed in the direction $\theta = 90°$ is

$$(1)(\cos^2\theta \sin\theta \, d\theta \, d\phi) \sin\theta \cos\phi \quad ,$$

and the intensity is given by

$$\cos^2\theta \sin\theta \, d\theta \, d\phi \sin^2\theta \cos^2\phi$$

$$= \cos^2\theta \sin^3\theta \cos^2\phi \, d\theta \, d\phi \quad .$$

Again, integration over a sphere gives

$$I_{||} = \int_0^{2\pi} \int_0^{\pi/2} \cos^2\phi \cos^2\theta \sin^3\theta \, d\theta \, d\phi \quad .$$

Evaluation of these two integrals gives

$$I_{\parallel} = \frac{2\pi}{5} \quad \text{and} \quad I_{\perp} = \frac{2\pi}{15} \quad .$$

Now if the degree of polarization is defined by

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

then,

$$P = \frac{1}{2} \quad .$$

Thus, integration over the angles θ and ϕ (Figure A1-1) shows that the maximum value of P observed in the XY plane with linearly polarized light is one half.

Now, if the molecules are subject to rotary brownian motion instead of being rigidly fixed, then the molecular rotation taking place between the time of absorption and emission may be expected to result in values of P lying between one half and zero. The extent of this rotation is a function of molecular dimensions and structure, solvent and temperature. Low molecular weight compounds, such as inorganic ions, will give rise to virtually completely depolarized fluorescence. Some polarization will be retained as molecular size increases and considering two molecules of equal size the fluorescence of the more asymmetric, rigid structure will be more highly polarized. If there is considerable internal flexibility within a molecule, very little polarization may be retained, because the fluorescent label then may assume a wide range of positions within the 10^{-8}-sec lifetime of the excited state, even if the entire structure does not rotate significantly as a unit.

REFERENCES

A1-1. Feofilov, P. P., The Physical Basis of Polarized Emission. Consultants Bureau, New York (1961).

APPENDIX 2

EQUILIBRIUM EXPERIMENTS

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EQUILIBRIUM EXPERIMENTS

In order to provide a complete set of data concerning the kinetics of the antibody-hapten reaction, a series of experiments to determine the equilibrium constant and the order of the reaction have been undertaken, and are reported in this Appendix.

Definition of the symbols used in the following discussion are:

I = net fluorescence intensity

H = net intensity of horizontally polarized component of fluorescence[a]

V = net intensity of vertically polarized component of fluorescence[a]

F = total molar concentration of 2-FNCS-ABZI in solution

Q = molar fluorescence, I/F

p = fluorescence polarization

R = molar concentration of unbound antibody[b]

f as a subscript denotes that unbound 2-FNCS-ABZI is involved

b as a subscript denotes that 2-FNCS-ABZI bound to antibody is involved.

---

[a]The vertical is perpendicular to the plane defined by the direction of observation and the exciting light.

[b]Actually it is the concentration of unbound binding sites.

In addition, identities that prove useful in the analysis are given in terms of the foregoing symbols by:

$$I = V+H = QF;\ I_f = V_f+H_f = Q_fF_f;\ I_b = V_b+H_b = Q_bF_b\ ,$$

$$V = V_f+V_b$$

$$H = H_f+H_b$$

$$F = F_f+F_b$$

$$p = \frac{V-H}{V+H}\ .$$

The basic equations that are used can be stated with the foregoing symbology and identified as follows.

$$\frac{I_b}{I_f} = \frac{p-p_f}{P_b-p} \tag{1}$$

$$\frac{F_b}{F_f} = \frac{Q}{Q_b}\left(\frac{p-p_f}{P_b-p}\right) \tag{2}$$

$$F_b = \frac{(F_b/F_f)F}{(F_b/F_f)+1} \tag{3}$$

The final polarization values at equilibrium obtained between various amounts of 2-FNCS-ABZI and antibody aliquots were measured to obtain titration curves. This was done for three different amounts of antibody, and the data are given in Table A2-1 and plotted in Figure A2-1. It can be seen that the titration curves intersect the polarization axis at a maximum polarization value of 0.328, which represents the polarization, P_b, of 2-FNCS-ABZI that is bound to antibody. Separate measurements of the fluor-tagged hapten in the absence of antibody gave a polarization value, p_f, of 0.045.

Comparison of 2-FNCS-ABZI fluorescence intensity with that of known amounts of fluorescein showed that 4 μL of the tagged hapten added to the 3.2 mℓ of buffer in the instrument cell gave a concentration of 2.5×10^{-10} M. Thus, the conversion of aliquot size to a concentration value in Table A2-1 is straightforward. In addition, it was observed that there was no change in fluorescent intensity as the fluorescent-labeled hapten was bound to the antibody. Consequently, Q_f/Q_b must be approximately unity. Hence, it is possible to obtain F_b/F_f from Eq. (2) and the data in Table A2-1, and thereafter F_b from Eq. (3). These values are given in Table A2-2, and plotted as F_b/F_f versus F in Figure A2-2 for the case of 200 μL of antibody. Extrapolation of this curve enables an estimation of 1.5×10^{-9} M for the maximum value of F_b to be made, i.e., $F_{bmax} \approx 1.5 \times 10^{-9}$ M.

