SOLID-PHASE ANTIBODY RADIOIMMUNOASSAY OF ILE^5-ANGIOTENSIN-I FOR RENIN ACTIVITY

by

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INTRODUCTION

Radioimmunoassay

In the latter part of the 1950's, Berson and Yalow demonstrated the presence of insulin antibodies in insulin resistant diabetic patients. These patients had been treated with other than species specific insulin preparations and presumably, had made antibodies against the foreign hormone.

It was found that these insulin antibodies had the ability to competitively bind insulin which had been labeled with ^{131}I as well as unlabeled insulin. From these observations, Berson and Yalow developed the first radioimmunoassay (RIA) in 1959 (8) and presented a clinically useful method in 1960 (65).

Since the RIA of insulin was first presented, these techniques have been applied to many areas of research and clinical methods. Due to the ability of the RIA to measure nanogram and picogram quantities of substances in complex mixtures, such as biological fluids, with little or no purification, the popularity of RIA increased rapidly.

As antibody production techniques improved, RIA was applied to hapten measurement. It was found to be directly applicable to assay substances for which there were specific receptors present, as in the case of certain steroid hormones.

The term radioassay is used to describe these methods generally, but terms which are frequently used are: radioimmunoassay, competitive protein binding (CPB), saturation analysis, and radiostereoassay. Some of the applications of RIA, at the present time, are listed in Table 1.

Generally RIA's employ similar methods (10). It is first necessary to have a pure substance available to be used as standard, against which unknowns can be compared and antibody produced. The substance to be measured must be able to be labeled, usually with ^{131}I or ^{125}I, to a specific activity that will allow accurate counting. Iodination occurs in the para position of tyrosyl residues by substitution. The first step in the RIA is to incubate the unknown and standards in a uniform medium, with constant amounts of antibody and labeled substance. The antigen-antibody reaction is described in terms of the law of mass action as follows:

(Ag) Antigen (AgAb) Antigen-Antibody

$$+ \text{ (Ab) Antibody} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$$

(Ag*) Antigen-^{125}I (Ag*Ab) Antigen-^{125}I-Antibody

and may be expressed in terms of the antibody binding constant (K), which is the relationship between the rate of association (k_1) and dissociation (k_2).

$$K = \frac{k_1}{k_2} = \frac{[AgAb]\ [Ag^*Ab]}{[Ag]\ [Ag^*]\ [Ab]}$$

In the region of antigen excess, when all the antibody binding sites are occupied, labeled and unlabeled antigen compete for available binding sites. An increase in unlabeled antigen displaces labeled antigen resulting in a measurable decrease in the amount of activity in the antibody bound fraction which is proportional to the antigen concentration in the standard curve. After an appropriate incubation

period, the antibody bound ("bound") and unbound ("free") labeled substance are separated, and one or both fractions are counted. Depending on the intent of the investigation, several methods of plotting the results may be used. For clinical assay purposes, the plot of CPM "bound" or "% bound" (CPM "bound" ÷ total CPM added x 100%) versus the log of standard substance concentration provides the most useful information. This plot results in a sigmoid dose-response curve in which the usable portion of the standard range is the linear part of the curve. Unknowns are read from the standard curve in the usual manner.

Methods of separation of bound and free antigen

One of the chief problems encountered in all RIA's comes at the point where the "bound" and "free" fractions are separated (11, 32). These methods vary widely, but generally are much less specific than the antigen-antibody reaction. This leads to problems of non-specific binding, coprecipitation, and changes in antigen-antibody equilibrium, resulting in decreased accuracy and precision. An ideal system for separation of "bound" and "free" would include the following:

1. specific separation of "bound" and "free";
2. no disturbance of antigen-antibody equilibrium;
3. no coprecipitation or trapping;
4. simplicity, speed and economy.

Some methods used for separation of "bound" and "free" are listed in Table 2.

The first method used for separation of "bound" and "free" was chromatoelectrophoresis (50). In this system, "bound" hormone migrates

in an electrical field on special Whatman paper, while "free" remains at the origin. Iodide migrates ahead of the "bound" fraction. After iodination, "damaged" hormone was demonstrated as that remaining at the origin after incubation with excess antibody. Separation of "bound," iodide, "free" and "damaged" hormones is the chief advantage to this method. However, it requires special strip scanners, poor counting of ^{125}I, excess time and labor, and only small volumes of samples (300-400 µl) may be separated.

Paper chromatography, polyacrylamide gel and cellulose acetate electrophoresis have been used in a manner similar to chromatoelectrophoresis, but do not give the same degree of resolution and suffer the same limitations (10).

Double antibody precipitation (50) is based on precipitation of the "bound" by an antibody against the anti-hormone. Separation may be by filtration or centrifugation. Sample processing is faster, higher count rates may be attained using less label, and larger volumes may be processed. Problems include production of a second antibody, which has to be added to the assay system, increasing work, and incubation time. There is also some coprecipitation and trapping of label in the precipitate.

