NWRI CONTRIBUTION 89-80

RADIOIMMUNOASSAY FOR THE DETECTION OF POLYCHLORINATED DIBENZO-P-DIOXINS IN ENVIRONMENTAL SAMPLES: INTRODUCTION, PRELIMINARY EVALUATION, AND DEVELOPMENT OF A WORKING ASSAY

by
J.P. Sherry[1], B.K. Afghan[2], J. ApSimon[3]
L Collier[3], F. Bishai[4] and P.W. Albro[5]

[1]National Water Research Institute,
867 Lakeshore Road, P.O. Box 5050
Burlington, Ontario, Canada, L7R 4A6

[2]National Water Quality Laboratory
Burlington, Ontario, Canada

[3] Department of Chemistry
Carleton University
Ottawa, Ontario K1S 5B6

[4]331 Nadine Crescent
Mississauga, Ontario L5A 3L4

[5] National Institute of
Environmental Health Sciences
North Carolina, U.S.A.

MANAGEMENT PERSPECTIVE

Conventional methods for the determination of polychlorinated dibenzo-p-dioxins (PCDDs) in environmental samples are time-consuming, expensive, and also require large-scale capital investment. A screening test would, by the elimination of PCDD free samples, help to reduce the number of samples requiring thorough analysis. In this preliminary study, the ability of the radioimmunoassay (RIA) technique to detect 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in a series of fish extracts was examined. A radio-labelled derivative of 1-amino-2,3,7,8-TCDD was synthesized and characterized. Assay calibration curves were established using known amounts of 2,3,7,8-TCDD. Using laboratory standards, the lowest concentration of 2,3,7,8-TCDD detected was 50-100 pg; it should be possible to improve the detection limit by optimizing the assay. The RIA for the detection of PCDDs should lead to an improved ability within the Department to screen environmental samples for the presence of PCDDs, which in turn should facilitate the surveillance and monitoring of the environment for this important group of contaminants.

Dr. J. Lawrence
Director
Research and Applications Branch

PERSPECTIVE DE GESTION

Les méthodes classiques de dosage des dibenzo-p-dioxines polychlorés (PCDD) dans des échantillons environnementaux prennent beaucoup de temps, sont coûteuses et nécessitent en outre des investissements de capital importants. La mise au point d'un test de sélection qui éliminerait les échantillons exempts de PCDD aiderait grandement à réduire le nombre d'échantillons à analyser. Dans cette étude préliminaire, la capacité de la méthode de radioimmunoessai (RIA) à déceler la présence de 2,3,7,8-tétrachlorodibenzo-p-dioxine (2,3,7,8-TCDD) dans une série d'extraits de poissons a été évaluée. Un dérivé radiomarqué de 1-amino-2,3,7,8-TCDD a été synthétisé et caractérisé. Les courbes d'étalonnage de l'essai ont été établies à partir de quantités connues de 2,3,7,8-TCDD. À l'aide des normes de laboratoire, la concentration la plus faible de 2,3,7,8-TCDD était de 50-100 pg; il serait possible d'améliorer la limite de détection en optimisant l'essai. L'utilisation du RIA pour la détection des PCDD devrait permettre d'améliorer la capacité du Ministère à trier les échantillons environnementaux en fonction de leur teneur en PCDD, ce qui en retour faciliterait la surveillance et le contrôle de cet important groupe de contaminants dans l'environnement.

Dr.J. Lawrence

Directeur

Direction de la recherche et des applications

ABSTRACT

Screening tests, such as radioimmunoassay (RIA) can facilitate the analysis of environmental samples for polychlorinated dibenzo-p-dioxins. A preliminary evaluation indicated that RIA could detect 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a variety of fish samples. A radio-labelled derivative of 1-amino-3,7,8-TCDD was synthesized de novo, and characterized. The synthesis was repeatable. Assay calibration curves were established using known amounts of 2,3,7,8-TCDD. The lowest quantity of 2,3,7,8-TCDD detected was either 50 or 100 pg, depending on the solubilization procedure used; it should be possible to improve the detection limit by optimizing the assay. The assay was repeatable between batches of labelled hapten. On the basis of the repeatability of the hapten labelling procedure, the assay's performance when challenged with low levels of 2,3,7,8-TCDD and its ability to detect PCDDs in fish, it appears that RIA has potential usefulness in the detection of PCDDs in the aquatic environment.

RÉSUMÉ

Les tests de sélection, comme les radioimmunoessais (RIA), peuvent faciliter le dosage des dibenzo-p-dioxines polychlorés dans les échantillons environnementaux. D'après une évaluation préliminaire, il apparaît que le RIA pourrait détecter 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) dans divers échantillons de poissons. Un dérivé radio-marqué de 1-amino-3,7,8-TCDD a été synthétisé et caractérisé. La synthèse était répétable. Les courbes d'étalonnage de l'essai ont été établies à partir de quantités connues de 2,3,7,8-TCDD. La quantité la plus faible de 2,3,8-TCDD détectée était de 50 ou de 100 pg selon la méthode de solubilisation utilisée. Il serait donc possible d'améliorer la limite de détection en optimisant l'essai. L'essai était répétable entre les lots de hapten marqués. En se fondant sur la répétabilité de la méthode de marquage de l'hapten, sur la performance de l'essai en présence de faibles teneurs de 2,3,7,8-TCDD et sur sa capacité à détecter les PCDD dans les poissons, il semble que le RIA puisse aider à détecter les PCDD contenus dans le milieu aquatique.

TABLE OF CONTENTS

1.0 INTRODUCTION

Widespread concern about the toxicity and environmental distribution of the polychlorinated dibenzo-p-dioxins (PCDDs) has led to a requirement for the quantitative analysis of ever-increasing numbers of environmental samples for the presence of these persistent contaminants. Such demands are likely to increase, rather than diminish, in the future. Furthermore, similar demands will probably arise for other classes of trace contaminants. 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the most notorious and the most toxic of the PCDDs. However, some of the other PCDDs are comparable to 2,3,7,8-TCDD in degree of toxicity, although the majority of them have yet to undergo toxicity testing. Confirmation of the suspected widespread occurrence of PCDDs, and an improved understanding of their environmental distribution await the deployment of analytical procedures that will facilitate the analysis of large and diverse sample sets.

2.0 CONVENTIONAL ANALYTICAL METHODS

Conventional methods for the analysis of PCDDs combine gas chromatography with mass spectrometry normally require extensive extraction and cleanup of samples coupled with some form of gas chromatographic/mass spectrometric determination; such methods are time consuming and expensive when used in the routine analysis of environmental samples. Gas chromatography-mass spectrometry laboratories usually have a low sample throughput (less than 10 samples a day); an individual analysis, including isomer confirmation, costs approximatey $1500 - serious limitations when considered from the perspective of an environmental surveillance or monitoring program. Knowledge and understanding of the environmental distribution of PCDDs will require the analysis of thousands of environmental samples from many and varied locations. Moreover, an incident involving the release of PCDDs into the environment, could create a requirement for the short-term analysis

of large numbers of environmental and biological samples to be followed in all probability by an intensive monitoring program. Such demands would severely stress existing analytical facilities.

2.1 Screening Strategy

The inclusion of an effective screening test in the analytical protocol for PCDDs could resolve some of the foregoing problems by eliminating PCDD free samples from further time-consuming conventional analysis. The screening method need not be isomer specific; rather, positive samples from the screening step could be subsequently analyzed for specific PCDD isomers, using conventional analytical techniques. Several screening methods have been proposed for the detection of PCDDs: radioimmunoassay (RIA) (Albro et al., 1979), aryl hydrocabon hydroxylase (AHH) induction assay (Bradlaw and Casterline, 1979) and cystol receptor assay (Poland et al., 1976). Of these methods RIA was considered to offer the best potential for eventual inclusion in an analytical protocol for the routine analysis of PCDDs.

2.2 Radioimmunoassay

Based on the classicial antigen-antibody reaction, RIA is a relatively simple, powerful, and adaptable technique for the rapid determination of trace levels of organic compounds. Originally developed for the micro-determination of proteinaceous substances (Yalow and Berson, 1960), RIA is also extensively used in the detection and determination of steroidal hormones and other low molecular weight organic molecules. Immunoassays have been proposed for the detection of several environmentally important compounds in addition to PCDDs (Albro et al., 1979): PCBs (Newsome and Shields, 1981), Dieldren and Aldrin (Langone and Vanakis, 1975), Atrazine (Huber, 1985), Benomyl and Methyl 2-Benzimidazolecarbamate (Newsome and Shields, 1981), Diclofop-methyl (Schwalbe et al., 1984), 2,3,7,8-tetrachlorodibenzofuran (Luster et al.,

1980, and 2,4-D and 2,4,5-T (Rinder and Flecker, 1981). Recently, the International Union of Pure and Applied Chemistry, reporting on improved cost effective approaches to pesticide residues analysis, concluded that immunochemical methods, while being traditionally unfamiliar to the residue chemist, offer exciting possibilities for newer cost effective approaches (Hemingway, 1984).

2.3 Developing a Radioimmunoassay

The development of a radioimmunoassay for a low molecular weight compound requires the preparation of two key reagents (Figure 1): antibodies to the compound of interest, and a radio-labelled derivative of the compound of interest that can be bound by those antibodies. Firstly, an antigen must be prepared by coupling the compound of interest, or one of its analogues, via a linkage group to a carrier protein. The antigen is then injected into a suitable animal, usually a rabbit, whose immune system responds by producing a family of antibodies (Figure 1) directed against the antigen. Unwanted antibodies can be removed from the serum using affinity chromatography. Radioactive hapten is usually prepared by either labelling the compound of interest directly with ^{125}I, or if the iodine interferes with the compounds immuno-reactivity, a suitable side chain can be used to separate the tracer from the main body of the molecule.

A typical RIA procedure is schematically represented in Figure 2. Antibodies and a sample containing analyte, are incubated together for about 30 minutes. Labelled hapten is then added to the assay tubes. The tubes contents are mixed, and then re-incubated until equilibrium binding has occurred. At this stage one of several mechanisms is used to separate the bound from the unbound radioactivity. The bound (usually) radioactivity is quantified using a gamma radiation counter, and a calibration curve is prepared. A reduction in the amount of radioactivity bound is theoretically proportional to the quantity of analyte present in the assay tube. Analyte concentration is interpolated from a standard curve.

