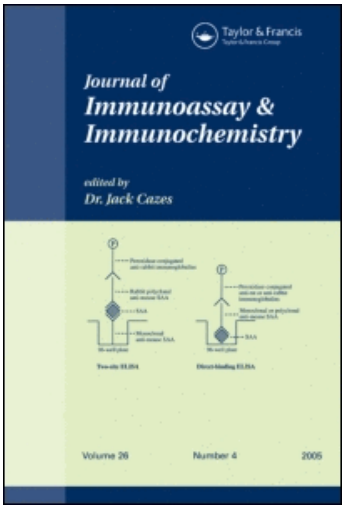

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A Novel Non-Separation Fluoroimmunoassay for Thyroxine

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A NOVEL NON-SEPARATION FLUOROIMMUNOASSAY FOR THYROXINE

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ABSTRACT

It has proved possible to develop a novel non-separation fluoroimmunoassay for thyroxine employing antibodies both to thyroxine and to fluorescein incorporated into immune complexes by addition of a common species-specific anti-immunoglobulin G serum. The labelled hapten could bind to antibody of either specificity but not to both at the same time. Binding to anti-fluorescein markedly reduced fluorescence whereas binding to anti-thyroxine did not. Unlabelled thyroxine competed with the labelled hapten only for anti-thyroxine binding sites and, as a result, more of the labelled thyroxine was bound to anti-fluorescein with a decrease in total fluorescence.

We suggest the term "alternative binding" fluoroimmunoassay for this type of non-separation technique which should be generally applicable to the assay of haptens.

(KEY WORDS: Fluoroimmunoassay. Non-separation. Alternative binding)

INTRODUCTION

An important advantage of fluoroimmunoassay (FIA) as compared with radioimmunoassay is the variety of possible analytical approaches,

some of which avoid the need to separate antibody-bound and free labelled antigen prior to fluorimetry. One example is a "direct quenching" FIA for gentamicin [1] in which antibody binding of fluorescein-labelled gentamicin causes a marked decrease in fluorescence. However, this approach has not proved applicable to the assay of all haptens or of proteins - probably because of the distance between the site of the antibody binding and of label attachment.

"Indirect quenching" non-separation FIA have been developed for albumin, immunoglobulin G and human placental lactogen [2] based on the observation that antibodies to fluorescein will bind to, and quench, the fluorescence of the free fraction but not of the bound. Others [3] have also used this approach, which they term "fluorescence protection immunoassay", and demonstrated by a series of elegant experiments that the inability of anti-fluorescein to bind the fluorescein in the bound fraction probably results from steric hindrnace by the antibodies present on the protein. A similar approach has been employed to detect antibodies [4] and it seemed reasonable to assess the possible application of indirect quenching to the FIA of haptens.

In studies with fluorescein-labelled thyroxine (T_4) and gentamicin, we found that antibodies to fluorescein were able to bind to, and quench, the label in both the antibody-bound and free fractions. Thus fluorescein-labelled haptens behave as other small bifunctional antigens formed by the covalent linkage of two different haptens, which have been shown to cross-link mixtures of antibodies against

the two haptens [5-7]. Furthermore, a recently described novel immunoassay technique depends on the ability of enzyme-labelled antibodies against the dinitrophenyl group to bind to the antibody-bound fraction of a dinitrophenyl-conjugated hapten, [8]. The success of indirect quenching FIA for proteins (as opposed to haptens) probably reflects the multiplicity of their epitopes so that the several specific antibodies bound to different sites on each labelled protein molecule effectively prevent binding of label by the anti-fluorescein serum added later.