The property of physical adsorption of the hormones is used widely. Active substances include silicates (Fuller's earth, Lloyd's reagent, fluorisil, talc), QuSO, dextran coated charcoal, and cellulose powder. Their primary advantage lies in being relatively cheap shelf-reagents which are stable and easily prepared. Separation is non-specific and

results in coprecipitation and trapping. Certain hormones are not adsorbed well making this method less generally adaptable (45, 11).

Several methods utilizing salt precipitation of "bound" with sodium sulfate or sulfite and ammonium sulfate have been devised (10, 30). Some precipitation of "free" may occur at higher salt concentrations in certain systems and coprecipitation and trapping may occur.

Dioxane (57) and ethanol-salt (10) have been used to precipitate the "bound." Larger volumes may be processed but again there is non-specific coprecipitation and trapping.

Ultrafiltration with cellulose acetate (30) or gel filtration with Sephadex columns (27) separate "bound" on the basis of molecular size. This is more selective than most precipitation methods but requires increased work, time and relatively expensive materials.

Recently polyethylene glycol was used to precipitate the "bound" (24). It was found necessary to add halide salts to decrease precipitation of "free" in the case of parathyroid and growth hormones.

Enzyme proteolysis of the "free" (44) is based on the protective effect of antibody against ficin. Enzymatically degraded fragments remain in the supernatant after precipitation of "bound" with trichloroacetic acid. The method requires additional time with an unstable enzyme preparation and there is some indication that bound peptide is also attacked.

The solid-phase antigen (SPAg) and antibody (SPA) methods have the theoretical advantage of complete specificity at the outset. If a solid support could be chosen to fit the criteria mentioned for an

ideal system, it would seem that solid phase would offer the most advantages. There are, however, additional considerations which need not be taken into account by other methods. The quantity of antibody or antigen required might increase and raise the cost prohibitively. Coupling to a solid phase may change the binding constant of the antibody or sterically hinder the antigen-antibody reaction. The inertness of the solid phase may be important, as would the ease of preparation and stability of the coupled complex.

Solid-phase RIA

Protopol solid phase:

The first use of a solid-phase antibody system was the method presented by Catt, Niall and Tregear in 1966 (13). Small, dense particles of the isothiocyanato derivative of the graft copolymer of styrene and polytetrafluoroethylene (Protopol polymer) were physically adsorbed to anti-growth hormone and used for RIA. These dense particles settled out of the incubation mixture and could be washed without centrifugation. The adsorbed antibodies were stable to mechanical treatment, weak acid and alkali. The binding of the antigen to the solid-phase antibody was nearly irreversible, even in the presence of excess antigen. Best results were obtained by washing the particles after incubation with the unknowns and then adding the labeled antigen, a technique referred to as disequilibration.

In 1967, Catt, Niall and Tregear (14) modified the solid phase to the form of small discs. Using quadruplicate analysis, precision was increased over the particle assay system. For growth hormone, repro-

ducibility ranged from 6.8 to 17.4%. Large amounts of label were required for each assay tube (100,000 CPM) and disequilibration was necessary to protect label from incubation damage. Incubation time was increased from 24 to 42 hours. A reported advantage was that the plot of the "bound" in the zero tube over the "bound" in the standard tubes, versus standard concentration, gave a straight line with an intercept on the abscissa equal to the mass of the labeled hormone (16). For the RIA of bovine luteinizing hormone, it was found that up to half of antibody coated discs were unusable and up to six washes were necessary to remove the "free" (54). It is felt that disequilibration may result in decreased sensitivity (28).

Sephadex or cellulose powder solid phase:

In 1966, Wide and Porath (59) reported a solid-phase method in which ammonium sulfate precipitated antibodies were covalently coupled to isothiocyanatophenoxyhydroxypropyl-Sephadex (5). Antisera to IgG, chorionic gonadotrophin, growth hormone and luteinizing hormone were coupled and used for RIA, and were found to be stable for eight months or more. The SPA's were resistant to mild acid and alkali treatment. The SPA-antigen complex was removed from solution by centrifugation. The major disadvantage reported for the system was the complexity and time required for the preparation of the SPA.

In 1967, Wide, Bennich and Johansson (60) presented a method for RIA of IgND allergen antibodies using SPAg. Common allergens were coupled to CNBr activated Sephadex (6). This new coupling procedure had similar stability but was much easier to prepare and activated

Sephadex could be prepared in advance and stored frozen (-20°C) for three months or more. ^{125}I-IgND was used as the label.

In 1969, Wide (62) applied the CNBr activated solid phase to SPA radioimmunoassay of luteinizing hormone and follicle stimulating hormone. Antibodies were purified with sodium sulfate and coupled to either Sephadex or microcrystalline cellulose which was activated with CNBr (61). The activated solid phases were reported to be stable for at least a year, as was the SPA. Sensitivity was 1 pg/ml for luteinizing hormone. Between day precision ranged from 7-9% for urine and 7-11% for serum. One ml of antiserum could be used for 100,000 reactions and overall antibody utilization was around 2-3 times that of the free antibody system. Incubation time ranged from 3-24 hours depending on the RIA system. A major reported advantage to the Sephadex or cellulose solid phases, which consist of polymerized glucose, is their inertness toward a wide variety of chemical and biological materials. In addition, the system appears almost universally applicable since activated solid phase requires only the presence of free amino groups for coupling to take place.