Table A2-1. TITRATION OF 2-FNCS-ABZI VERSUS ANTIBODY IN 3.2 mℓ OF BUFFER, POLARIZATION VALUES

2-FNCS-ABZI		Antibody Aliquot, μL		
μL	Concentration, in Units of 10^{-10} M	60	120	200
4	2.5	0.263	0.311	0.318
6	3.75		0.300	
8	5.0	0.195	0.283	0.308
12	7.5	0.166	0.262	0.303
16	10.0	0.137	0.234	
20	12.5		0.209	
22	13.75			0.267
32	20.0			0.227

Table A2-2. TOTAL, FREE, AND BOUND 2-FNCS-ABZI IN THE PRESENCE OF VARIOUS AMOUNTS OF ANTIBODY: CONCENTRATIONS, F, GIVEN IN UNITS OF 10^{-10} M

2-FNCS-ABZI, F	Antibody Aliquot, μL					
	60		120		200	
	F_b/F_f	F_b	F_b/F_f	F_b	F_b/F_f	F_b
2.5	3.52	1.95	19.0	2.38	39.0	2.44
3.75			10.2	3.42		
5.0	1.15	2.67	5.67	4.25	15.5	4.70
7.5	0.761	3.24	3.44	5.81	11.7	6.91
10.0	0.489	3.28	2.06	6.73		
12.5			1.40	7.29		
13.75					3.83	10.9
20.0					1.86	13.0