Nontheless, a novel non-separation FIA for haptens has been devised using the same reagents as for an indirect quenching assay with the important difference that the antibodies to the hapten and to fluorescein are incorporated into a complex with a common species-specific anti-immunoglobulin G serum. The principle of this "alternative binding" FIA is illustrated in Figure 1. The labelled hapten can bind to only one antibody and if this is directed against fluorescein then there is a marked decrease in fluorescence whereas if it is directed against the hapten fluorescence is not impaired. Unlabelled hapten competes only for the anti-hapten binding sites so that its addition results in more of the labelled hapten becoming bound to anti-fluorescein with a decrease in total fluorescence.

MATERIALS AND METHODS

L-T_4, as the free acid, was obtained from Sigma and labelled with fluorescein isothiocyanate as described previously [9]. Rabbit anti-fluorescein serum was prepared as described in an earlier paper [2] and sheep anti-rabbit immunoglobulin G serum was provided by

(A) Incubation of Mixed Antibody Complex with Labelled Hapten

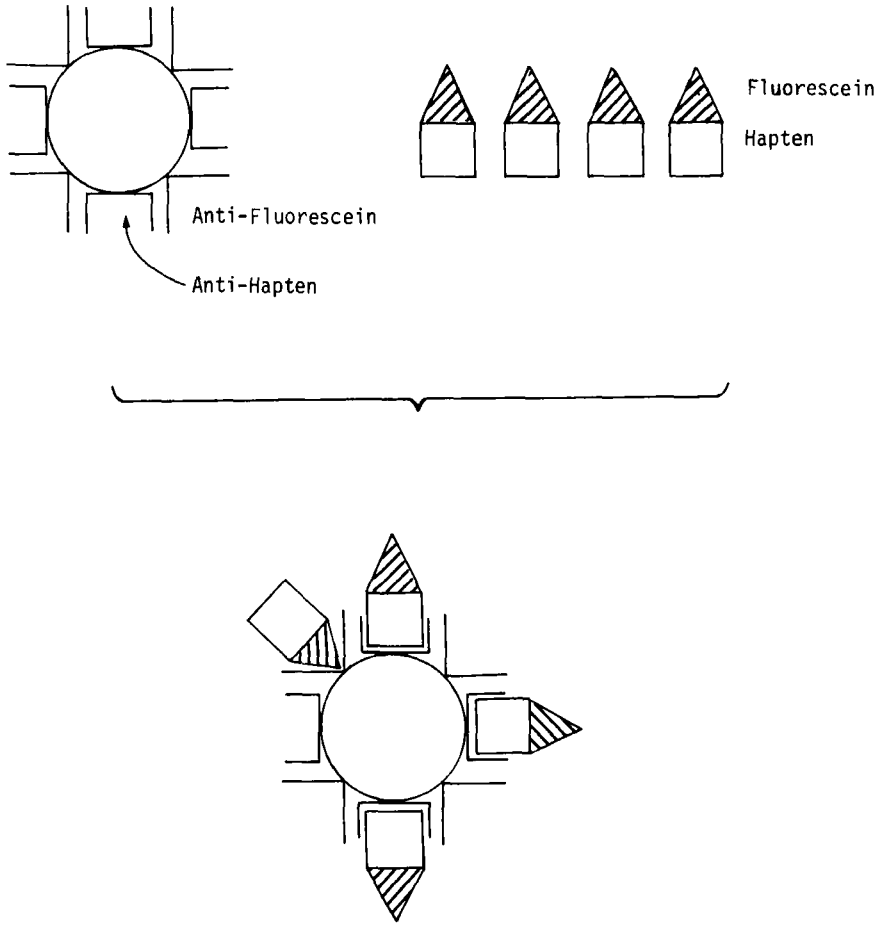

Figure 1 A. A diagramatic representation of alternative binding fluoroimmunoassays. When a fluorescein-labelled hapten is incubated with a complex of anti-fluorescein and anti-hapten binding sites some will be bound to the anti-hapten and continue to fluoresce while some will be bound by the anti-fluorescein with a resultant decrease in fluorescence.