In 1971, Wide and Killander (63) presented a method for RIA of vitamin B_{12} using CNBr activated Sephadex coupled to intrinsic factor or transcobalamin (20). The new method was found to be simpler, more rapid, and had better reproducibility (6.4-12%) when compared to the Euglena gracilis assay. Sensitivity was 25 pg/ml and recovery was 80-111%.

Arends (4) evaluated the sensitivity and efficiency of antibody utilization of the Sephadex coupled SPA and sodium sulfate precipi-

tated free antibody for chorionic gonadotrophin. He estimated that 2-15% of antibody activity was lost by sodium sulfate fractionation alone. In addition, he found that 95% of antibody activity was lost by coupling to the solid phase, as indicated by an apparent decrease in the SPA binding constant.

Plastic tube solid phase:

In 1967, Catt and Tregear (15) presented a SPA method for RIA utilizing polypropylene and polystyrene plastic tubes. Antibodies were bound to plastic tubes at pH 9.0-10.0 and used for RIA of chorionic gonadotrophin, placental lactogen, bovine luteinizing hormone, fibrinogen and tetanus toxin. One ml of antiserum was used repeatedly for 100-5,000 tubes. Coated tubes were stable to 0.2M NaOH, 0.2M HCl, and mechanical stress. The labeled antigen-antibody tube complex was found to be reversible in the presence of excess antigen, and thus the disequilibration step was found to be unnecessary. A large amount of label was required for each assay tube (95,000-140,000 CPM). Non-specific binding measured with nonimmune serum coated tubes was 0.5%.

Goodfriend, Ball and Farley (30) evaluated several RIA systems for the determination of angiotensin-II levels in plasma. They found that antibody coated tubes and Protopol polymer gave poorer precision than most methods, but the steepness of the slopes of the standard curves and procedural simplicity made them most useful for this RIA.

In 1969, Salmon, Mackey and Fundenberg (52) modified the antibody coated tube method by first adsorbing antigen to the tube and then coupling antibody to the antigen. The so-called "sandwich" solid phase was applied to the RIA of the IgG, A, and M heavy chains. The accuracy

of the assay using duplicates was ± 10%. The method was compared against the antibody coated tube and Protopol polymer disc methods and found to give better results, even when using quadruplicates for analyses by the other two methods. Less than 10% of "bound" label was displaceable by excess antigen but 80% of activity was lost in the presence of 2% sodium dodecylsulfate. From this they concluded that the adsorption to the solid phase was a charge dependent phenomenon. Each assay tube required 50,000 CPM and non-specific binding was 0.5% or less. Best results were obtained with the disequilibration technique.

In 1970, Abraham, Odel, Edwards and Purdy (1) compared three modifications of the antibody coated tube method for the RIA of 17β-estradiol (E_2-17β). Antibodies to E_2-17β-bovine serum albumin conjugates were adsorbed to polystyrene tubes and the RIA performed using E_2-17β-6,7-^{3}H as the label. Methods using prior extraction and chromatography were found to give the best results with all biological fluids tested. Between day reproducibility was 4-16% and sensitivity was 5 pg. It was necessary to run triplicates or quadruplicates.

Ceska, Grossmüller and Lundkvist (19) applied the antibody coated tube method to RIA of insulin. They found that the nonionic detergent Tween-20 enhanced binding of antigen. Best results were obtained when sodium sulfate precipitated antibodies were used to coat the tubes. Prepared tubes were stable for at least 20 weeks if freeze dried and stored frozen at -20°C. Precision and accuracy were said to be acceptable for clinical purposes.

Certain antisera are said not to bind well to plastic tubes (58); an example of this was cited where rabbit anti-human thyrotropin failed to be adsorbed (16).

Immunoradiometric solid phase:

In 1968, Miles and Hales (42) introduced a labeled antibody method which utilized solid-phase insulin for the RIA of insulin. Unknown and standard insulin were allowed to react with labeled antibodies and the free labeled antibody was later removed by incubation with insulin coupled to cotton wool. Theoretically the larger number of possible labeling sites on the antibody might produce greater specific activities, and therefore, increase the sensitivity of the RIA. In practice, it was found that the more the antibody was labeled the more the antibody binding affinity was decreased. The labeled antibody preparation was relatively difficult to prepare and the method did not appear to be routinely practical (41, 43).

Addison and Hales, 1971 (2) presented a modification of the immunoradiometric method for RIA of growth hormone. Anti-growth hormone was coupled to cellulose filter paper, incubated with growth hormone and then with ^{125}I-labeled anti-growth hormone. This "two-site" RIA could detect 10 pg/ml of growth hormone. With 10,000 CPM of labeled antibody the sensitivity was 62.5 pg/ml. One ml of anti-serum could be used for 20,000 assays.

Summary:

The solid-phase method is the only method for separation of "bound" and "free" in the RIA that maintains the specificity which

is inherent in the assay. This specificity should allow for separation of "damaged" hormone and iodide, especially if the solid phase alone is counted.

The most generally applicable, successful and practical solid-phase systems appear to be the antibody coated tube and the Sephadex or cellulose powder coupled antibody. The latter method appears to offer more versatility for experimentation in terms of manipulation of antibody concentration.