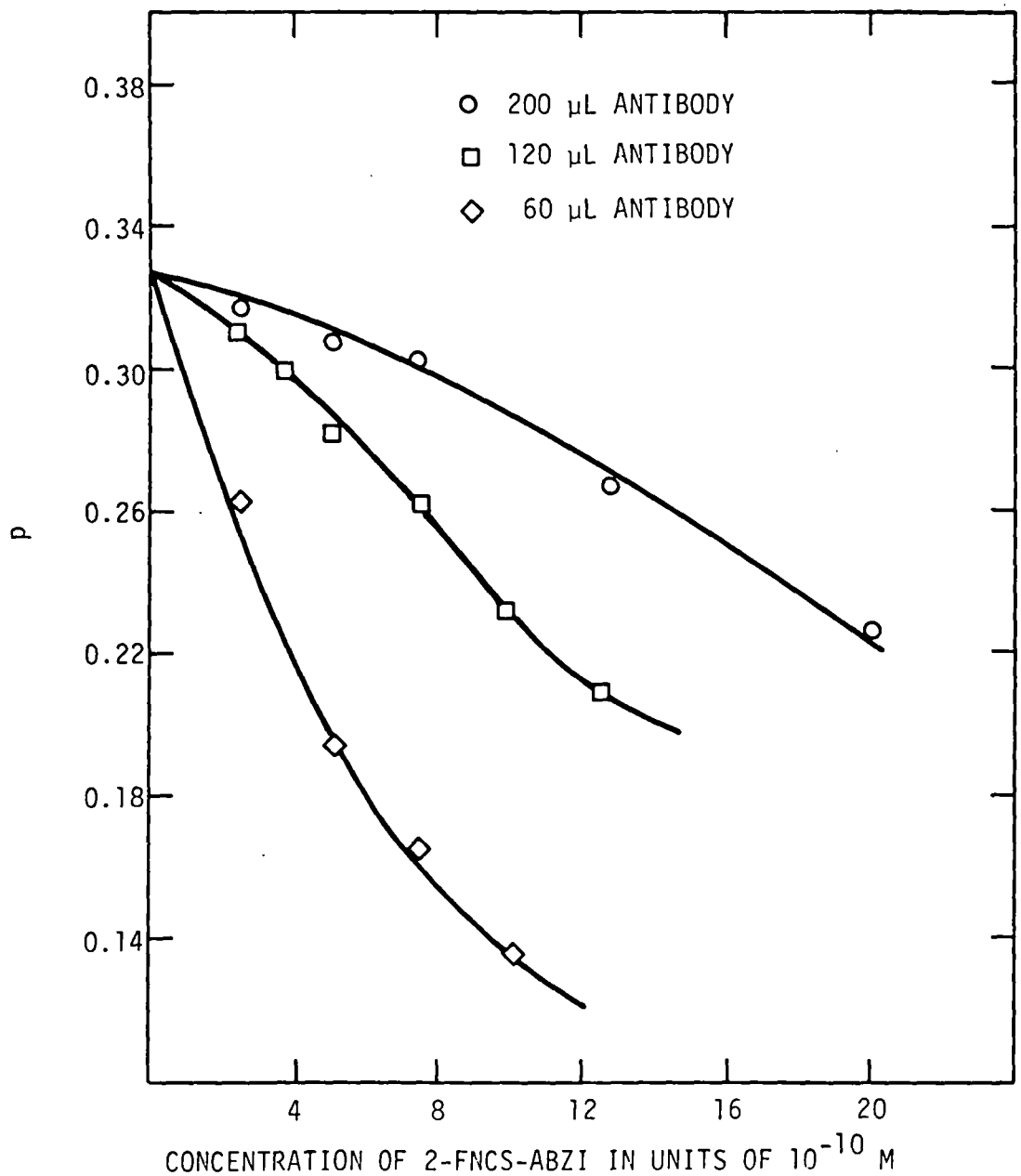

Figure A2-1. Titration Curves 2-FNCS-ABZI vs Antibody

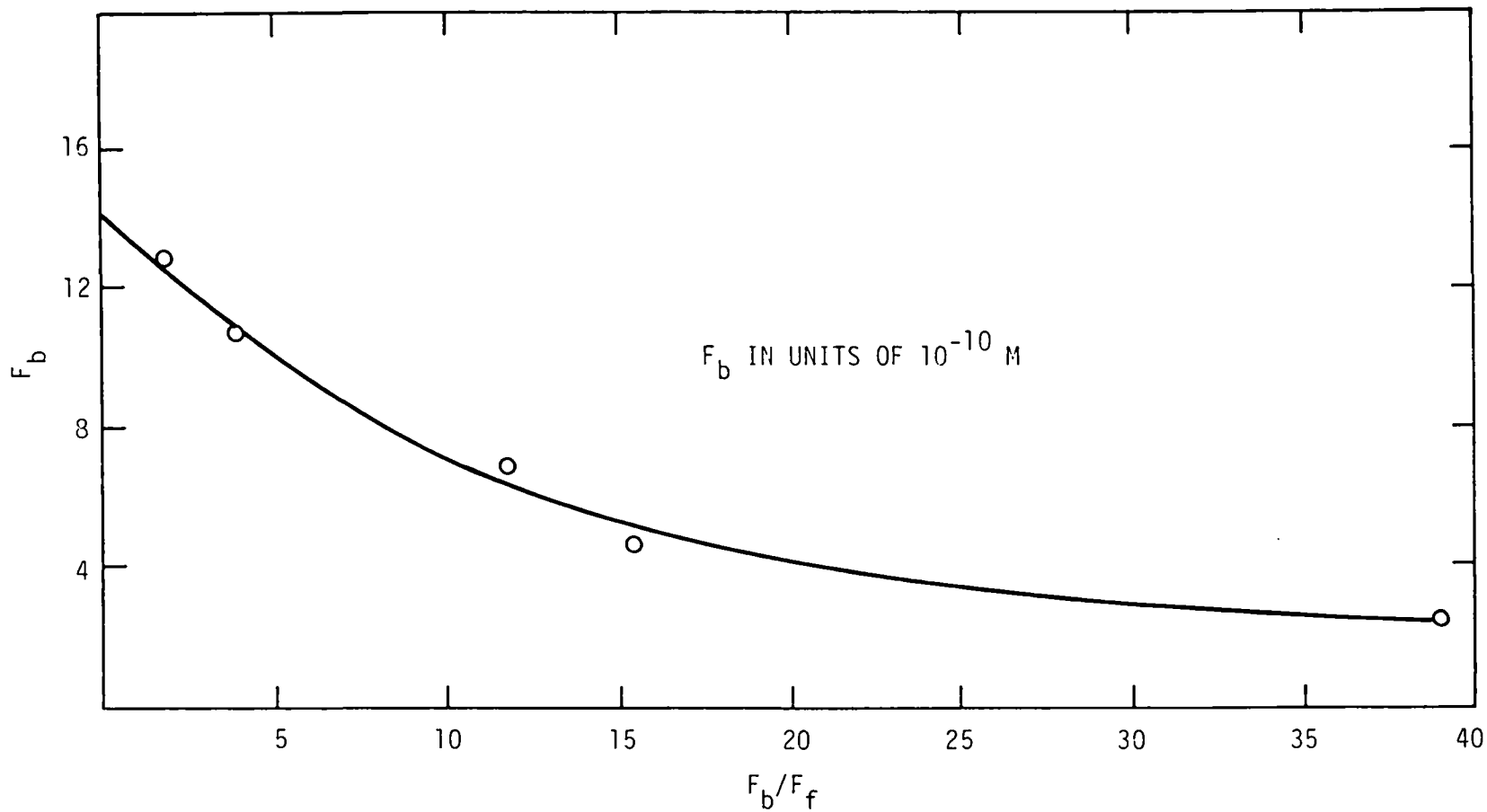

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Figure A2-2. F_b/F_f vs F_b, for the Case of 2-FNCS-ABZI vs 200 µL of Antibody (see Table A2-2)

It should be noted that the maximum molar concentration of binding sites, R_{max} (i.e., the binding sites in free antibody), is the same as F_{bmax}. Therefore, when 200 µL of the antibody preparation is added to 3.2 mℓ of buffer, the concentration of binding sites for 2-FNCS-ABZI is 1.5×10^{-9} M. It is also true that $R = F_{bmax} - F_b$.

EQUILIBRIUM CONSTANT

Ordinarily, one might expect the equilibrium constant for the reaction, $F+R \rightleftarrows FR$ to be given by,

$$K = \frac{(FR)}{(F)(R)} = \frac{F_b}{F_f(F_{bmax}-F_b)} \quad . \tag{4}$$

However, the curvature of Figure A2-2 indicates a nonlinear function. Therefore, it is necessary to use the generalized isotherm of Sips (Ref. A2-1 after the method of Dandliker et al. (Ref. A2-2) to properly express the mass law. Specifically,

$$\text{Log } F_f = \frac{1}{a} \log \left(\frac{F_b}{F_{bmax}-F_b} \right) - \log K_0 \quad , \tag{5}$$

where K_0 is the average association constant, and a is a heterogeneity factor.

Equilibrium data taken from Table A2-2 were used to calculate F_f and $F_b/(F_{bmax}-F_b)$, plotted in Figure A2-3, which is known as a Sips plot.

Least-squares analysis indicates

$$\text{Log } F_f = 1.38 \log \left[\frac{F_b}{F_{bmax}-F_b} \right] - 10.1592 \quad .$$

The slope is 1/a, which indicates a heterogeneity factor of 0.72. The value of the intercept is $-\log K_0$, which indicates that the equilibrium constant, K_0, is 1.44×10^{10}.