(B) Incubation of Mixed Antibody Complex with Labelled and Unlabelled Hapten

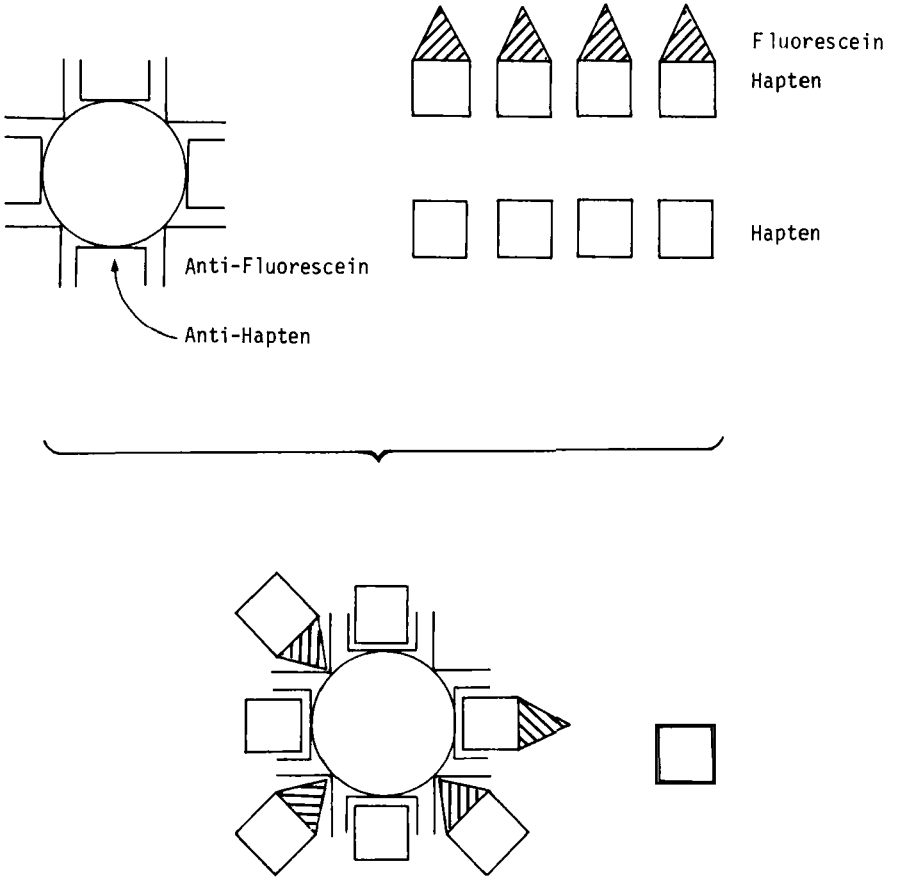

Figure 1 B. A diagramatic representation of alternative binding fluoroimmunoassays. If the unlabelled hapten is also present in the reaction mixture this will compete for anti-hapten binding sites, more labelled hapten will be bound by anti-fluorescein, and there will be a further decrease in fluorescence. Thus the fluorescence of the incubation mixtures will be inversely related to the initial amount of unlabelled hapten present.

Technia Diagnostics. Rabbit and sheep anti-T_4 sera were gifts from Dr. G. Zborowski (Technicon Instruments Corp., Tarrytown, New York, U.S.A.) and Dr. T.G. Merrett (Benenden Chest Hospital, Cranbrook, Kent), respectively.

All experiments were performed using 75 mmol/l barbital buffer, pH 8.6, at ambient temperature.

Fluorimetry

Fluorescence was measured using a Perkin-Elmer Model 1000 fluorimeter, equipped with filters as described elsewhere [10]. In all experiments a correction was made for the background signal contributed by reactants other than fluorescein-labelled T_4. This was determined by fluorimetry of incubation mixtures containing no labelled hapten. Results were expressed relative to an arbitrary scale of fluorescence intensity.