The Sephadex coupled antibody method produces better precision with less tubes and makes more efficient use of antibody, which is an expensive reagent. Less labeled hormone is used, reducing the cost of this reagent. Assays are performed in one tube with constant geometry, which simplifies performance and counting variability. Solid-phase antibody appears to be simply prepared and has demonstrated good stability. Antigen-antibody equilibrium should not be disturbed to the degree found in other non-specific methods of separation. Coprecipitation and trapping should be minimal especially if a high dilution of the solid phase is possible. Potential loss of antibody or antibody activity appear to be the only real drawbacks to this method. Except for the experience of Arends (4) the actual losses are much more acceptable than for any other solid-phase system.

Statement of problem

Since a RIA for renin activity had recently been developed in this laboratory by Lehfeldt and Hutchens, 1971 (39), it was suggested to apply the solid-phase system of Wide (62) to this method. Insofar

as is known, the RIA of angiotensin-I (A-I) has not presently been applied to this system.

In addition, the solid-phase system presents the possibility of preparing a specific solid-phase antigen. SPAg might be used to isolate high affinity antibodies and purify the antibody preparation to a degree not possible with sodium sulfate fractionation. Use of this pure antibody preparation in the above method could result in a more sensitive RIA.

MATERIALS AND METHODS

MATERIALS

Equipment

1. Nuclear-Chicago dual channel automatic gamma spectrometer, model 4230
2. Packard Tri-carb scintillation spectrometer, model 2002
3. Column for Sephadex, 2.5 x 45 cm, water-jacketed, PE-200 outlet tubing, Pharmacia Fine Chemicals
4. Fractomat automatic fraction collector, Buchler Instruments, model 3-4200, with Drop Counting Photocell Search Unit 3-4022
5. pH meter, Instrumentation Laboratory, model 245, combination electrode #14043
6. Rotary mixer, modified Big Boy barbecue spit (Fig. 1 & 2) on plywood base

   Dimensions: 16 x 6.5 x 11.5 inches with both supports

   Refrigerator space requirement: 17 x 12 x 13.5 inches

   Mallinckrodt 72-tube support: P613-0072

   Capacity: 72 - 144 tubes

   Speed: ca. 5 RPM
7. International Portable Refrigerated centrifuge, model PR-2, 12 place horizontal head #253, 8 place multiple carriers #381
8. Sonicator, Bronwill Biosonik, Biosonik II probe
9. Aerometrics sonicator, model GC 75.5, bath type
10. Lyophilizer, Virtis, model 10-010

11. Falcon polystyrene plastic test tubes

    #2052: 12 x 75 mm tubes without caps

    #2057: 17 x 100 mm tubes with caps

    #2032: polyethylene caps for 12 x 75 mm tubes

12. Buchner funnel, Coors 03, 51 mm

13. Glass fiber filter, Type A, 2 inch, 100 per box, 0.3 μ pore size, Gelman

14. Erlenmeyer flask, 250 ml, polyethylene-lined screw cap, Kimax #26505

15. Centriflo ultrafiltration membrane cones, #2100 CF 50 with conical support and centrifuge tube, Amicon Corp.

16. Beckman Microzone electrophoresis system, densitometer, Clifford Instruments, model 345

17. Refractometer, AO TS Meter, American Optical, model AO-10400

Reagents

1. Sephadex G-25-40, G-15-120, Pharmacia Fine Chemicals
2. ^{125}I-asp^{1}-ile^{5}-angiotensin-I, ca. 400 μCi/μg, Schwarz/Mann #0750-53
3. ^{125}I-asp^{1}-ile^{5}-angiotensin-I, ca. 1,000 μCi/μg, Cambridge Nuclear
4. asp^{1}-ile^{5}-angiotensin-I, Schwarz/Mann #1388
5. Na^{125}I, iodine-125, NEZ-033, >99% radiochemical purity, in 0.1N NaOH, New England Nuclear
6. Na^{125}I, iodine-125, NES-050 solution, 1 μCi standard, New England Nuclear
7. Na^{131}I, Mallinckrodt Nuclear
8. ^{129}I, iodine-129, NES-135A rod, 0.2 μCi simulated ^{125}I standard, New England Nuclear
9. CNBr, cyanogen bromide, Eastman #919
10. $C_7H_7ClNO_2SNa \cdot 3H_2O$, Chloramine-T, Eastman #1022, 96% by spectroanalysis
11. Tween-20, polyoxyethylene sorbitan monolaurate, Sigma P-1379
12. Dextran T-70, 100 g, Pharmacia Fine Chemicals
13. Blue Dextran 2000, Pharmacia Fine Chemicals
14. Bovine Serum Albumin, crystallized and lyophilized, grade V, Sigma
15. Lysozyme, Grade I, 3X crystallized and lyophilized from egg white, Sigma

All other reagents are Reagent Grade or better.