2.4 Radioimmunoassay for PCDDs

The RIA for PCDDs is a double antibody procedure with a reported detection limit of approximately 25 pg of 2,3,7,8-TCDD (Albro et al., 1979), and a reliable sensitivity in human fat of 100 pg ($P < 0.05$) (McKinney et al., 1982). The assay uses detergent micelles to solubilize the highly insoluble PCDD molecules which are then incubated, in a competitive binding reaction, with an ^{125}I labelled dioxin derivative and antibodies raised in rabbits against 1-amino,3,7,8-TCDD. When equilibrium binding has been reached, bound and free radioactivity are separated using a second antibody procedure, and the bound radioactivity is subsequently quantified.

2.5 Study Objectives

Our objectives were to assess the ability of the RIA to detect PCDDs in fish samples, to develop a working capability in the RIA method by synthesizing, evaluating, and characterizing a labelled dioxin derivative, and to appraise the assay's performanmce using a 2,3,7,8-TCDD reference standard.

3.0 MATERIALS AND METHODS

3.1 Antibodies

Antiserum (Alb-5), raised in rabbits against an antigen that had been prepared by conjugating 1-amino-3,7,8-trichlorodibenzo-p-dioxin to bovine thryoglobulin by means of an adipamide linkage. Alb-5 was lyophilized and stored at 4°C. Portions of the antiserum were reconstitued in distilled water and diluted 1:50 (v/v) or 1:100 (v/v) in antibody diluent. The diluted antiserum was stored in 0.5 mL portions at -80°C until use. Because Alb-5 had been originally selected on the basis of high specificity toward PCDDs, low recognition of the linkage arm, and high affinity for dioxin, it was not considered necessary to

remove antibodies to the adipamide linkage arm by means of affinity chromatography (M.I. Luster, personal communication).

3.2 Hapten

1-N-(5-iodovaleramido)-3,7,8-trichlorodibenzo-p-dioxin (8) was synthesized using the following procedure: an initial attempt to synthesize 2,5,-dichloro-1,3-dinitrobenzene (1) using conditions and procedures described by Holleman (1920) yielded a mixture of compound 1 (the desired product) and compound 2 (Figure 3). The two materials gave almost identical ^{1}H NMR spectra, and the same mass spectra. The desired product was purified using HPLC and was selected on the basis of its solubility and ^{13}C NMR spectrum.

Condensation of compound 1 with 4,5,-dichlorocatechol dianion (3) using the method of Chae et al. (1977), yielded 1,3,7,8-TCDD (4) rather than the desired nitro-dioxin (5) (1-nitro-3,7,8-trichlorodibenzo-p-dioxin), which was produced only as a minor side product. The reaction was repeated with the same results. It was considered possible that a metal catalyzed removal of the nitro group and subsequent replacement by a chlorine atom might have occurred. The glassware was pre-treated with an EDTA solution to remove trace metals, and the reaction was repeated under the same conditions. The desired nitrodioxin (5) was obtained in a 70% yield and was greater than 90% pure on GC/MS analysis. The elemental composition was confirmed using high resolution mass spectroscopy. The subsequent reduction of the nitrodioxin produced the 1-amino-3,7,8-trichlorodibenzo-p-dioxin) (6) in a 60% yield. The elemental composition was again confirmed using high resolution mass spectroscopy. The production of the 1-(5-bromovaleramido)-3,7,8-trichlorodibenzo-p-dioxin) (7) and subsequent conversion to the desired hapten (8) proceeded as described by Albro et al. (1979). GC analysis indicated the presence of a single compound. An electron impact mass spectrum indicated the correct mass (less HI) for the hapten. The hapten was further characterized using thin layer

chromatography on pre-coated silica gel TLC plates (Sybron Brinkman, Si1G-UV254) using benzene as the solvent. After solvent removal, the plates were examined under UV light (254 nm).

Following some initial stability problems, the following procedure was adopted for the routine storage and manipulation of the hapten:

a) The hapten was stored dry at -80°C under nitrogen.
b) The hapten was equilibrated to room temperature in a dessicator, before being dissoved in $CHCl_3$.
c) A portion of the hapten solution (approximately 10 mL) was removed and added to a solvent rinsed glass beaker. Dry acetone (90 mL) was then added to the beaker. The solvent was removed under a stream of nitrogen until approximately 20 mL remained in the beaker. Dry acetone (50 mL) was again added to the beaker and the solvent removal was repeated until about 6 mL of solvent remained.
d) The hapten solution was then transferred to a volumetric flask and adjusted to 10 mL.
e) Ultrasonication was used throughout the foregoing procedure to assist solubilization.

3.3 Labelled Hapten

The hapten was rendered radioactive by exchanging its iodo group for ^{125}I. The radio-tracer, $Na^{125}I$, and hapten were reacted in a molar ratio of 2.33:21.5; an equi-molar reaction would have involved the use of excessive radioactivity: 46 mCi. Unlabelled hapten (10 µg) dissolved in approximately 250 µL of dry acetone was injected into a vial that contained 5 mCi of $Na^{125}I$ (Amersham, IMS300, carrier free). The vial and its contents were incubated at 50°C for 66 hr in a thermostatically controlled heating block. The reaction product was then extracted and purified using a previously described procedure (Sherry et al., 1988).

The labelled hapten was characterized using both TLC and immunoassay procedures. Thin layer chromatography was carried out as previously described for the unlabelled hapten. The developed plates were scanned for gamma radiation using a Packard TLC plate scanner.

3.4 Antiserum Standardization

The quantity of antiserum required to bind 30-40% of the added radioactivity was determined for each batch of labelled hapten using the following serum standardization procedure:

a) The serum standardization was carried out in triplicate. The first three tubes were assay blanks and received only antibody diluent. The second triplicate (TOTAL) received only labelled hapten.
b) 0.2 mL of 1% (w/v) cutscum in methanol was added to each tube.
c) The methanol was removed using a vortex evaporator.
d) 0.2 mL of phosphate buffered saline (PBS) was added to each tube. The tubes were stoppered and ultra-sonicated for 20 minutes; the ultra-sonic bath contained sufficient water to cover the bottom 0.5 cm of the tubes. The tubes were then vortex mixed, re-sonicated for 10 minutes, and then allowed to equilibrate to room temperature.
e) 0.2 mL of antibody diluent was added to tubes 1-3. The remaining tubes with the exception of the TOTAL tubes, received the following dilutions of antiserum in antibody diluent: 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000.
f) The contents of the tubes were mixed using a vortex mixer.
g) The tubes were incubated with shaking in a water bath for 30 minutes at 37°C.
h) 7000 cpm of ^{125}-I-labelled hapten was added to each tube.
i) The tubes were re-stoppered, mixed on a vortex mixer, and incubated with shaking in a water bath for 60 minutes at 37°C.
j) The tubes were re-incubated without shaking for 68-72 hours at 4°C.

k) 0.2 mL of second antibody reagent was added to each tube. The tubes were kept on ice during these manipulations.

l) The tubes were gently mixed using a vortex mixer, and incubated statically for six hours at 4°C.

m) The tubes were then centrifuged for 30 mintues at 4°C.

o) The tubes were drained for 10 minutes, following which the inside walls of the tubes were wiped with tissue paper and the tubes were re-stoppered.

p) The amount of radiation in each tube was determined using a gamma radiation counter. The arithmetic mean of each set of replicates was calculated. The blank tube value was subtracted from those of the other tubes. The percentage of radioactivity bound was plotted against antiserum dilution and the antiserum dilution required to bind 35-40% of the radioactivity was interpolated.

3.5 Radioimmunoassay Procedure

a) Initial assays were run in triplicate.

b) The first three sets of triplicate tubes (12 x 75 mm pre-treated glass tubes) were labelled BLANK (for the estimation of non-specifically bound radioactivity), REFERENCE (for the estimation of the amount of radioactivity bound in the absence of PCDDs) and TOTAL (the total amount of radioactivitiy added) respectively. Subsequent sets of triplicates received known amounts of 2,3,7,8-TCDD, and were arranged in order of increasing concentration. Unknown samples were analyzed after the TCDD standards.

c) TCDD reference standards and unknown samples were added to the appropriate tubes in of benzene (200 µL). BLANK, REFERENCE and TOTAL tubes received 200 µL of benzene.

d) The benzene was removed from the tubes using a vortex evaporator.

e) 200 µL of 1% Cutscum or Triton X-305 in absolute methanol (w/v) was added to each tube.

f) The tubes were then vortex mixed three times so as to ensure adequate solubilization of the PCDD residues.

g) The methanol was removed from the tubes using a vortex evaporator.

h) 200 µL of PBS was added to each tube. The tubes were then stoppered and sonicated for 30 minutes in an ultrasonic bath, after which the tubes were vortex mixed and re-sonicated for a further ten minutes.

i) The tubes were equilibrated to room temperature, and 200 µL of previously standardized antiserum was added to all except the BLANK and TOTAL tubes which received 200 µL of antibody diluent each.

j) The tubes were re-stopperd, vortex mixed, and incubated with shaking in a water bath for 40 minutes at 30°C.

k) 7000 cpm of ^{125}I labelled hapten was then added to each tube.

l) The tubes were re-stoppered, vortex mixed, and re-incubated with shaking in a water bath for 60 minutes at 37°C.

m) The tubes were then incubated statically for 68-72 hours at 4°C.

n) Bound and unbound radioactivity were separated using the procedure described under "antiserum standardization".

o) The amount of radiation in the tubes was determined using a gamma radiation counter. The arithmetic mean of each set of replicates was calculated.

p) The counts from the BLANK tubes were subtracted from the counts for the other tubes. The readings from the standard tubes were plotted either as cpm or as $B/B_0 \times 100$ versus amount of TCDD on semi-log paper (B = cpm in standard or sample tube minus cpm in BLANK tubes; B_0 = cpm in REFERENCE tubes minus cpm in BLANK tubes). The standard curves were usually plotted using spline techniques. Amounts of PCDD in sample extracts were interpolated from the standard curve.

3.6 Other Equipment and Reagents

All chemicals used were of Analar or equivalent grade. PCDD standards were obtained from KOR and Cambridge Isotope Laboratories. All other equipment and reagents have been previously described (Sherry et al., 1988).

4.0 EXPERIMENTAL

A preliminary evaluation was made of the ability of the RIA for PCDDs to detect PCDDs in fish, a common environmental matrix. A set of fish samples (Table 1) consisting of a variety of tissue homogenates that had been previously extracted and prepared for TCDD analysis using a deactivated florisil cleanup procedure (R. Thomson, personal communication) was analyzed using RIA and GC-MS. The RIA analyses were undertaken by Dr. C. Mituma, SRI International, Menlow Park, California, USA. The data presented in Table 1 are from single replicate analyses; no level of statistical significance is implied. The detection limit of the RIA will be the subject of a future report.