Fluorescence of labelled T_4 in the presence of antibody excess

To 500 µl of fluorescein-labelled T_4 (30 µg/l) was added 500 µl of antiserum, control serum or buffer followed, after at least 5 min, by the addition of 500 µl of a different antiserum, control serum or buffer. Fluorescence was determined as above. Rabbit anti-fluorescein and rabbit control serum was present at a final dilution of 1:6400 and sheep anti-T_4 serum and sheep control serum at a final dilution of 1:400.

Fluorescence of labelled T_4 in the presence of doubling antiserum dilutions

To 500 µl aliquots of doubling dilutions of rabbit anti-T_4, sheep anti-T_4, rabbit anti-fluorescein and control sera from sheep

and rabbits was added 1 ml of fluorescein-labelled T_4 (15 μg/l). Fluorescence was determined after an incubation period of at least 5 min.

Formation of mixed antibody complex

To 500 μl aliquots of doubling dilutions of sheep anti-rabbit immunoglobulin G serum was added 500 μl of a mixture of rabbit anti-T_4 and rabbit anti-fluorescein serum. After 1 h, 500 μl of fluorescein-labelled T_4 (30 μg/l) was added and the fluorescence measured as above.

Alternative binding fluoroimmunoassay using a mixed antibody complex

The mixed antibody complex was first prepared as above. To 100 μl aliquots of standard solutions of T_4 in buffer was added 650 μl of fluorescein-labelled T_4 (23 μg/l) followed by 750 μl of the mixed antibody complex and the fluorescence was then determined as above. A control experiment was performed in an identical manner except that a sheep control serum was used in place of the sheep anti-rabbit immunoglobulin G serum.

RESULTS

Fluorescence of labelled T_4 in the presence of antibody excess

The results are summarised in Table 1. Addition of sheep anti-T_4 serum in place of buffer resulted in the expected enhancement of fluorescence (Smith, 1977) (from 16 to 42 fluorescence units) which was not affected significantly by the presence of rabbit control serum. Conversely, addition of rabbit anti-fluorescein serum in place of buffer caused a marked decrease in fluorescence (from 16 to 2 untis) unaffected by sheep control serum. Irrespective of the

TABLE 1

Fluorescence of Fluorescein-Labelled T_4 in the Presence of Antibody Excess

Order of reagent addition to labelled T_4		Fluorescence intensity
First reagent	Second reagent	
Buffer	Buffer	16
Anti-T_4 serum*	Buffer	42
Anti-T_4 serum	Rabbit control serum	41
Anti-fluorescein serum	Buffer	2
Anti-fluorescein serum	Sheep control serum	2
Anti-fluorescein serum	Anti-T_4 serum	6
Anti-T_4 serum	Anti-fluorescein serum	10

* The antiserum dilutions employed are given in the Methods section.

order of addition, the fluorescence of fluorescein-labelled T_4 was largely quenched in the presence of both the anti-T_4 and anti-fluorescein sera, relative to the signal when bound by antibodies to T_4 alone. In all cases the reaction was complete within 1 min as judged by attainment of stable fluorescence readout.

Fluorescence of labelled T_4 in the presence of doubling antiserum dilutions

Rabbit anti-T_4 and rabbit anti-fluorescein dilution curves were obtained in order to choose appropriate dilutions of these antisera for use in forming the mixed antibody complex. A sheep