METHODS

Preparation of SPA

1. CNBr activation of Sephadex G-25-40

   Sephadex G-25-40 was activated after the method of Wide (62) with minor modifications. CNBr (5g) was dissolved in 200 ml of distilled water by magnetic stirring in a 250 ml Erlenmeyer flask fitted with a polyethylene-lined screw cap under a hood. After the CNBr was dissolved, it was placed in a 600 ml beaker on a magnetic stirrer. Sephadex G-25-40 (5g) was added with stirring and the pH was adjusted to 10.5 with 2M NaOH from a 50 ml burette. The pH was monitored with a pH meter. The pH was maintained at 10-11 for 2 minutes. The activated Sephadex was transferred to a Buchner funnel in a 2 liter suction flask and filtered on a glass fiber filter. The activated Sephadex was washed once with 500 ml of distilled water (4°C) and then with acetone and water as follows: one wash of 50% acetone to water (200 ml), one wash of 60% acetone to water (200 ml), one wash of 70% acetone to water (200 ml), one wash of 80% acetone to water (200 ml), one wash of 90% acetone to water (200 ml), and two washes of 100% anhydrous acetone (200 ml). The activated Sephadex was allowed to dry (30 minutes) on the Buchner funnel with the vacuum running, weighed, divided into 1g aliquots, and stored at -20°C in a desiccator jar in 17 x 100 mm plastic tubes.

2. Purification of anti-A-I with Na_2SO_4

   Anti-A-I was precipitated with Na_2SO_4 after the method of Wide

(62). All antiserum was prepared by Lehfeldt and Hutchens (39), using rabbit III, lot 3. One ml of this anti-A-I was placed in a 17 x 100 mm plastic tube, and Na_2SO_4 (180 mg) plus 1 ml of 18% (w/v) Na_2SO_4 was added, mixed in a 25°C water bath until all the Na_2SO_4 was dissolved, and incubated at 25°C for 1 hour. The white precipitate was centrifuged (2,000 RPM for 10 minutes) and the supernatant discarded. The precipitate was washed with 2.5 ml of 18% (w/v) Na_2SO_4, centrifuged as before, and the supernatant discarded. The precipitate was dissolved in 0.1M $NaHCO_3$ to a total volume of 1 ml. The purified anti-A-I solution (0.1 ml) was dispensed into 17 x 100 mm plastic tubes, capped and stored at -20°C.

Anti-A-I was characterized by cellulose acetate electrophoresis (7) before and after Na_2SO_4 precipitation. Total protein was determined on serum by direct refractometry of total serum solids. Anti-A-I serum was compared against "normal" rabbit serum.

3. Coupling of purified anti-A-I to activated Sephadex G-25-40

Anti-A-I purified with Na_2SO_4 was covalently coupled to activated Sephadex G-25-40 after the method of Wide (62). One gram of activated Sephadex G-25-40 was added to 0.1 ml of purified anti-A-I in a 17 x 100 mm plastic test tube containing 4 ml of 0.1M $NaHCO_3$. It was mixed on the rotary mixer for either 1 day at ambient temperature, or 3 days at 4°C. The mixture was centrifuged (2,000 RPM for 5 minutes) and the supernatant discarded. The SPA was then washed on the rotary mixer in various solutions: twice with 10 ml

of 0.5M $NaHCO_3$, mixed 20 minutes, centrifuged and supernatant discarded as before; once in 10 ml of 0.1M sodium acetate buffer pH 4.0 for 1 hour as before. The SPA was then sonicated with a probe-type (15 seconds at a setting of 60) or bath-type (5 minutes) sonicator, in acetate buffer and mixed overnight as before. The acetate buffer was discarded after centrifugation and the SPA was washed for 20 minutes as before in 2 x 10 ml of "assay buffer" (0.2M NaH_2PO_4, pH 8.0, containing 1 mg/ml lysozyme). The SPA was diluted to 10 ml, total volume, in "assay buffer" and dispensed (0.5 ml) from a magnetically stirred suspension, into 12 x 75 mm plastic tubes using a 1 ml Cornwall syringe, capped and stored at -20°C.

Preparation of ^{125}I-A-I

1. Iodination

A-I was labeled with ^{125}I after the method of Nielson et al. (47). A-I (10 µg in 20 µl) was added to 50 µl of 0.5M NaH_2PO4, pH 7.4 in a 12 x 75 mm plastic tube. Mixing was performed using a vortex mixer after the addition of each reagent. $Na^{125}I$ (200 µCi in 100 µl) was added to the A-I in phosphate buffer and mixed. Chloramine-T (10 µg in 25 µl of 0.5M NaH_2PO_4, pH 7.4) was added and mixed for 20 seconds. After 1 minute, sodium metabisulfite (10 µg in 25 µl of 0.5M NaH_2PO_4, pH 7.4) was added, mixed, and 20 seconds later 1 ml of distilled water was added and mixed. An aliquot was removed, and the entire mixture transferred to the top of the Sephadex G-15-120 column with a Pasteur pipette and washed into the column with two, 1 ml portions of 0.1M NaH_2PO_4, pH 7.4.