Three of the fish extracts (lake trout #2 fraction D, rainbow trout fraction D, and lake trout #7 fraction E) contained high TCDD levels, observations that were confirmed by the RIA analyses. Overloading of the low capacity florisil column probably caused carryover of TCDD from the D fraction, where it would have been expected to elute from the column, to the F fraction where it was detected using RIA in the lake trout #2 sample. The discrepancies between the TCDD levels detected using GC-MS and RIA may have been caused by the RIA's responsiveness to PCDD isomers other than 2,3,7,8-TCDD. Such discrepancies are unimportant, since RIA is proposed as a screening method for the detection, not the determination, of PCDDs in environmental samples.

Several contradictions are apparent between the GC-MS and RIA results. No TCDD was detected in the lake trout #1 fraction D, and

ocean haddock fraction D samples using GC-MS, whereas > 20 and 12 ng respectively were detected using RIA. These observations may result from the presence of positive interferences, or PCDD isomers other than 2,3,7,8-TCDD, in the sample extract; the anti-dioxin antibodies are known to cross-react with other PCDD and PCDF isomers. Fraction E of the acid digested lake trout #7 sample, was the only sample tested that gave a false negative result using RIA. The remainder of the samples, in which no TCDD was detected using GC-MS, yielded zero or low readings using RIA.

The foregoing results illustrate the importance of interfacing the RIA for PCDDs with a sample preparation procedure that adequately removes low level matrix interferences. Furthermore, a means of differentiating pg quantities of PCDDs from background matrix interferences must be devised so as to reduce the impact of low level false positive results. The success of these two measures, which are the subject of current research, will influence the assay's detection limit and its ultimate usefulness.

4.1 Preparation, Characterization, and Evaluation of Labelled Hapten

4.1.1 Hapten stability

The hapten (1-N-(5-iodovaleramido)-3,7,8-dibenzo-p-dioxin) was unstable in dry acetone at room temperature. TLC analysis of the hapten after two weeks storage under such conditions indicated the presence of a main spot (R_f .43 and a weak spot (R_f .38). Dry storage of a second batch of hapten at -80°C under nitrogen appeared to correct the instability problem; TLC analysis of the unlabelled hapten indicating the presence of a single spot (R_f 0.71).

4.1.2 Hapten degradation during labelling reaction

Over a period of several months, four batches of hapten were labelled and evaluated using chemical and immunoassay procedures; the results of these experiments are summarized in Table 2. Initially, the first batch of hapten, which had been stored at room temperature, was labelled with ^{125}I. The fractions described in Table 3 were eluted from the silica gel chromatogrphy column: TLC analysis of fraction 4, which contained 91% of the organically bound radioactivity, indicated the presence of two radioactive spots (Figure 5a): one spot (R_f .48) contained 59%, and the other (R_f .21) 41% of the applied radioactivity. The specific activity of the labelled hapten (Table 2) was substantially less than was obtained by Albro et al. (1979) (78 Ci/mmol).

Sixty-nine percent of the radioactivity from fraction 4 was lost during solvent removal, which indicated the presence of a volatile breakdown product: possibly a portion of the hapten's aliphatic side chain. Forty percent of the radio-tracer was bound at an antiserum dilution of 1:250 (Figure 4), which was lower than the expected dilution of approximately 1:9000 (Luster, personal communication). There are several possible explanations for the latter observation. Deterioration of the serum could have reduced the ability of the antibodies to bind the labelled hapten, however, this possibiity was at least partially eliminated by the assay calibration curve subsequently prepared using unlabelled hapten (Figure 4(b)). Either or both of the labelled compounds detected in fraction 4 may have had a low affinity for the antibodies to PCDD. The presence in the labelled hapten reagent of unlabelled molecules (such as amino-3,7,8-TCDD or deiodinated hapten) capable of being preferentially bound by antibodies to either TCDD or the adipamide spacer molecule, could also have been a contributory factor. Such impurities could have arisen through deterioration of the hapten during either the labelling reaction or storage, and could, through competitive binding, adversely affect the amount of labelled hapten bound by the anti-dioxin antibodies.

The labelled and unlabelled hapten were tested for the ability to compete for antibody binding sites; a characteristic standard curve (Figure 4(b)) was obtained. Thus, one or both of the compounds identified in the unlabelled hapten preparation competed effectively for antibody binding sites with one or both of the radioactive components of fraction 4. Since Alb5 was originally selected for low recognition of the adipamide spacer arm, it may be that the anti-dioxin antibodies have a high affinity for 1-amino-3,7,8-TCDD. An attempt to prepare a calibration curve using TCDD standards yielded a flat curve that showed only a slight response to TCDD at high concentrations (Figure 4(c)). Thus, TCDD was unable to compete effectively for antibody binding sites with the labelled components of Fraction 4. These observations suggest that the antibodies may have a higher affinity for 1-amino-3,7,8-TCDD than for 2,3,7,8-TCDD.

A second hapten preparation that had been stored at -85°C under nitrogen yielded qualitatively different results than those obtained using the first preparation.

1. Fraction 2 (Table 4) yielded an apparent doublet spot (R_f .6) (Figure 5b) that contained 45% of the applied radioactivity. The observed loss of radioactivity (70%) from this fraction during solvent removal probably explains the spot's low activity; the volatile radioactivity was presumably a portion of the hapten's aliphatic side chain. The doublet nature of the peaks suggests that more than one labelled compound with similar R_f values were present.
2. Fraction 3 yielded no peaks and presumably contained a volatile component that evaporated from the plate during solvent removal.
3. Fraction 4 yielded a single peak with an R_f value of .35 which contained 95% of the applied activity (Figure 5d).
4. Fraction 5 yielded a single peak with an R_f value of .34 which was probably the tail of fraction 4 (Figure 5c).

The TLC data indicate that the hapten underwent degradation during the labelling reaction. The ability of the antiserum to bind the radioactive component of fraction 4, but not of fraction 2 indicates that the nondegraded hapten was eluted from the silica column in fraction 4.

A third batch of hapten yielded similar results when labelled with ^{125}I. TLC analysis of fraction 3 (Table 5), before solvent removal, yielded a single spot (R_f .48); however, the peak had two apparent shoulders which indicate that it may have contained more than one closely related compound (Figure 6a). Analysis of fraction 4 yielded (Figure 6b), minor (R_f .33), and major (R_f .41) peaks. Thirty-five percent of the radioactivity in fraction 3, and 75% of the radioactivity in fraction 4 were bound by an antiserum dilution of 1:250.

4.2 Improvement of Hapten Purificaton Procedure

During the purification of the third batch of labelled hapten, the radioactivity was eluted from the column in two main peaks, which were collected in fractions 3 and 4 (Table 5) respectively. Refractionation of fraction 3 indicated that the bulk of the volatile radioactivity was located in fractions 4 and 5 (10 mL fractions) (Table 6). Fractions 7 and 8 contained a radioactive compound with high affinity for Alb5, and yielded similar assay calibration curves to the unfractionated fraction 4 - indicating that they contained the same compound. Henceforth, all post labelling chromatographic separations were collected in 10 mL fractions, the radioactivity of each fraction was determined, the fractions were then examined for the presence of volatile components and finally tested for the presence of labelled hapten by challenging with anti-dioxin antibodies.

The repeatability of the labelling procedure and the performance of the revised separation procedure were further assessed by labelling a fourth batch of hapten with ^{125}I. Each fraction was

counted, examined for the presence of volatile components and tested for its ability to bind with excess antiserum (Table 7).

Fractions #5 and #8 were analyzed using TLC. Fraction 5 was found to contain at least three peaks (R_f .25, .33, and .39) (Figure 7b). Fraction 8 contained a single peak (R_f .48) with an apparent shoulder at R_f .51 (Figure 7a), and contained a compound with a high affinity for the antidioxin antibodies. The foregoing results demonstrate the adequacy of the separation procedure.

4.3 Antiserum Standardization

The antiserum was standardized against each batch of labelled hapten. A curve typical of those obtained during the earlier experiments is presented in Figure 8a. The amount of antiserum required to bind 40% of the radioactivity in the assay tubes decreased with the improved hapten purification procedure used in the later experiments (Table 2). However, the specific activity of the reaction product was variable (Table 2).

4.4 Assay Calibrations

Typical assay calibration curves for the second, third and fourth batches of hapten are presented in Figures 8 and 9 respectively. It was possible to detect 100 pg of 2,3,7,8-TCDD using the Cutscum version of the assay (Table 2). Considering that the assay has yet to be brought to the limits of its performance capabilities, the levels of 2,3,7,8-TCDD detected were satisfactory. The similarity of the assay calibration curves obtained using the different hapten preparations illustrates the repeatability of the RIA for PCDDS.

4.5 Elimination of Volatile and Unbindable Hapten Breaddown Products

In an effort to eliminate the production of radioactive impurities during the labelling reaction, an hydroxide free I preparation was evaluated (New England Nuclear NEZ-33L); the labelling procedure was otherwise unmodified. The fractions described in Table 8 were eluted from the silica gel column. The bindable radioactivity was eluted in a single peak. No peaks of unbindable or volatile organically bound radioactivity were recovered. Fractions 13 and 14 yielded assay calibration curves similar to those obtained using the third hapten preparation.

4.6 Triton Based Assay

The RIA was also evaluated using the Triton based assay system, in which Triton replaced Cutscum as the TCDD solubilization agent. The antiserum standardization curve (Figure 10a) differed from those obtained for the Cutscum assay system (Figure 8a); 35% binding occurred at an antiserum dilution of 1:13,888, which indicates that Triton may inhibit antibody binding less than Cutscum. The Triton assay's calibration curve had a steeper slope (Figure 10b) than the Cutscum assays; it was possible to distinguish 50 pg of TCCC from zero concentration ($P < 0.05$) with the Triton assay.

5.0 DISCUSSION

The preliminary data presented, while not conclusive, demonstrated that the RIA can detect PCDDs in fish, thus complementing the observation of McKinney et al., (1981) that RIA could be used to detect 2,3,7,8-TCDD in human adipose tissue. The false positive results might have been caused by matrix interferences, assay variability, or the presence in the samples of PCDD isomers other than 2,3,7,8-TCDD. Selection

and optimization of an appropriate sample preparation method should help to eliminate positive interferences in sample extracts. The cleanup procedure used during the initial evaluation has since been superseded by an improved method (Afghan and Wilkinson, 1984). The use of matrix blanks and control samples covering a range of analyte concentrations would further reduce the effect of low level positive interferences, assay variability, and assay instability. The detection of PCDD isomers other than 2,3,7,8-TCDD or of PCDFs, is not considered to be detrimental, since the presence of such compounds in environmental samples is also cause for concern. In an environmental monitoring program, samples containing such cross-reacting compounds would also have to be further analyzed using GC/MS.