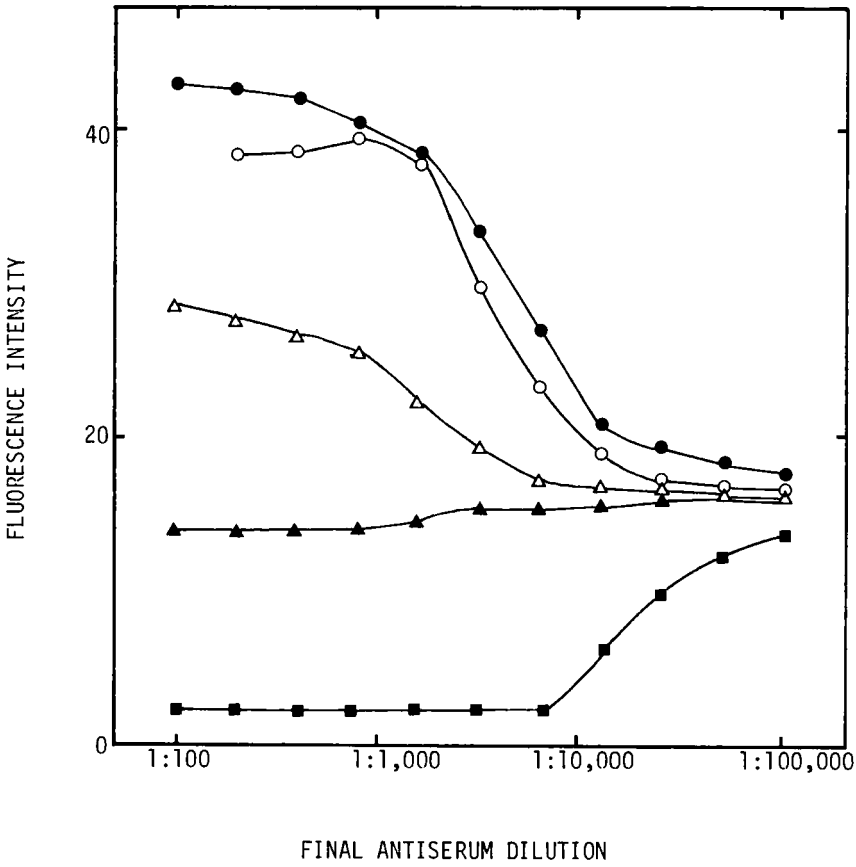

Figure 2. Anti-T_4 and anti-fluorescein dilution curves. Closed circles, sheep anti-T_4 serum; open circles, rabbit anti-T_4 serum; closed triangles, control sheep serum; open triangles, control rabbit serum; closed squares, anti-fluorescein serum.

anti-T_4 dilution curve was also obtained and the results are shown in Figure 2, together with the results using control rabbit and sheep sera. Binding of fluorescein-labelled T_4 by antibodies to T_4 and to fluorescein resulted in the expected enhancement and quenching of fluorescence respectively. Non-specific effects were negligible. On the basis of these studies a final dilution of rabbit anti-T_4 of