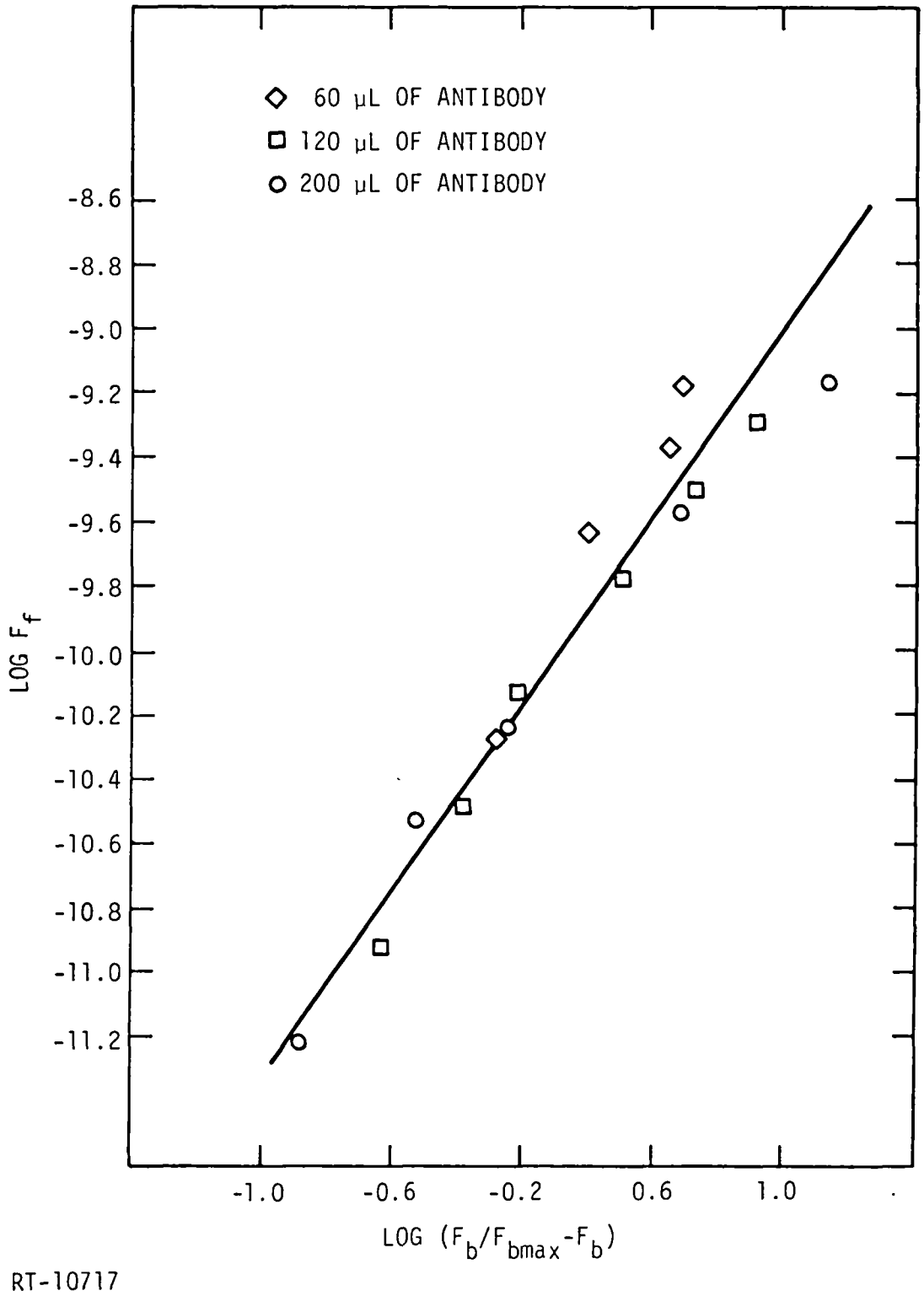

Figure A2-3. Sips Plot Based on Data From Table A2-1

INITIAL RATE DATA

Initial rate data are presented in Figures A2-4 and A2-5, where it can be seen that the rate of change of polarization, dp/dt, is linear over the first few minutes; thus the initial values for dp/dt are easily defined and obtained.

The equation governing the initial rate of change of polarization, $(dp/dt)_o$, has been formulated by Levison and Dandliker (Ref.A2-3),and may be expressed as follows.

$$\left(\frac{dp}{dt}\right)_o = \frac{Qb}{Qf} (P_b - P_f) k (R)_o^{N_1} (F_f)_o^{N_2 - 1} \quad . \tag{6}$$

A number of additional initial rate experiments were carried out with a different level of concentrations of antibody and 2-FNCS-ABZI, than was used in the detection sensitivity experiment, and are summarized in Table A2-3. Plotting log $(R)_o$ versus log $(dp/dt)_o$, as shown in Figure A2-6, demonstrates that the value for N_1 is essentially unity. By plotting log (F_f) versus log $[(dp/dt)_o/(R)_o]$, as shown in Figure A2-7, N_2 is also shown to be essentially unity. Substituting $N_2 \approx 1 \approx N_1$ and $Q_b/Q_f \approx 1$ in Eq. (6) gives

$$(dp/dt)_o = (P_b - P_f) k (R_o)(F_f)_o \quad . \tag{7}$$

Table A2-3. INITIAL RATE EXPERIMENTS

Reactants, Concentration in Units of 10^{-10} M		Initial Rate (min)
Antibody $(R)_o$	2-FNCS-ABZI $(F_f)_o$	$(dp/dt)_o \times 10^3$
15.0	3.3	31.5
2.4	3.5	4.8
7.7	1.3	15.7
2.4	1.3	4.2
7.7	4.4	12.5
7.4	12.0	12.7
7.7	4.4	12.1
7.7	1.3	10.7
7.7	1.3	13.5
3.8	13.0	5.5
3.9	4.4	6.8