The observed instability of the hapten in acetone at room temperature is probably a consequence of the weak C-I bond. Because iodide is a good leaving group. special procedures must be employed for the manipulation and storage of the hapten. Loss of iodide from the hapten would cause a decrease in the specific activity of the labelled hapten, an effect that is consistent with the results obtained using the first batch of labelled hapten. Cyclization of the hapten, which is thought to occur in acetone, could lead to a decreased ability to take up label, and might also interfere with the ability of the antibodies to bind the hapten by a stearic hindrance mechanism.

Deterioration of the hapten during the labelling reaction may also have contributed to the initial difficulties encountered with the labelling procedure. The iodide tracer solution supplied by Amersham contains hydroxide ion as a stabilization agent; hydrolysis of the hapten, by this hydroxide ion, would yield a radio-labelled product that could probably be bound by antibodies to the adimpamide linkage group, against which TCDD would be unable to compete for binding sites. Furthermore, the unlabelled 1-amino,2,3,7,8-TCDD so generated could also be bound by anti-dioxin antibodies, thus further interfering with assay kinetics. The losses of radio-tracer observed during the removal of solvent from the labelled hapten preparation probably resulted from the

production of volatile breakdown products during the labelling reaction.The labelled compounds that were unbindable by Alb5 were probably generated in a similar manner. The problem was solved by the use of an hydroxide free tracer preparation.

Arising from the foregoing observations, several improvements, mainly designed to protect the hapten's iodide component were made to the hapten storage procedure; the result was a functioning assay with which it was possible to prepare a TCDD calibration curve. However, both the curve's slope and the assay's performance at low concentrations indicated that further improvements were required.

The key to further progress was the observation that finer fractionation of the reaction products facilitated the separation of the desired radio-labelled dioxin derivative from volatile breakdown products and from compounds that were unbindable by the antibodies to TCDD. The improved procedure resulted in a calibration curve that had an LDC of 100 pg of 2,3,7,8-TCDD. The indications are that the Triton based assay can outperform the Cutscum based assay at low 2,3,7,8-TCDD levels (LDC = 50 pg).

In conclusion, the data demonstrate that RIA can be used to detect 2,3,7,8-TCDD, the most toxic and environmentally important of the PCDDs, in a variety of fish species. The hapten labelling procedure was found to be repeatable and a working capability in the assay procedure was established. Future reports will describe the detailed characterization of the RIA in terms of assay repeatability, precision, detection limits and cross reactivity. The assay will then be interfaced with an appropriate extraction and cleanup procedure as a pre-requisite for the use of the RIA in routine environmental analysis. Efforts are being made to modify or optimize the assay so as to obtain as low a detection limit as possible in view of the growing concern about ultra-trace levels of PCDDs in the environment. These investigations should lead to a useful means of screening a variety of environmental matrices for the presence of PCDDs, in addition to being a valuable confirmatory procedure for GC-MS.

REFERENCES

Afghan, B.K. and R.J. Wilkinson. Cleanup and Isolation Procedure for the Analysis of Dioxins and Furans in Environmental Samples. NWRI Internal Report #112-AMD-3-84-BKA. 1984.

Albro, P.W., M.I. Luster, K. Chae, S.K. Chaudhary, G. Clark, L.D. Lawson, J.T. Corbett and J.D. McKinney. 1979. A Radioimmunoassay for Chlorinated Dibenzo-p-Dioxins. Toxicol. App. Pharmacol. 50:137-146.

Albro, P.W. M.I. Luster, K. Chae, G. Clark and J.D. McKinney. 1982. Radioimmunoassay of Chlorinated Dibenzo-p-Dioxins. Methods Enzymol. 84:619-628.

Bradlaw, J.A. and J.L. Casterline, Jr. 1979. Induction of Enzyme Activity in Cell Culture: A Rapid Screen for Detection of Planar Polychlorinated Organic Compounds. J. Assoc. Off. Anal. Chem. 62:904-916.

Chae, K. L.K. Cho and J.D. McKinney. 1977. Synthesis of 1-amino-3,7,8-trichlorodibenzo-p-dioxin and 1-amino-2,3,7.8-tetrachloro-dibenzo-p-dioxin as haptenic compounds. Agricu. Food Chem. 25: 1207-1209.

Hemingway, R.J., N. Aharonson, P.A. Greve, T.R. Roberts and H.P. Thier. 1984. Improved Cost-Effective Approaches to Pesticide Residues Analysis. Pure & App. Chem. 56:1131-1152.

Holleman, A.F. 1920. Recl. Trav. Chim. Pay-Bas 39:435.

Huber, S.J. 1985. Improved Solid-Phase Enzyme Immunoassay Systems in the ppt Range for Atrazine in Fresh Water. Chemosphere 14:1795-1803.

Langone, J.L. and H. Van Vunakis. 1975. Radioimmunoassay for Dieldrin and Aldrin. Res. Commun. Chem. Pathol. and Pharmacol 10:163-171.

Luster, M.I. P.W. Albro, K. Chae, L.D. Lawson, J.D. Corbett, and J.D. McKinney. 1980. Radioimmunoassay for Quantitation of 2,3,7,8-tetrachloridibenzofuran. Anal. Chem. 52:1497-1500.

McKinney, J., P. Albro, M. Luster, B. Corbett, J. Schroeder, and L. Lawson. Development and Reliability of a Radioimmunoassay for 2,3,7,8-tetrachlorodibenzo-p-dioxin. In: Chlorinated Dioxins and Related Compounds. Ed. O. Hutzinger et al., Pergamon Press Oxford and New York, pp. 67-75. 1982.

Newsome, W.H. and H.B. Shields. 1981a. Radimmunoassay of PCBs in Milk and Blood. Intern. J. Environ. Anal. Chem. 10:295-304.

Newsome, W.H. and J.B. Shields. 1981b. A Radioimmunoassay for Benomyl and Methyl 2-benzimidazolecarbamate on Food Crops. J. Agric. Food Chem. 29:220-222.

Poland, A., E. Glover, and A.S. Kende. 1976a. Sterospecific High Affinity Binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by Hepatic Cytosol. J. Biol. Chem. 251:4936-4946.

Rinder, D.F. and J.R. Fleeker, 1981. A Radioimmunoassay to Screen for 2,4-dichlorophenoxyacetic acid and 2,4,5-trichloroacetic Acid in Surface Water. Bull. Environm. Contam. Toxicol. 26:375-380.

Schwalbe, M., E. Dorn and K. Beyermann. 1984. Enzyme Immunoassay and Fluoroimmunoassay for the Herbicide Diclofop-methyl J. Agric. Food Chem. 32:734-741.

Sherry, J.P., J. Apsimon, L. Collier, B.K. Afghan and P.W. Albro. 1988. Radioimmunoassay for the Detection of Polyclorinated Dibenzo-p-Dioxins in Environmental Samples: Method Descritpion. NWRI Contribution 88-73.

Yalow, R.W. and S.A. Berson. 1960. Immunoassay of Endogenous Plasma Insulin in Man. J. Clin. Invest. 39:1157.

LIST OF FIGURES

TABLE 1

Analysis of Fish Samples for 2,3,7,8-TCDD Using RIA and GC-MS

Sample	Lipid %	Fraction	GC-MS (ng)	RIA (ng)
Lake Trout #1	18.5	D[1]	ND[2]	> 20
Lake Trout #1	18.5	F[3]	ND	0
Ocean Haddock	0.4	D	ND	12.0
Ocean Haddock	0.4	F	ND	0.3
Lake Trout #2	24.7	D	3.4	> 20
Lake Trout #2	24.7	F	-	> 20
Rainbow Trout	19.8	D	3.7	> 20
Rainbow Trout	19.8	F	ND	0.6
Lake Trout #7[4]	35.6	E	0.8	0
Lake Trout #7[4]	35.6	F	ND	0.19
Lake Trout #7[4]	35.6	E+F	ND	0
Lake Trout #7	35.6	E	4.0	> 20
Lake Trout #7	35.6	F	ND	0.16
Fish Sample #10	-	E+F	ND	0.5
Fish Sample #13	-	E+F	ND	0.5
2,3,7,8-TCDD	-	([5])	10.0	> 20

1 15% ethyl acetate fraction, passed through Al_2O_3 on 40% $MeCl_2$/hexane

2 ND: none detected

3 25% ethyl acetate fraction, passed through Al_2O_3 on 40% $MeCl_2$/hexane

4 Hexane extraction of HCl digested fish

5 Standard run through GPC column, fraction 30-40 min at a flow rate of 5 mL/min, collected.

TABLE 2

Repeatability of the RIA for PCDDs

Hapten Batch	Specific Activity (Ci/mmol)	40% Dilution	LCD[2] (pg)
1	1.3	1:250	nd
2	64.5	1:2900	470
3	26.9	1:4760	200
3b	-	1:6896	100
4	40.2	1:8300	50[1]

[1] Triton Assay

[2]Lowest concentration of 2,3,7,8-TCDD distinguishable from control ($P < 0.05$)

TABLE 3

Fractionation of Eluent from Chromatography Column; Hapten Preparation #1

Fraction	Volume (mL)	Activity (cpm)
2	15	1.4×10^6
3	30	2.6×10^6
4	35	3.9×10^7

TABLE 4

Fractionation of Eluent from Chromatography Column; Hapten Preparation #2

Fraction	Volume (mL)	Activity (cpm)
2	20	1.4×10^7
3	17	6.6×10^7
4	40	2.0×10^9
5	11.5	9.9×10^7

TABLE 5

Fractionation of Eluent from Chromatography Column; Hapten Preparation #3

Fraction	Volume (mL)	Activity (cpm)
1	15	4.3×10^6
2	14	6.34×10^6
3	40	7.92×10^8
4	27	1.12×10^6

TABLE 6

Refractionation of Fraction 3

Fraction	Activity (cpm)	Blow-Down Loss %	% Bound
1	8.6×10^4	-	-
2	2.4×10^5	-	-
3	6.6×10^5	-	-
4	1.0×10^6	69	3.5
5	1.3×10^6	49	47.4
6	3.4×10^6	0	72.1
7	8.8×10^6	0	77.0
8	8.4×10^6	0	77.0
9	1.7×10^6	0	72.4
10	5.4×10^5	0	-

TABLE 7

Fractionation of Eluent from Chromatography Column; Hapten Preparation #3, Second Labelling Experiment