2. Purification of ^{125}I-A-I

a. Column preparation and apparatus setup

Sephadex G-15-120 was hydrated in distilled water for 3 hours at ambient temperature. "Fines" were removed by swirling and aspiration. Hydrated Sephadex G-15-120 was poured into the column, settled by tapping, and mounted on the equipment frame of the automatic fraction collector. The column was connected to the drop counting photocell search unit with PE-200 tubing (8 inches), and to a 1 liter buffer reservoir, mounted on a ring stand, by standard Tygon tubing (ca. 36 inches). The flow rate was adjusted by raising and lowering the reservoir on the ring stand. Before use the column was primed with 1 ml of 0.2M NaH_2PO_4, pH 8.0 containing 1 mg of lysozyme and 13.8 µg of A-I.

b. Void volume

The void volume was estimated according to Determan (22) using Blue Dextran-2000.

c. Fraction collection

Fractions (1-4 ml) were collected in 12 x 75 mm plastic tubes, on the automatic fraction collector, using the drop counting mode.

d. Counting of ^{125}I and ^{131}I

Fractions were usually counted directly on either of the two automatic gamma spectrometers in 12 x 75 mm tubes. Both gamma spectrometers were recalibrated for ^{125}I. One channel

of the Nuclear-Chicago gamma spectrometer was set for ^{131}I. The efficiency of the Nuclear-Chicago gamma spectrometer was determined with a $Na^{125}I$ standard solution for volumes of 0.5 to 4.5 ml, in steps of 0.5 ml, in 12 x 75 mm plastic tubes. The Nuclear-Chicago gamma spectrometer has an efficiency of 63% for volumes less than 2.0 ml. The Packard gamma spectrometer has an efficiency of 60% in this range. Between day reproducibility and accuracy were monitored for both instruments with a ^{129}I simulated ^{125}I (ca. 0.2 μCi) sealed standard source.

e. Location of the iodide peak

The iodide peak was located with ^{131}Iodide (1.2 μCi), eluted as usual and counted directly.

f. Location of A-I peak

The A-I peak was located using stock standard A-I (50 μg in 0.1 ml) by SPARIA, and by direct O.D. 210 nm (46) measurement of the fractions.

g. Location of ^{125}I-A-I peak

The ^{125}I-A-I peak was located using ^{125}I-A-I (ca. 1 μCi, Schwarz/Mann) and direct counting of the fractions.

3. Procedure for purification of ^{125}I-A-I

The ^{125}I-A-I iodination mixture was transferred to the top of the Sephadex G-15-120 column with a Pastuer pipette and rinsed with 2-3 ml of 0.1M NaH_2PO_4, pH 7.4. The above buffer was used to develop the column at a rate of 20 drops per minute (ca. 0.66

ml/min), and 3 ml fractions were collected in 1 ml of buffer containing 8 mg/ml of lysozyme. Fractions were mixed well, as soon as possible, counted directly and frozen (-20°C). If the count rate exceeded 10^6 CPM, a 2 µl aliquot was removed, with a self-filling Drummond Microcap, and counted directly in a 12 x 75 mm plastic tube. Fractions were later selected for binding and specific activity determinations.

Solid-phase radioimmunoassay (SPARIA)

1. General methods

a. RIA

SPA was diluted in "assay buffer" (62) to give 20-60% binding of 0.1 ml of ^{125}I-A-I (10,000-15,000 CPM) diluted in "assay buffer." Diluted SPA (0.8 ml) was dispensed with a 1 ml Cornwall syringe, with or without an automatic adapter, from a magnetically stirred suspension. RIA was performed in 12 x 75 mm plastic tubes with caps, and mixed on the rotary mixer overnight at 4°C. Tubes were washed according to Wide (62) by centrifuging (2,000 RPM, 3 minutes, 4°C), removing the caps, and washing with "wash buffer," with the tubes still in the centrifuge. Washing was done by adding 3 x 2.5 ml washes of "wash buffer," using a 5 ml Cornwall syringe with automatic adapter, centrifuging as before, and aspirating the supernatant down to 0.3 ml with a 16 gauge needle fitted with a cork retainer. The SPA was then counted in the gamma spectrometer. The SPARIA method and reagents were varied as the experiments

required.

b. Standard A-I dilutions

Stock A-I standard was prepared as described previously (39), except that it was diluted in "assay buffer." Eleven standards (more if required) were run with each standard curve. Dilutions were made in 12 x 75 mm plastic tubes with assay buffer. The first dilution was made with a 25 µl capillary (TC, Scientific Products) micropipette, subsequent serial dilutions were with a 1 ml serological pipette as follows:

	Dilution	pg/50 µl	ng/ml
1.	1:100 (stock 25-2,500 µl)	5,000	100
2.	1:2 (1-2 ml)	2,500	50
3.	1:2	1,250	25
4.	1:2	625	12.5
5.	1:2	313	6.25
6.	1:2	156	3.13
7.	1:2	78	1.56
8.	1:2	39	0.78
9.	1:2	20	0.39
10.	1:2	10	0.20
11.	1:2	5	0.10

The "zero" tube was prepared by substituting 50 µl of "assay buffer" for diluted standard. The "% bound" for each standard was plotted versus A-I concentration on 4 cycle semi-log graph paper (39). Dilutions were altered as required by the experiments.