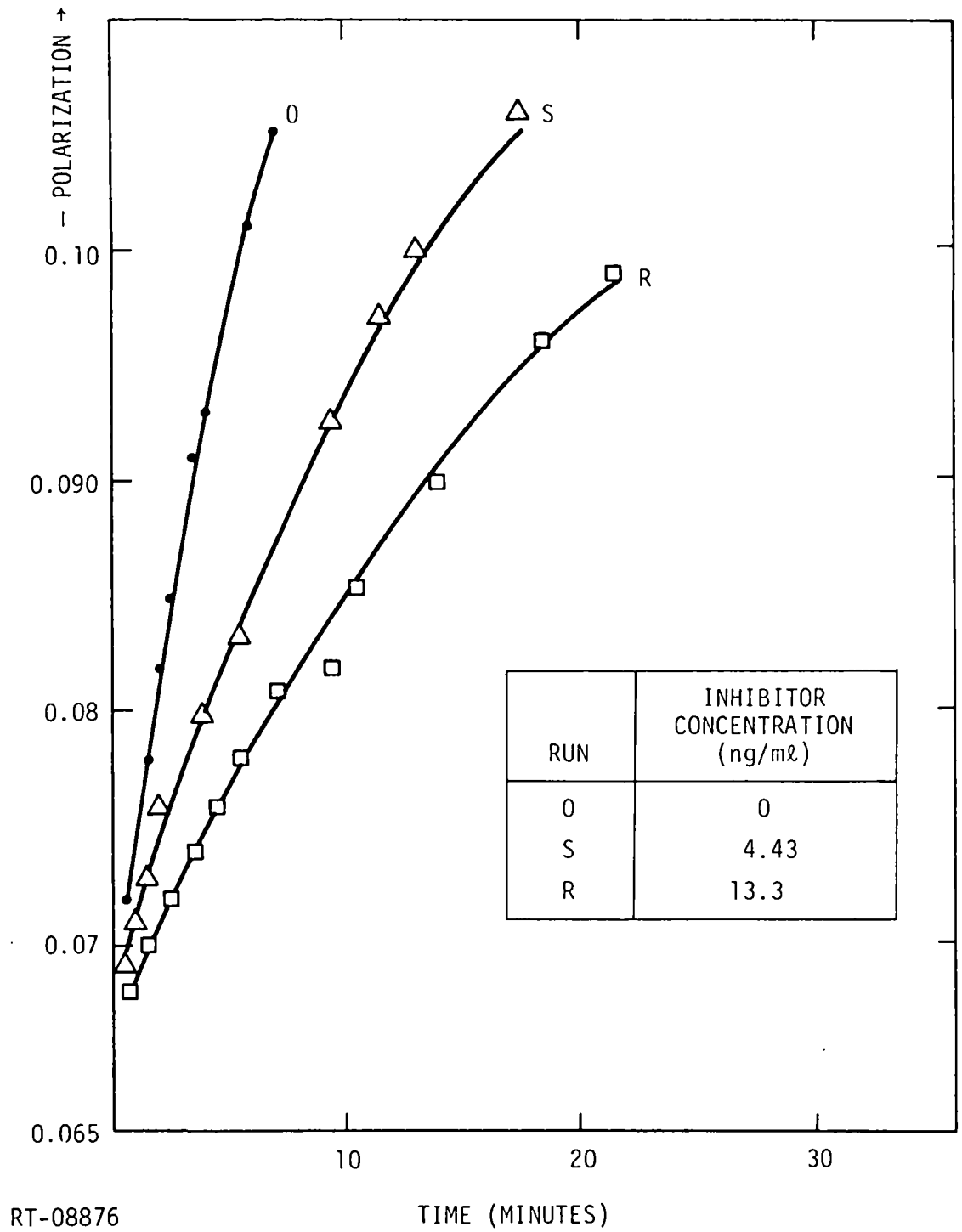

Figure A2-4. Standard Inhibition Curve

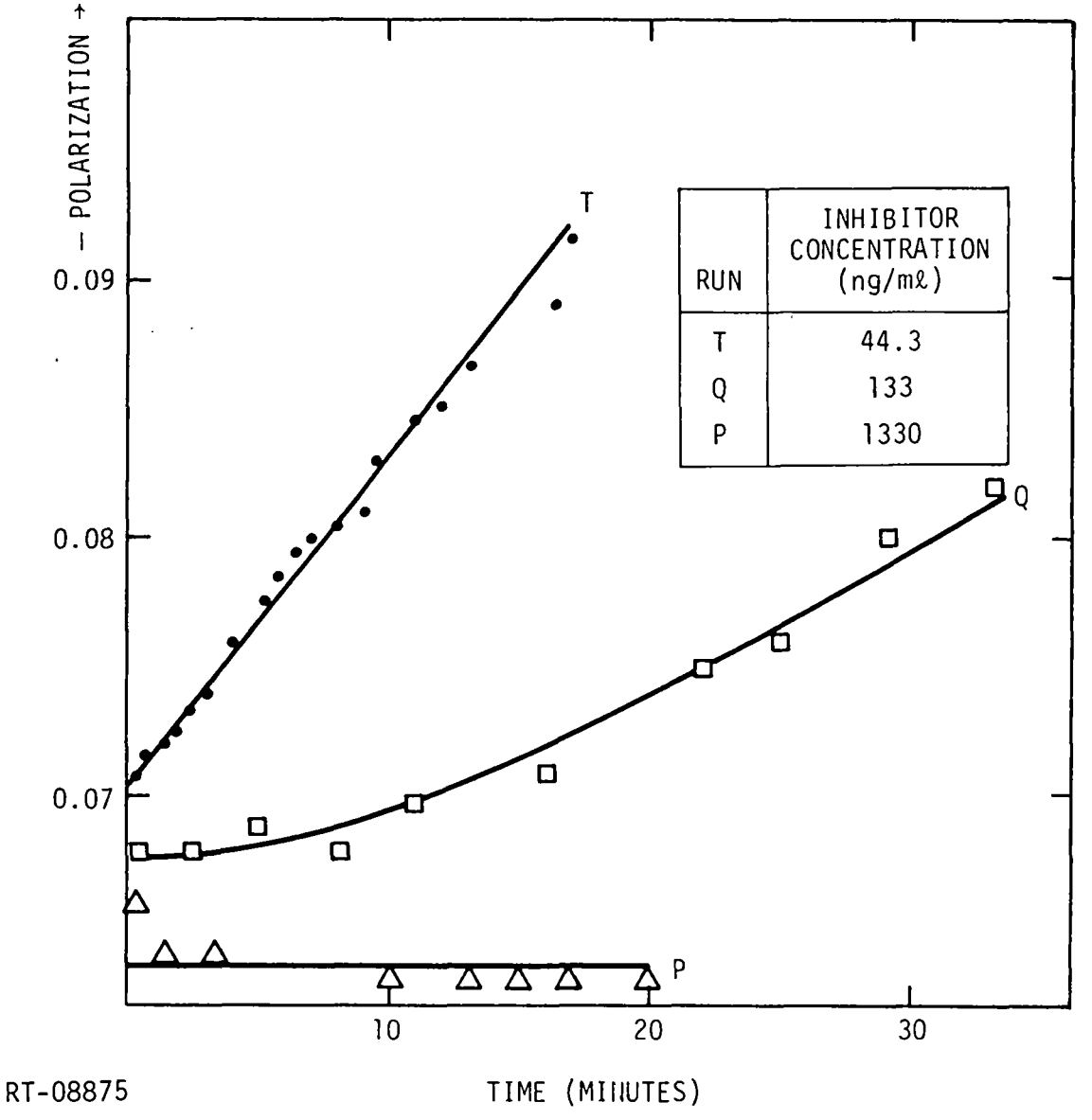

Figure A2-5. Rate of Change of Polarization as a Function of Inhibitor Concentration