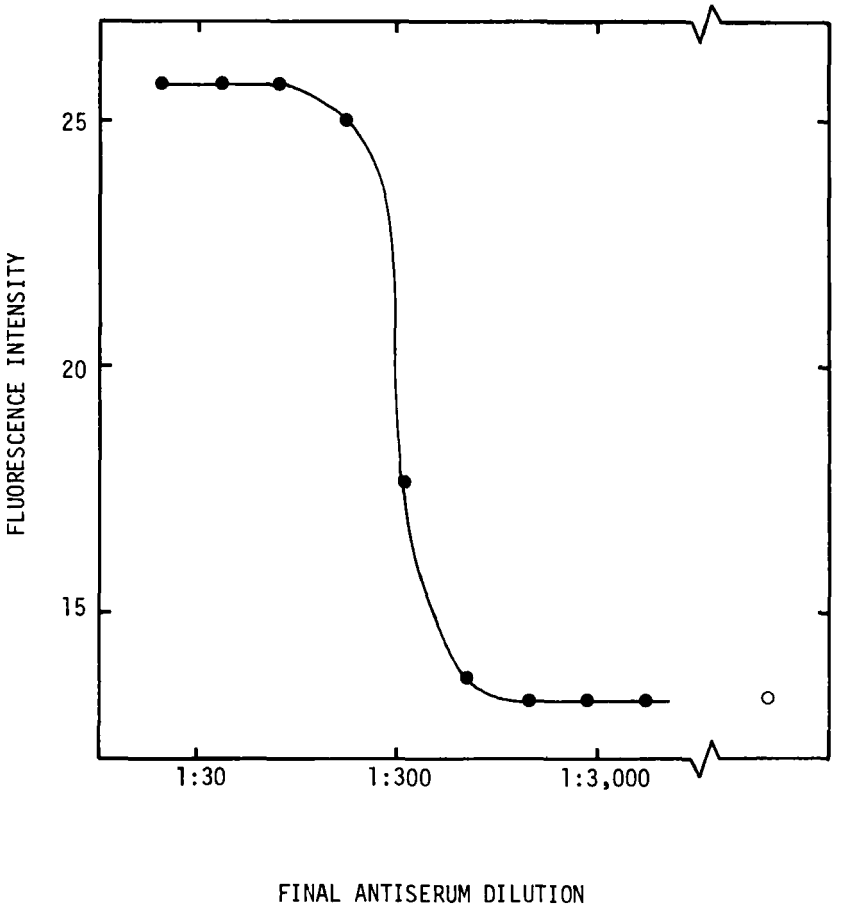

Figure 3. Anti-immunoglobulin G dilution curve in formation of mixed antibody complex. Final dilutions: anti-T_4 serum 1:1600; anti-fluorescein serum 1:6400. Open circle shows fluorescence in absence of added anti-immunoglobulin G serum.

1:1600 and of rabbit anti-fluorescein of 1:6400 was chosen to form the mixed antibody complex.

Formation of mixed antibody complex

The fluorescence of fluorescein-labelled T_4 added to a mixture of rabbit anti-T_4 and anti-fluorescein sera was increased some two-