2. Washing "used" SPA

   SPA was collected at the end of experiments with a Pasteur pipette, pooled and stored at -20°C. SPA (ca. 1 ml) was washed for 5 days on the rotary mixer, at ambient temperature, in a 50 ml, round bottom, screw capped, centrifuge tube. The wash buffer was changed daily and consisted of 40 ml of glycine-HCl buffer pH 2.0 (0.4M glycine, 0.1M NaCl, adjusted to pH 2.0 with concentrated HCl). The washing process was followed by direct counting of the SPA radioactivity.

3. Lyophilization of SPA

   SPA (0.5 ml) was washed with distilled water (3 x 4 ml) and stored at -70°C. Frozen SPA was lyophilized overnight and stored at -20°C.

Determination of renin activity, general method of A-I generation

A-I was generated in plasma, containing 1 mg/ml disodium or dipotassium EDTA, and buffered to pH 6.0 with maleic acid/ammonium maleate as described by Lehfeldt and Hutchens (39). "Control plasma," a plasma pool collected from the hematology department and frozen in 3.3 ml aliquots, was added to all standard tubes and used as a control. Plasma (1 ml, 4°C) was mixed with 3M ammonium maleate buffer (10 µl, pH 6.0) and 2M maleic acid inhibitor reagent (10 µl, containing 1 mg of 8-OH quinoline and 0.25 mg of BAL), mixed on the vortex mixer, placed in the 37°C shaking waterbath and sampled at 0, 1, 2 and 3 hours.

The tubes for RIA were set up, as described previously (SPARIA), in an ice bath. After the 3 hour incubation, the pH of the plasma was determined with the pH meter. Renin activity was calculated graphically by plotting the A-I (ng/ml) generated on the ordinate, time (h) on the abscissa, and drawing a straight line to best fit the points. The result was expressed as ng/ml/h.

Anti-A-I purification with solid-phase antigen (SPAg)

1. Coupling A-I to activated Sephadex G-25-40

   Lysozyme-free ^{125}I-A-I was prepared by collecting several fractions of the ^{125}I-A-I preparation in 1 ml volumes, without 1 ml of 8 mg/ml lysozyme added. These tubes were frozen immediately. Lysozyme-free ^{125}I-A-I (8.57 µCi in 50 µl) was coupled to activated Sephadex G-25-40 (100 mg) in phosphate or carbonate-bicarbonate buffers of various pH's. Coupling was done in 12 x 75 mm plastic tubes with caps, at 4°C, on the rotary mixer, for 2, 19 and 41 hours. The reaction was stopped by washing with "wash buffer" (3 x 4 ml). The "% bound" by the activated Sephadex, and the plastic tubes, for each time were compared.

2. Anti-A-I purification with SPAg

   SPAg (100 mg) containing ca. 48 ng of A-I was washed at 4°C, in 17 x 100 mm plastic tubes, on the rotary mixer, in the following solutions: 0.1M $C_2H_3O_2Na$, pH 4.0 (3 x 13 ml) for 2, 6 and 18 hours; "wash buffer" (3 x 4 ml) for 20 minutes; glycine/HCl, pH 2.0 buffer (4 ml) for 24 hours; and "assay buffer" (3 x 4 ml) for 20 minutes.

Washed SPAg was incubated with Na_2SO_4 purified anti-A-I (10 µl) in 1 ml of "assay buffer," for 24 hours, at 4°C, on the rotary mixer. Then it was washed with "assay buffer" (3 x 4 ml) for 20 minutes; and the bound A-I eluted with glycine/HCl, pH 2.0 buffer (4 ml) for 24 hours, at 4°C, on the rotary mixer. The eluate was separated by centrifugation (2,000 RPM for 5 minutes), removed with a Pasteur pipette and stored at -20°C.

Procedure for free-antibody RIA

1. Place the following in 12 x 75 mm plastic tubes and incubate overnight at 4°C:

	Standard (µl)	Blank (µl)
"assay buffer"	800	900
A-I standards	50	---
anti-A-I	50	---
^{125}I-A-I	100	100

Mix well by lateral shaking.

2. Add 1.0 ml of dextran-coated charcoal solution, at 4°C, from a magnetically stirred suspension, using a 5 ml Cornwall syringe, and mix on the vortex mixer.

3. Centrifuge at 2,500 RPM, at 4°C, for 20 minutes.

4. Prepare duplicate set of tubes and decant supernatant (bound), being careful to transfer all liquid possible.

5. Count "bound" in the automatic gamma counter for 1 minute.

Reagents

1. "Assay buffer": NaH_2PO_4, 0.1M, pH 8.0 adjusted with saturated NaOH. Add 1 mg/ml lysozyme prior to use.

2. Dextran-coated charcoal solution: add 1 g of Norit SG extra activated charcoal to 100 ml of 0.1M NaH_2PO_4, pH 8.0 containing 1.2 g of Dextran T-70. Stir for 30 minutes prior to use, on a magnetic stirrer in an ice bath.

3. A-I standards: dilute the same as for SPARIA.

4. Anti-A-I: dilute Na_2SO_4 purified anti-A-I as required, in "assay buffer."

5. ^{125}I-A-I: 10,000-15,000 CPM, diluted in "assay buffer" as for SPARIA.