Fraction	Activity (cpm)	Blow-Down Loss %	Bound %
1	1.26×10^6	nd	nd
2	4.81×10^6	nd	nd
3	8.8×10^7	98.5	0.6
4	4.5×10^8	98.2	1.5
5	4.8×10^8	98.5	15.4
6	1.95×10^8	94.0	65.7
7	4.98×10^7	22.5	88.4
8	6.05×10^7	0.0	84.9
9	4.02×10^7	13.0	61.3
10	8.1×10^6	nd	nd
11	2.3×10^6	nd	nd

TABLE 8

Fractionation of Eluent from Chromatography Column; Hapten Preparation #3, Labelled Using OH^- Free Radio-Tracer

Fraction	Activity (cpm)	Blow-Down Loss %	Bound %
8	Trace	0	nd
9	Trace	0	nd
10	Trace	0	nd
11	7.1×10^6	0	nd
12	1.59×10^7	0	77
13	2.52×10^7	0	76
14	2.51×10^7	0	76
15	1.65×10^7	0	68.6
16	8.6×10^6	0	nd

FIGURE 1

Key Reagents Required for the Development of an RIA for the Detection of a Low Molecular Weight Compound

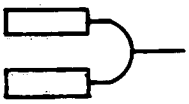
a)  COMPOUND OF INTEREST

b) 

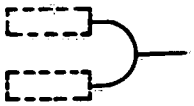
LINKAGE GROUP

c)  = ANTIGEN 'A'

d) ANTISERUM CONTAINS A FAMILY OF ANTIBODIES:

1. TO  Ab_1

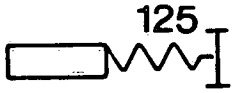
2. TO  Ab_2

3. TO  Ab_3

AND TO VARIOUS SUBCOMPONENTS.

e) REMOVE Ab_2 FROM SERUM BY AFFINITY CHROMATOGRAPHY

f) LABELLED HAPTEN:



γ EMITTER

$\frac{1}{2}$ LIFE = 2 MONTHS

FIG.1 PREPARATION OF ANTIBODY AND LABELLED HAPTEN

FIGURE 2

Schematic Representation of an RIA for the Detection of a Low Molecular Weight Compound

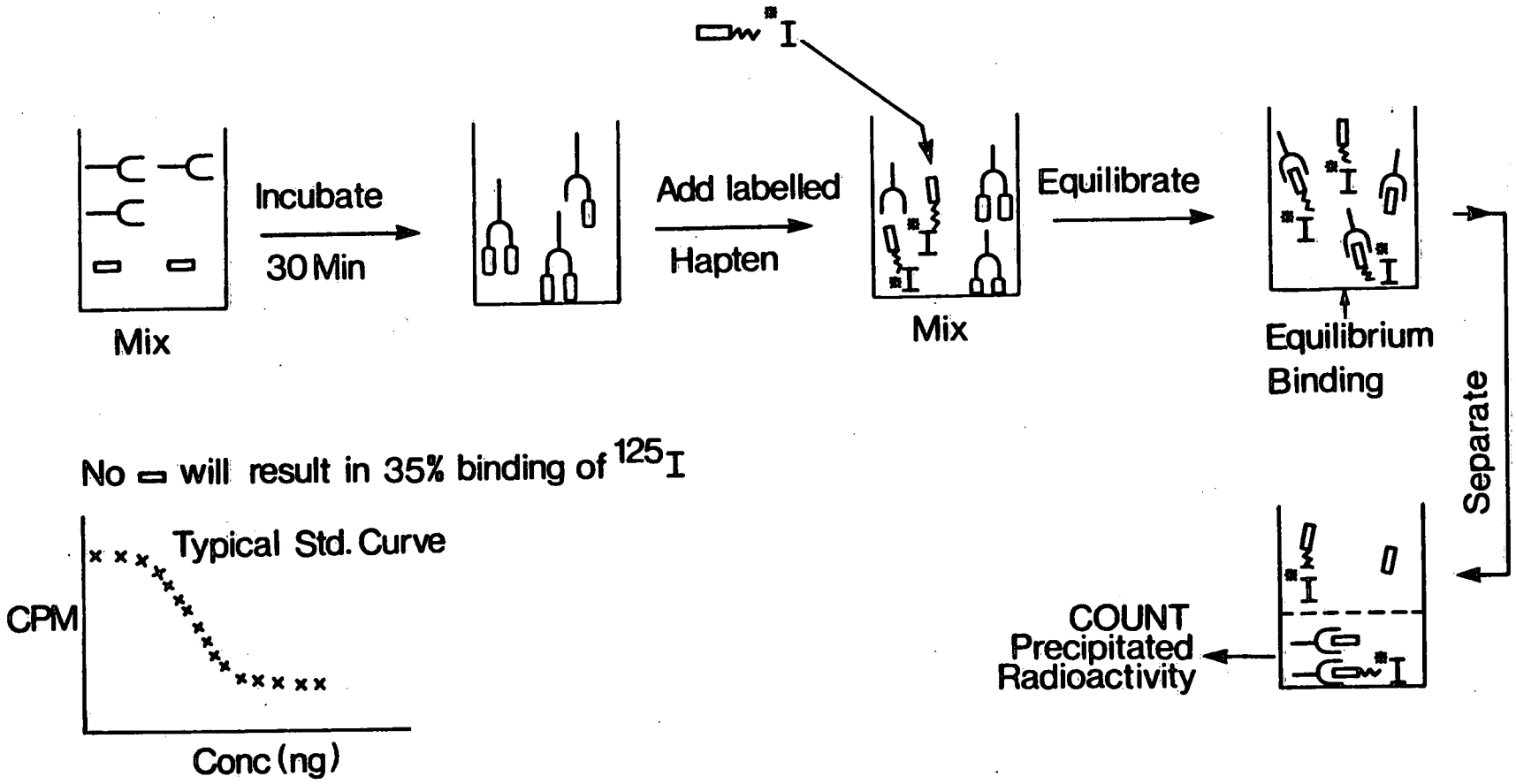


FIG 2: RIA ASSAY PROCEDURE (SCHEMATIC)

FIGURE 3

Molecular Formulae of Hapten Related Compounds

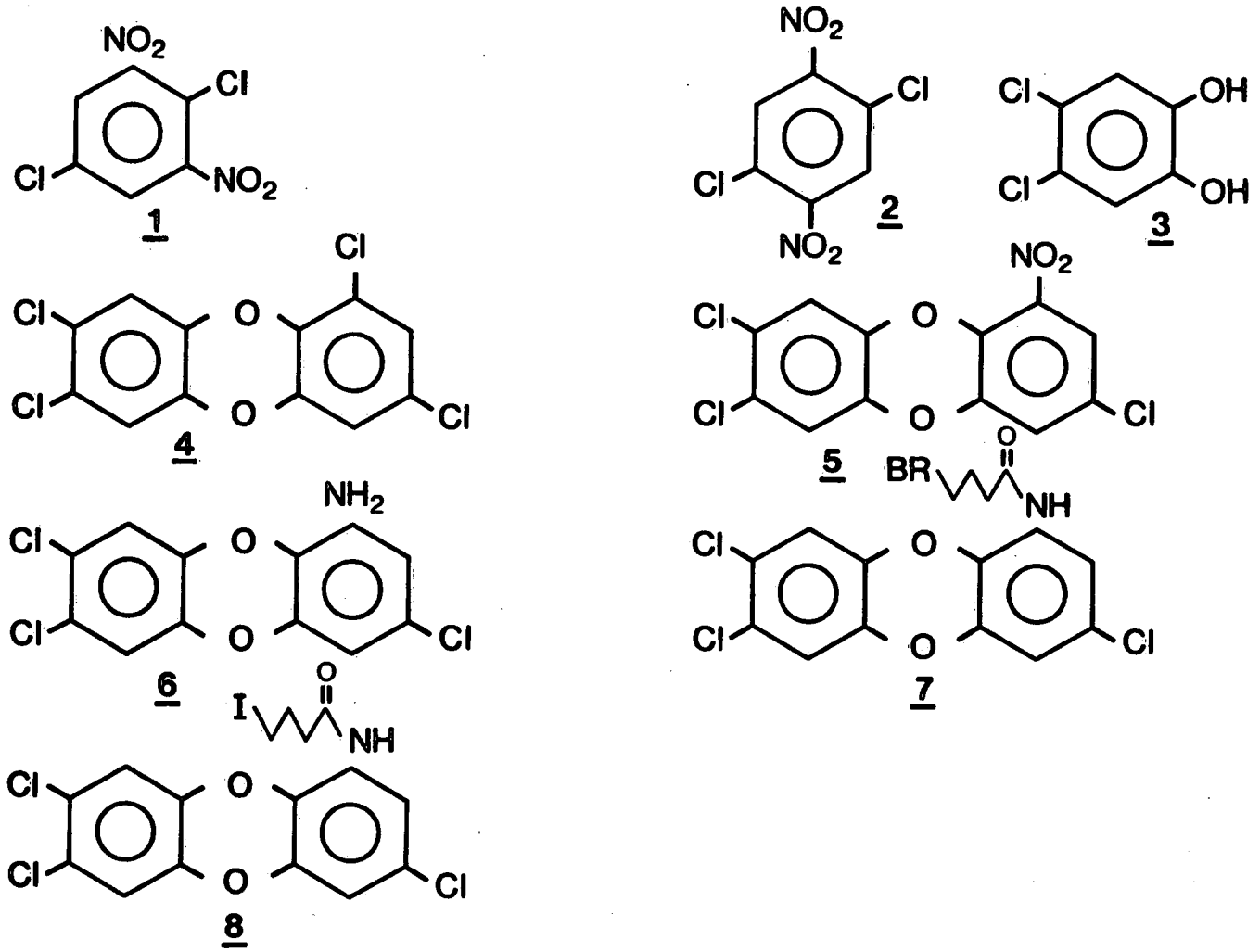

NO2
Cl
Cl
NO2
1
Cl
Cl
O
Cl
O
Cl
4
NH2
Cl
Cl
O
O
Cl
6
O
I
NH
Cl
Cl
O
O
Cl
8
NO2
Cl
Cl
NO2
2
Cl
OH
Cl
OH
3
NO2
Cl
Cl
O
O
Cl
5
O
BR
NH
Cl
Cl
O
O
Cl
7

Figure 3.

FIGURE 4

First Batch of Labelled Hapten

a) Standardization of Antiserum Against Fraction 4

b) Determination of Unlabelled Hapten Using the RIA for PCDDS

c) Determination of 2,3,7,8-TCDD Using the RIA for PCDDs

Figure 4.