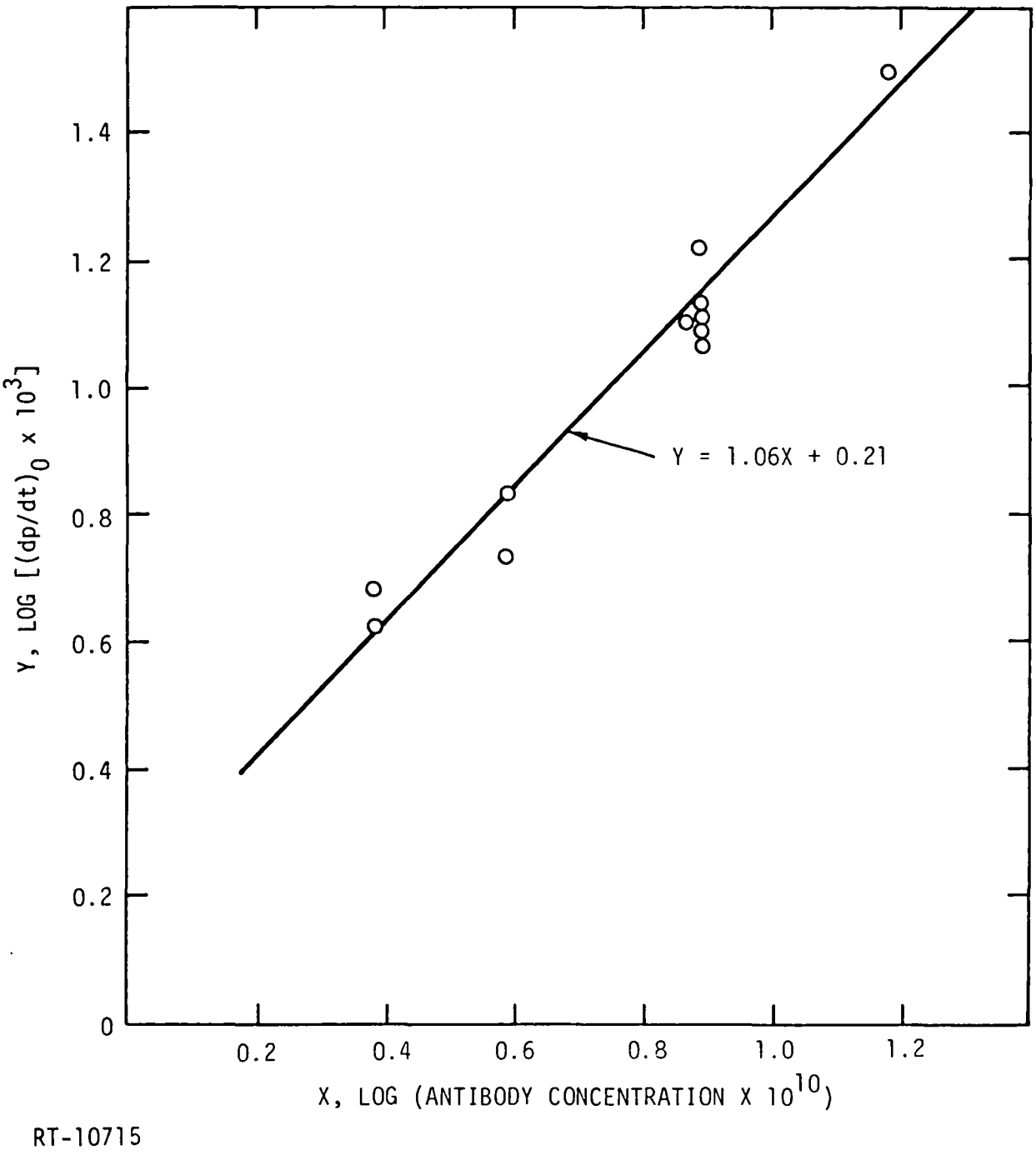

Figure A2-6. Reaction Order with Respect to Antibody

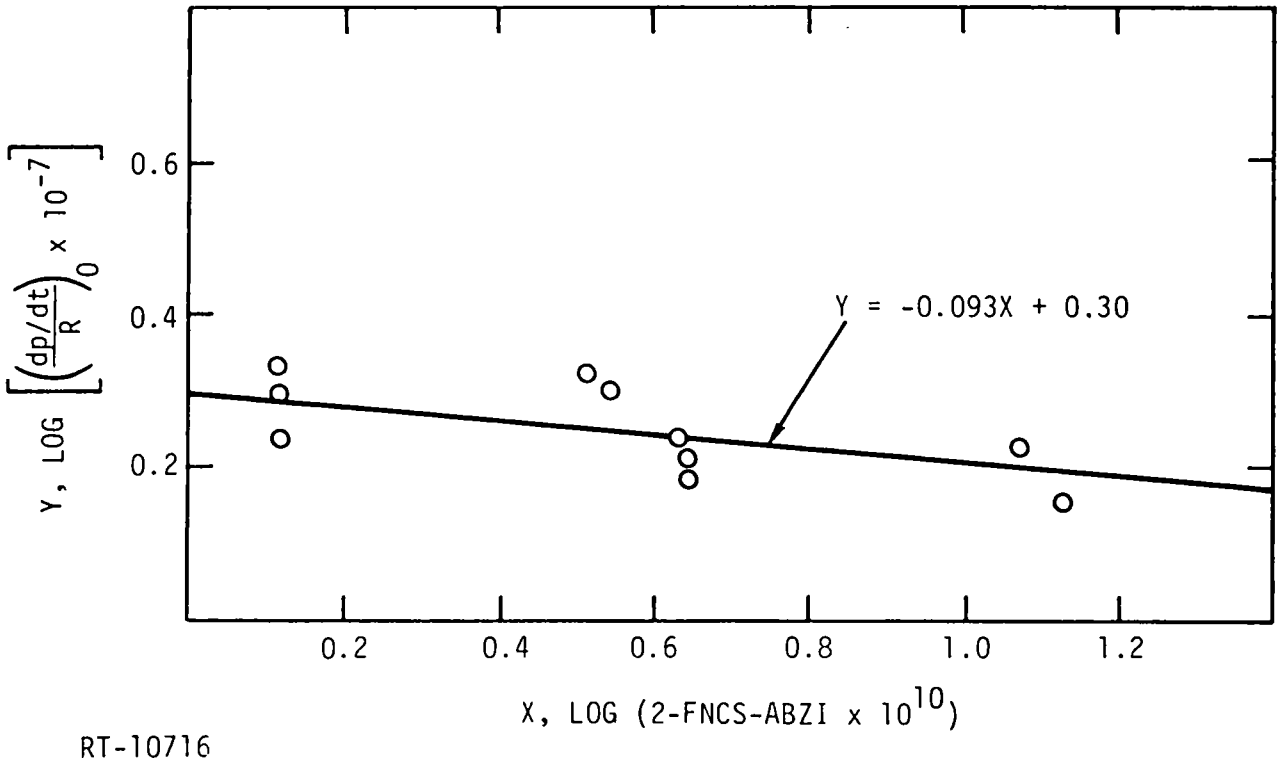

Figure A2-7. Reaction Order with Respect to Fluor-Tagged Hapten

INTEGRATED RATE DATA

One can effect a psuedo-first-order condition by using sufficient excess of antibody to have $(R) \approx (R)_o$ over any time, t. Then, the relationship

$$-(dF_f/dt) = K'F_f \quad , \quad \text{can be applied where} \tag{8}$$

$K' = k(R_o)$ is the pseudo-first-order rate constant.

Integration of Eq. (8) gives

$$\ell n\left(\frac{F_{f_0}}{F_f}\right) = K't \quad , \tag{9}$$

and since

$$\frac{F_{f_0}}{F_f} = \frac{F}{F_f} = \frac{F_f + F_b}{F_f} = 1 + \frac{F_b}{F_f} \quad ,$$

substitution from Eq. (2) in Eq. (9), with $Q_f/Q_b = 1$, gives

$$\ell n\left(1 + \frac{P - P_f}{P_b - P}\right) = K't \quad . \tag{10}$$

Thus, K't can be calculated from polarization data.

A run involving $R_o = 2.32 \times 10^{-9}$ M and 2-FNCS-ABZI $= 2.5 \times 10^{-10}$ M gave the data listed in Table A2-4 and the calculated values of K't, also listed. Figure A2-8 is drawn with K't versus t, and allows the estimation of K' as 5.8×10^{-3} sec^{-1}, from which it follows that the second-order rate constant, k, is approximately 2.5×10^{6} M^{-1} sec^{-1}.