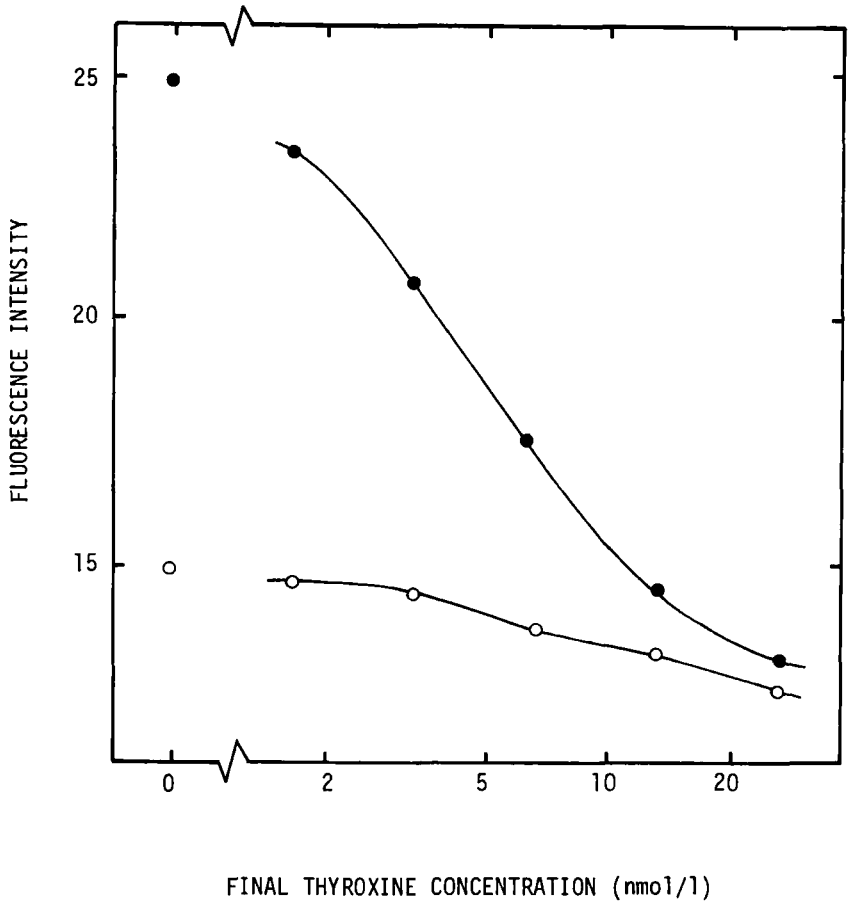

Figure 4. Alternative binding fluoroimmunoassay standard curve. Closed circles, using mixed antibody complex; open circles, anti-immunoglobulin G serum replaced by control sheep serum. Final dilutions: anti-T_4 serum 1:1600; anti-fluorescein serum 1:6400; anti-immunoglobulin G serum or control sheep serum 1:160.

fold by prior complexation of the antibodies with sufficient amounts of sheep anti-rabbit immunoglobulin G serum (Figure 3). On the basis of this experiment a final dilution of 1:160 was chosen to form the mixed antibody complex. Prior incubation of the two rabbit antisera with serum from a control sheep had no significant effect.

Alternative binding fluoroimmunoassay using a mixed antibody complex

It proved possible, using the mixed antibody complex, to obtain a standard curve for T_4 (Figure 4) with the addition of increasing amounts of unlabelled T_4 causing a progressive decrease in the final fluorescence reading. The control experiment confirmed the dependence of the observed effects on the presence of specific sheep anti-rabbit immunoglobulin G serum.

The background signal from reagents other than the labelled T_4 was 10 units and was largely contributed by the intrinsic fluorescence of the relatively high concentration of sheep antiserum present. The standard curve obtained employing a mixed antibody complex 24 h after preparation was the same as that obtained after 1 h although the mixed antibody reagent had developed visible turbidity and the background signal had increased to 12 units.

DISCUSSION

Fluorescein-labelled haptens behave similarly to other small bifunctional antigens comprising two different haptens, in that antibodies directed against the two are able to diffuse sufficiently close together to bind both simultaneously. This is supported by the present finding that antibodies to fluorescein quenched the fluorescence of fluorescein-labelled T_4 even when the labelled hapten was already bound by antibodies to T_4 (Table 1). Thus indirect quenching FIA of the type suitable for proteins [2] would not, in general, be expected to be applicable to hapten assay. Similar failure of the indirect quenching approach was found with gentamicin using all possible combinations of one rabbit and two different sheep anti-

gentamicin sera and five different rabbit anti-fluorescein sera (Smith, D.S., unpublished observations).

We predicted that a non-separation FIA could be made possible by preventing the close approach of the combining sites of antibodies directed against the label and against the hapten. Thus, for example, an individual molecule of fluorescein-labelled T_4 might bind to a molecule of anti-fluorescein or of anti-T_4 but not to both. This was achieved by incorporating mixed rabbit antibodies into immune complexes formed by addition of sheep anti-rabbit immunoglobulin G serum. Fluorescein-labelled T_4 partitions between the anti-T_4 and anti-fluorescein binding sites and the proportions of the two antisera can be selected such that an intermediate final fluorescence signal is given. Added unlabelled T_4 competes for the anti-T_4 binding sites so that more of the labelled T_4 is bound by anti-fluorescein sites leading to reduced fluorescence and the ability to construct a standard curve for T_4.

Alternative binding FIA of this type involve one fewer step than an indirect quenching assay and are comparable in simplicity with other non-separation FIA methods such as direct quenching [1], fluorescent excitation transfer [11] and substrate-labelled FIA [12]. Potential alternatives to the use of second antibody in forming the mixed antibody complex include protein A [13], chemical cross-linkage [14, 15], complexation of hapten-conjugated antibodies with anti-hapten antibody [16] and covalent coupling of antibodies to suspendable polyacrylamide microspheres [17] or other supports.

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