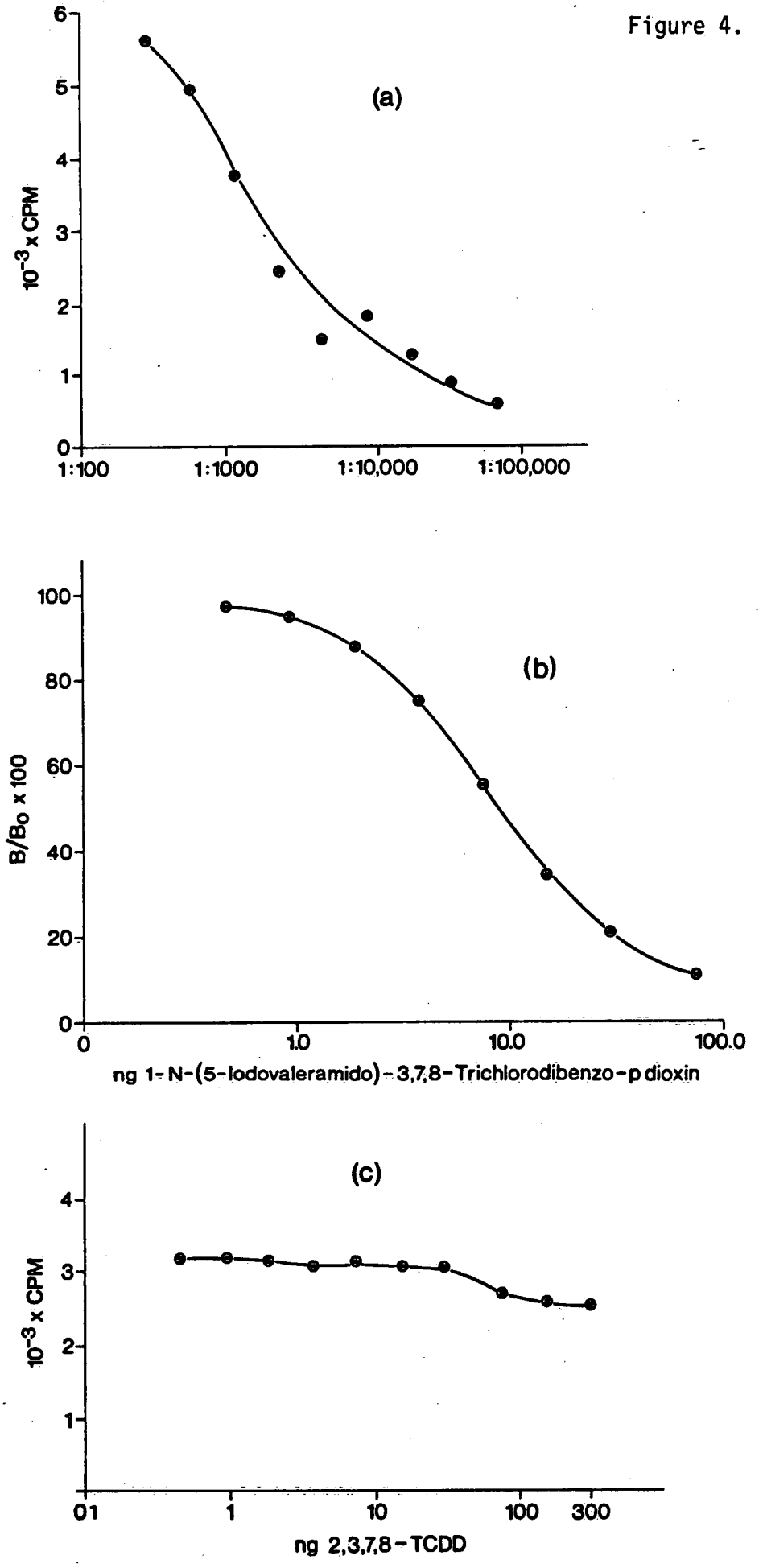

(a)
10^{-3} x CPM
6
5
4
3
2
1
0
1:100
1:1000
1:10,000
1:100,000
(b)
B/Bo x 100
100
80
60
40
20
0
0
1.0
10.0
100.0
ng 1-N-(5-Iodovaleramido)-3,7,8-Trichlorodibenzo-p dioxin
(c)
10^{-3} x CPM
4
3
2
1
01
1
10
100
300
ng 2,3,7,8-TCDD

FIGURE 5

Tlc Analysis of Labelled Hapten Preparations:

a) First Batch of Labelled Hapten, Fraction 4
b) Second Batch of Labelled Hapten, Fraction 2
c) Second Batch of Labelled Hapten, Fraction 5
d) Second Batch of Labelled Hapten, Fraction 4

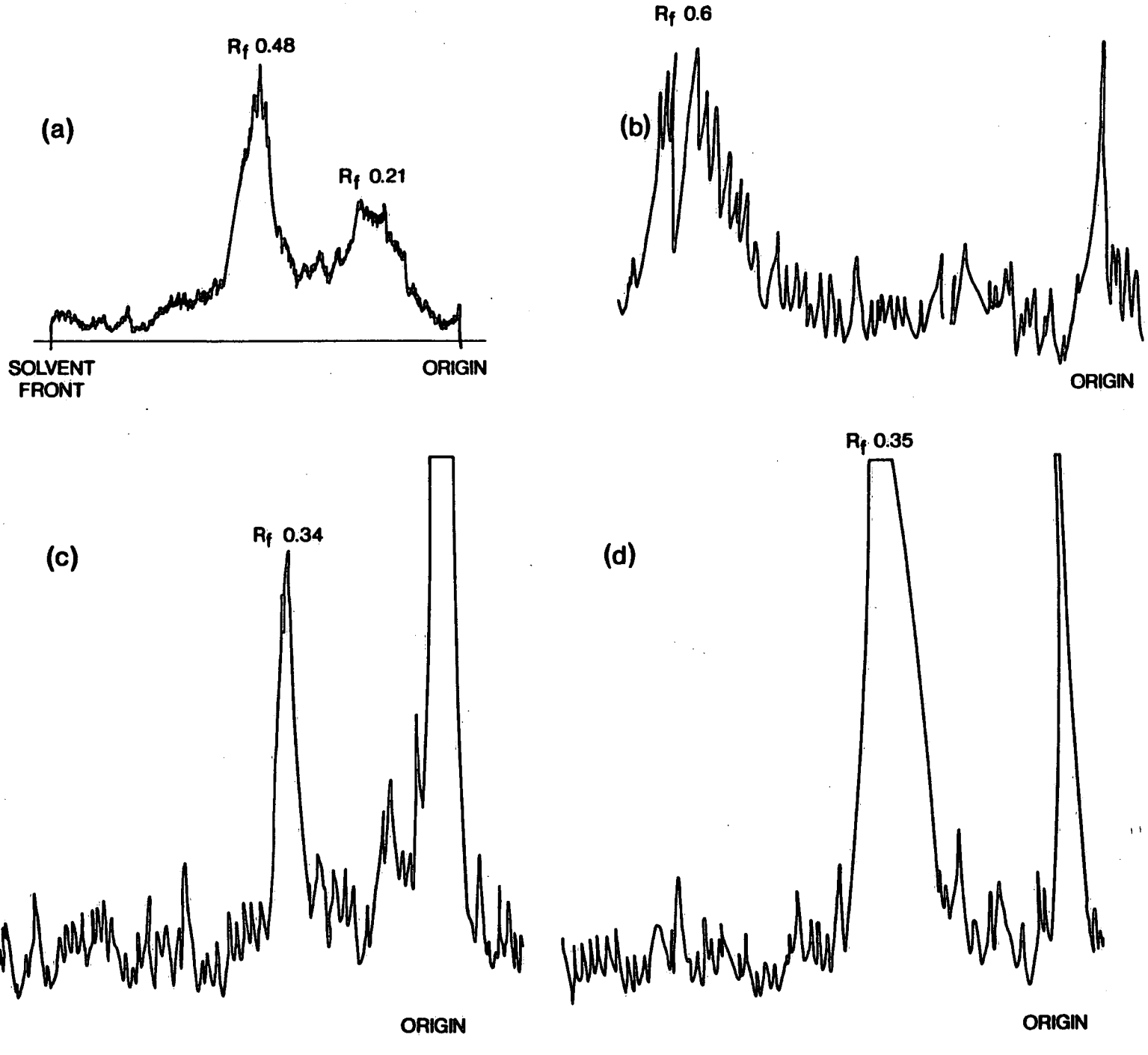

(a)
Rf 0.48
Rf 0.21
SOLVENT FRONT
ORIGIN
(b)
Rf 0.6
ORIGIN
(c)
Rf 0.34
ORIGIN
(d)
Rf 0.35
ORIGIN

Figure 5.

FIGURE 6

Tlc Analysis of Third Batch of Labelled Hapten:

a) Fraction 3

b) Fraction 4

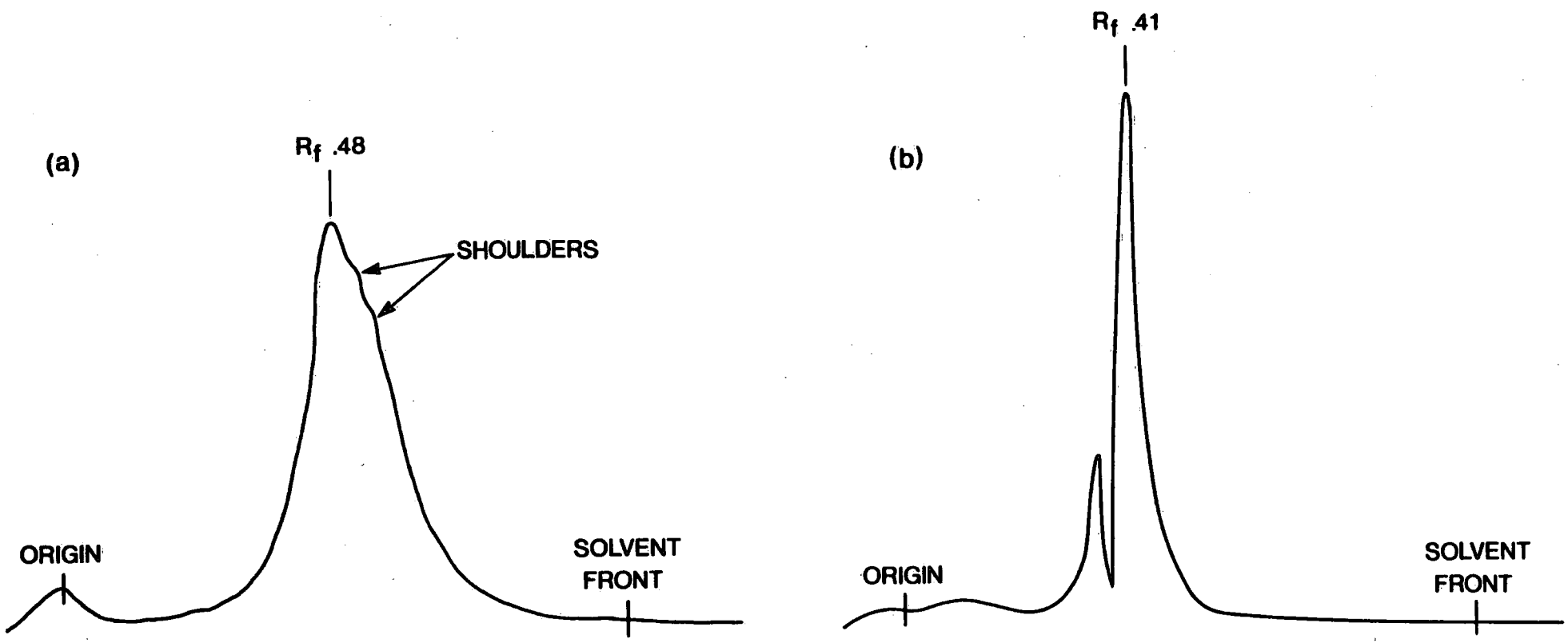

(a)
R_f .48
SHOULDERS
ORIGIN
SOLVENT
FRONT
(b)
R_f .41
ORIGIN
SOLVENT
FRONT

Figure 6.

FIGURE 7

Tlc Analysis of Fourth Batch of Labelled Hapten:

a) Fraction 8

b) Fraction 4

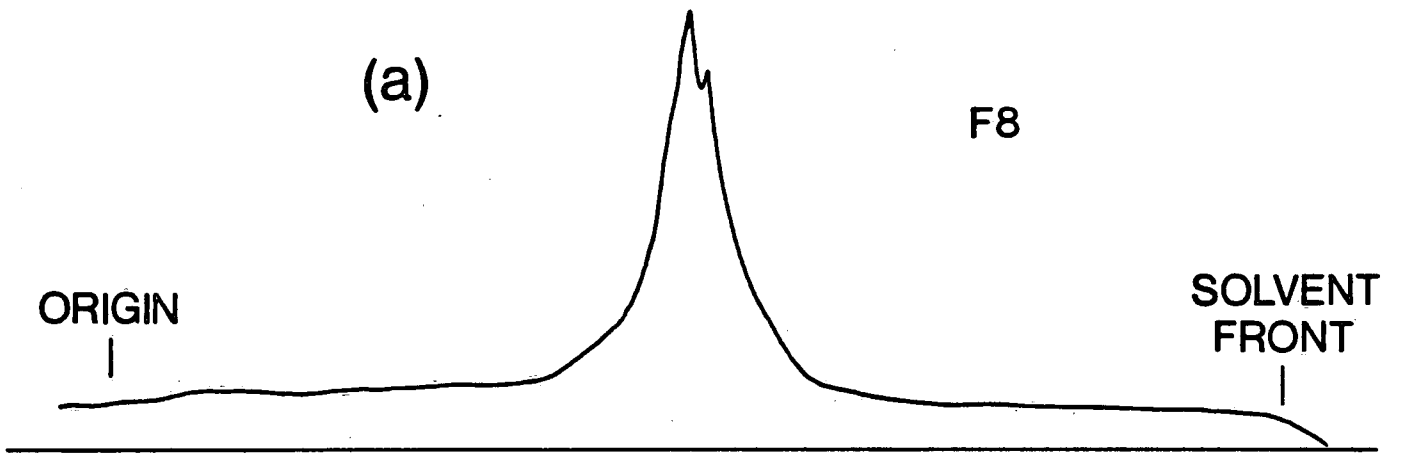


Figure 7.

FIGURE 8

a) Standardization of Antiserum Against Fraction 4 of the Third Batch of Labelled Hapten

b) Determination of 2,3,7,8-TCDD,; Assay Calibration Curve

Figure 8.

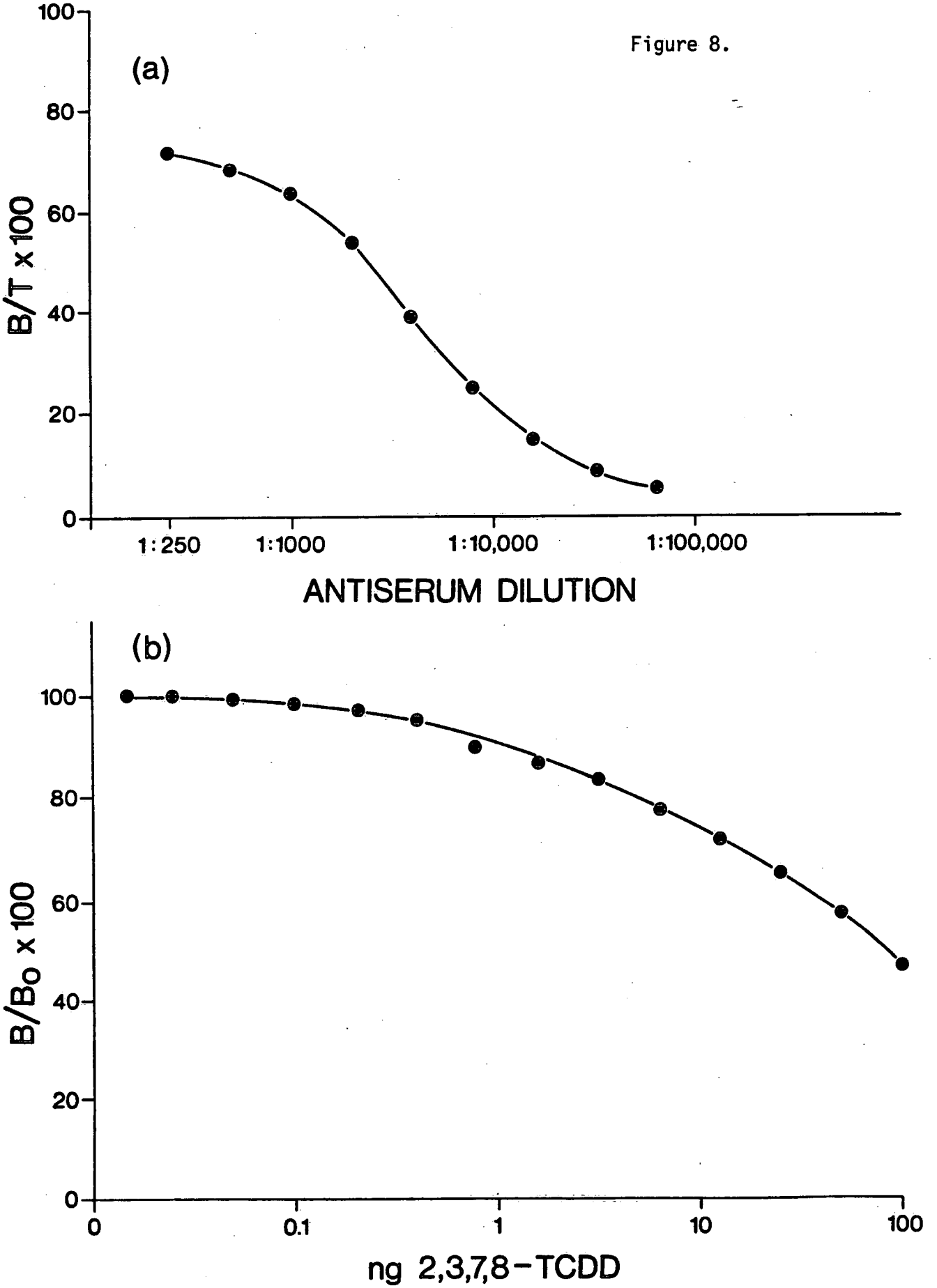
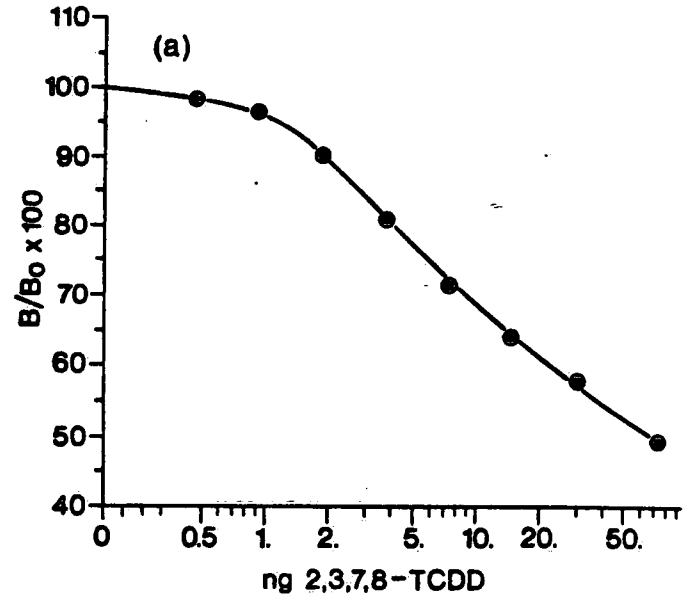
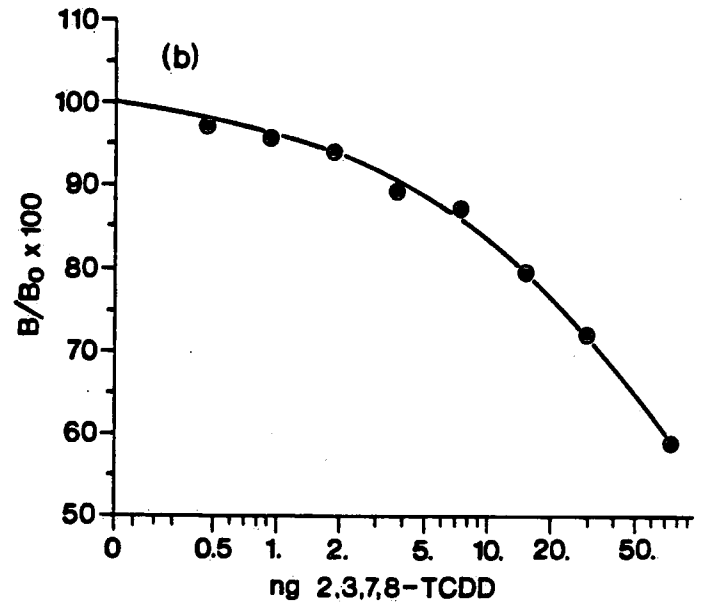