Table A2-4. PSEUDO-FIRST-ORDER KINETIC DATA

WHERE $R_0 = 2.32 \times 10^{-9}$ *M,* $F = 2.5 \times 10^{-10}$ *M,*

$P_f = 0.040$, *AND* $P_{bmax} = 0.328$

t (sec)	P	K′t	t (sec)	P	K′t
21	0.127	0.357	150	0.245	1.238
30	0.140	0.424	159	0.246	1.256
39	0.156	0.513	171	0.252	1.332
48	0.167	0.579	183	0.255	1.373
60	0.181	0.673	198	0.262	1.473
72	0.191	0.739	213	0.267	1.544
81	0.200	0.811	258	0.281	1.813
87	0.205	0.851	270	0.284	1.879
96	0.211	0.901	282	0.287	1.949
102	0.214	0.927	294	0.288	1.974
108	0.217	0.949	318	0.300	2.151
117	0.224	1.014	342	0.298	2.245
135	0.234	1.120			

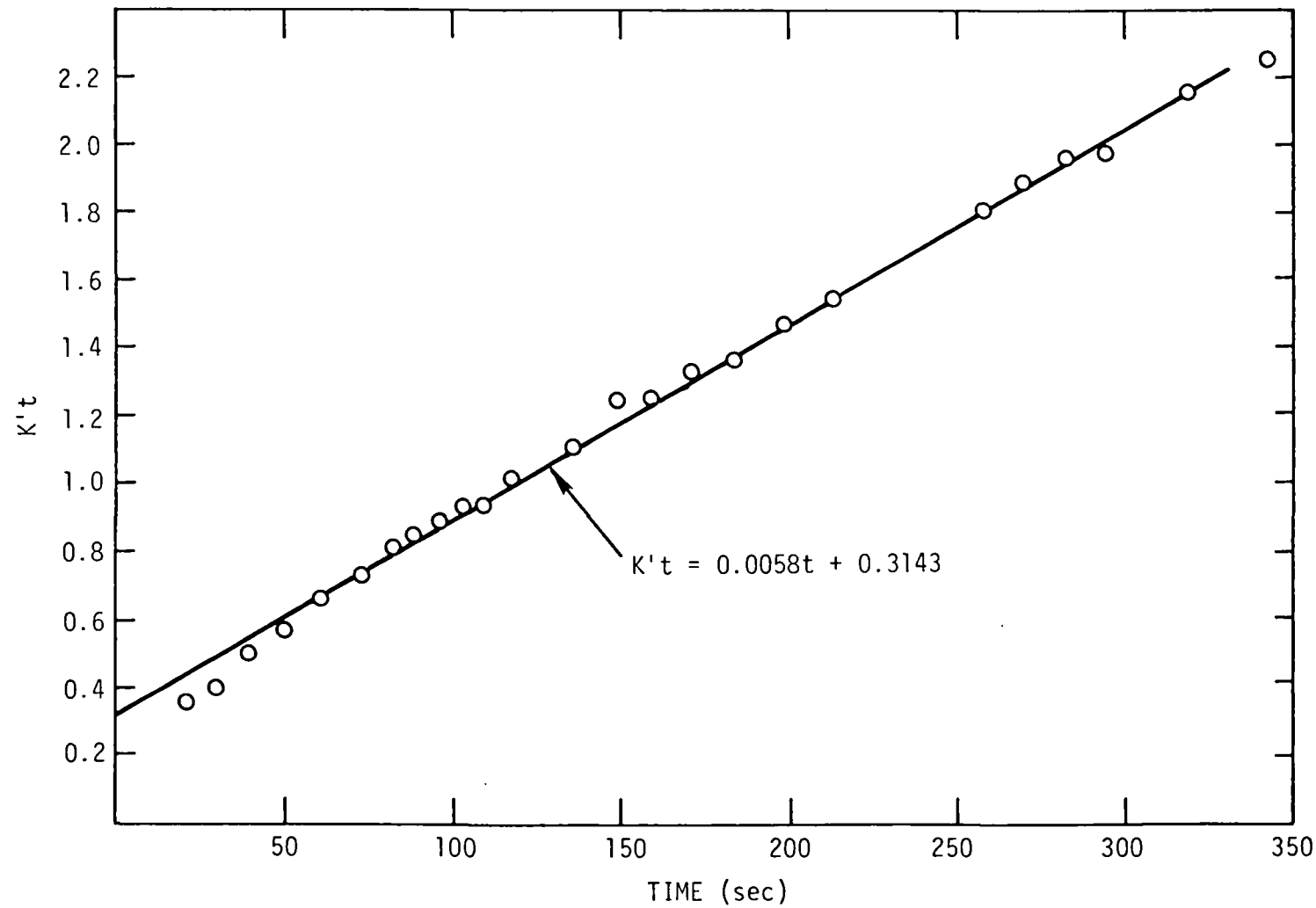

Figure A2-8. Fluorescence Polarization, Pseudo-First-Order Representation

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16. ABSTRACT

This report describes the development and successful demonstration of an immunological assay for the detection of low molecular weight organic contaminants of environmental concern. The specific technique described is a fluorescence polarization immunoassay, the theory of which is presented. The preparation of the two required reagents, namely a fluorescent conjugate of the contaminant of interest, together with an antibody to the contaminant, is described in detail. The specific contaminant chosen for this study was 2-aminobenzimidazole (MW = 133), a metabolite of certain fungicide agents used in agriculture. The particular fluorescent moiety chosen to form the conjugate with 2-aminobenzimidazole was fluorescein. A successful demonstration of the assay has been accomplished, and a detection sensitivity in the sub-nanogram/mℓ range obtained. A high degree of specificity of the antibody for the hapten has been demonstrated, and a successful quantitative recovery from an unknown solution has been obtained. This report is submitted in fulfillment of Contract 68-02-1266 by IRT Corporation, San Diego, California, under the sponsorship of the Environmental Protection Agency. The program was initiated in November 1973 and successfully completed in November 1974.

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