(a)
100
80
60
40
20
0
B/T x 100
1:250
1:1000
1:10,000
1:100,000
ANTISERUM DILUTION
(b)
100
80
60
40
20
0
B/Bo x 100
0
0.1
1
10
100
ng 2,3,7,8-TCDD

FIGURE 9

Determination of 2,3,7,8-TCDD; Assay Calibration Curves:
a) and b) Prepared with Third Batch of Labelled Hapten
d) Prepared with Fourth Batch of Labelled Hapten


(a)
110
100
90
80
70
60
50
40
B/Bo x 100
0
0.5
1.
2.
5.
10.
20.
50.
ng 2,3,7,8-TCDD


(b)
110
100
90
80
70
60
50
B/Bo x 100
0
0.5
1.
2.
5.
10.
20.
50.
ng 2,3,7,8-TCDD

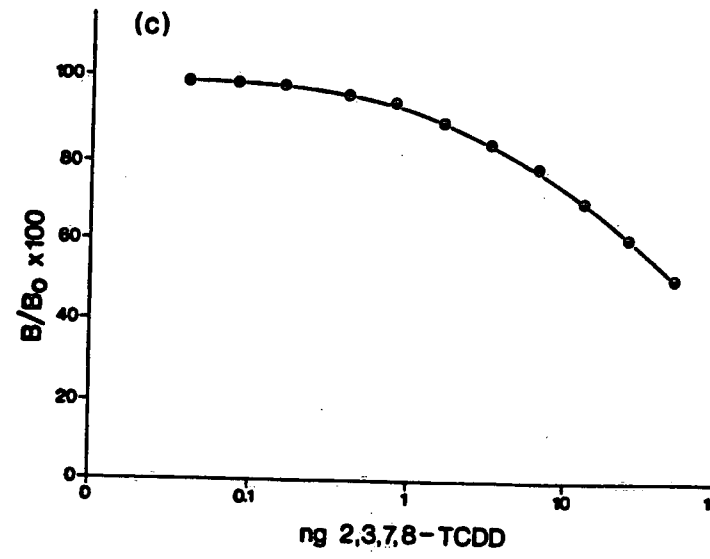

(c)
100
80
60
40
20
0
B/Bo x100
0
0.1
1
10
100
ng 2,3,7,8-TCDD

Figure 9.

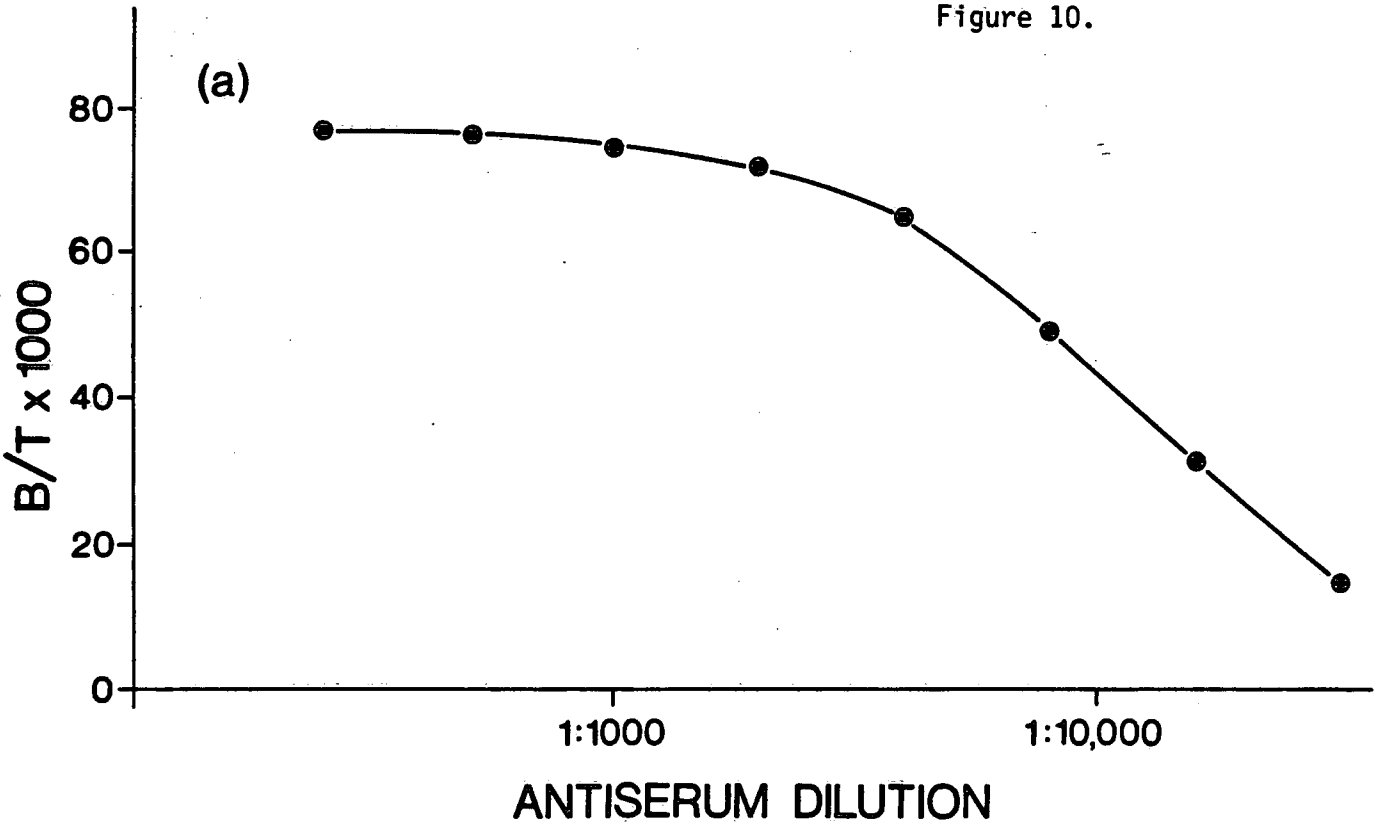
FIGURE 10

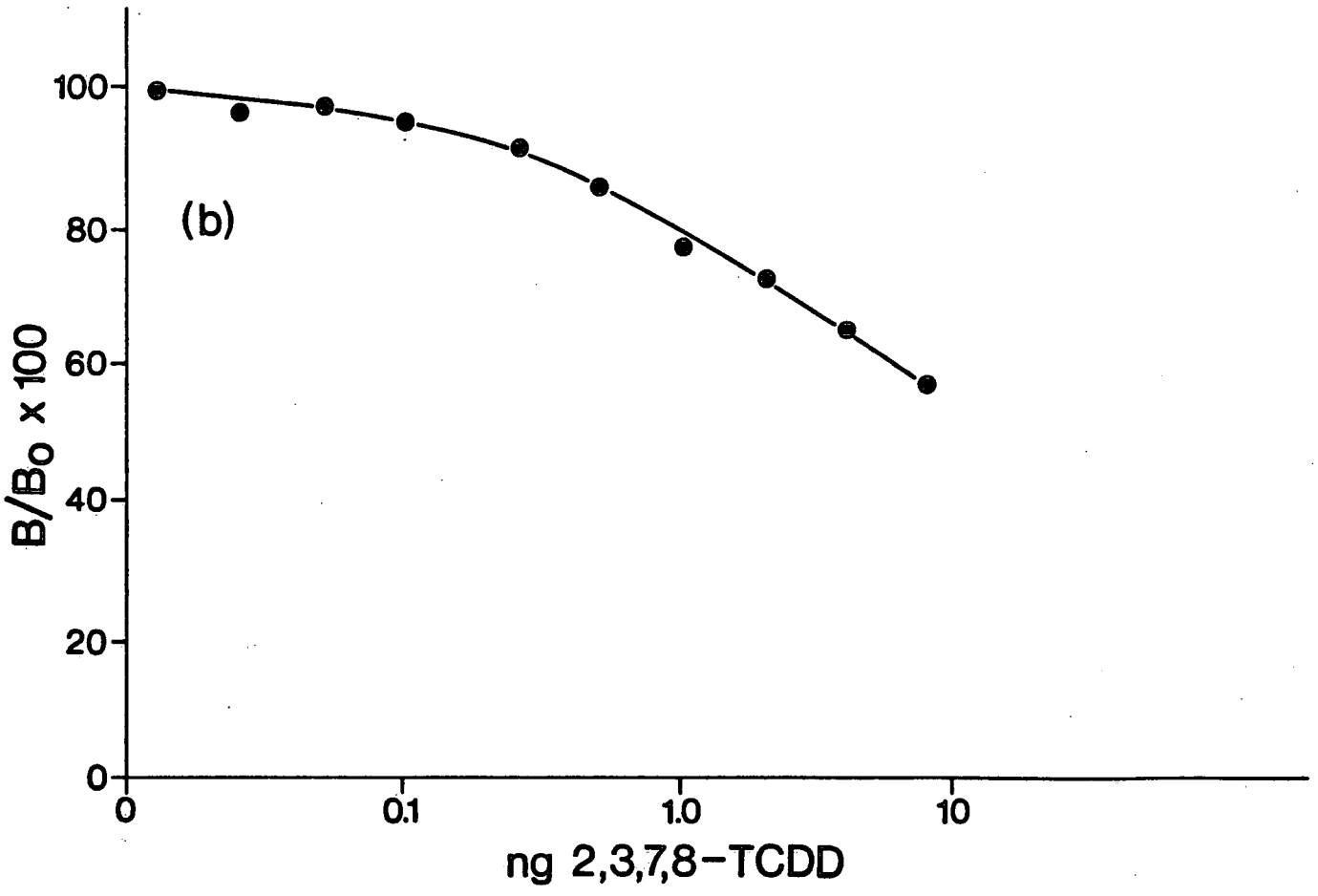
Triton Based Assay System:

a) Standardization of Antiserum Against Combined Fractions 7 and 8 from the Re-Fractionated Fraction 3.

b) Determination of 2,3,7,8-TCDD; Assay Calibration Curve

Figure 10.


(a)
80
60
40
20
0
B/T x 1000
1:1000
1:10,000
ANTISERUM DILUTION


100
80
60
40
20
0
(b)
B/Bo x 100
0
0.1
1.0
10
ng 2,3,7,8-TCDD