RESULTS

Preparation of SPA

1. CNBr activation of Sephadex G-25-40

   Activation of Sephadex G-25-40 with CNBr results in a marked gain in weight (average 26.9%). Table 3 lists some typical results. Activated Sephadex G-25-40 has the same appearance as unactivated Sephadex G-25-40.

2. Purification of Anti-A-I with Na_2SO_4

   The results of Na_2SO_4 fractionation of "normal" and anti-A-I rabbit serums are presented in Figures 3-6. Precipitated globulins are uniform and white in appearance. Addition of 0.1M $NaHCO_3$ completely redissolves the precipitate into a clear, colorless solution.

   The total protein of anti-A-I antiserum is higher than for "normal" rabbit serum (7.4 g% vs. 5.9 g%). The gamma globulin fraction of anti-A-I is higher than for "normal" rabbit serum (25.8% vs. 7.3%) (Figs. 3 & 5).

   After Na_2SO_4 precipitation and reconstitution to the original volume of anti-A-I, the peak height of the gamma region, on cellulose acetate electrophoresis, appears to be reduced. Proteins other than gamma globulin appear to be almost completely removed (Figs. 4 & 6).

3. Coupling of purified Anti-A-I to activated Sephadex

   The appearance of SPA is that of normal Sephadex G-25-40.

a. Sonication time

Table 4 shows the effect of sonication time, using probe-type sonicator, on SPA maximum binding.

b. Stability, time, temperature and sonication

Table 5 shows the relative amounts of maximum binding of ^{125}I-A-I by SPA (1:200) under various conditions of CNBr activation, time, temperature and the use of bath or probe sonicators. Preparation number one has the lowest maximum binding (6.4%), and was prepared using the wrong concentration of CNBr for activation. The highest binding is observed in the second preparation (27.0%) which was prepared with the correct CNBr concentration, incubated at ambient temperature overnight, and sonicated using the probe sonicator. The third preparation is similar to the second in maximum binding (25.7%) and was prepared at 4°C, for 72 hours using the bath-type sonicator. The three preparations were 8, 5.5, and 2.5 months old, respectively, at the time of evaluation.

c. Buffer

Figure 7 shows standard curves run with SPA prepared in 0.1M NaH_2PO_4 at pH 8.0, or in 0.1M $NaHCO_3$. The curves are similar, but the curve set up using 0.1M $NaHCO_3$ shows increased binding (3.9%) in the low standards.

Preparation of ^{125}I-A-I

1. Column characteristics

The properties of the Sephadex G-15-120 column are presented in Table 6.

2. Void volume

   The appearance of Blue Dextran-2000 was at approximately 71 ml.

3. Location of the iodide peak

   The location of the iodide peak from the Sephadex G-15-120 column is shown in Figure 8. ^{131}Iodide (ca. 1.2 µCi) was applied to the column, eluted as usual, and counted directly. There was one peak beginning at 260 ml, reaching an apex at 283 ml, dropping back down rapidly to 305 ml and then slowly to the baseline.

4. Location of the A-I peak

   The position of standard A-I (50 µg) on the Sephadex G-15-120 column is shown in Figure 9. The column was developed as usual, and the fractions were evaluated by SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a). There is one peak beginning at 80 ml, reaching an apex at 131 ml, dropping off rapidly to 165 ml and then slowly toward the baseline. The position of the peak was confirmed by reading the $O.D._{210}$ of the fractions on a Beckman Du, against 0.1M NaH_2PO_4, pH 7.4.

5. Location of the ^{125}I-A-I peak

   The position of ^{125}I-A-I (ca. 1 µCi) peak is shown in Figure 10. ^{125}I-A-I was purchased from Schwarz/Mann for use as a reference, and developed on the column in the usual manner. There are two peaks in the chromatogram; a first major peak and a second minor peak. The major peak begins at 80 ml, reaches its apex at 190 ml, and drops to a nadir at 250 ml. The minor peak begins at 250 ml,

reaches its apex at 270-275 ml, and drops slowly toward the baseline.

6. Purification of ^{125}I-A-I

The chromatogram for purification of ^{125}I-A-I (182 μCi) is shown in Figure 11. There are four peaks, two major and two minor. The first and smallest peak begins at 65 ml, reaches its apex at 70 ml and ends with a nadir at 80 ml. The second minor peak begins at 80 ml, reaches its apex at 110 ml and ends with a nadir at 125 ml. The third and largest peak begins at 125 ml, reaches its apex at 190 ml and ends with a nadir at 345 ml. The fourth and next largest peak begins at 345 ml, reaches its apex at 365 ml, drops rapidly to 390 ml and then more gradually toward the baseline.

The amounts of ^{125}I activity appearing in the four peaks are given in Table 7.

RIA of A-I

1. SPA dilution curve and displacement of ^{125}I-A-I

Figure 12 shows the amounts of ^{125}I-A-I bound by SPA dilutions in the original SPA preparation. Binding was determined using the SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a). Binding decreases parabolically from 48.8% (SPA 1:2), with increased dilution, and approaches 0.8% asymptotically.

Figure 13 shows the amounts of ^{125}I-A-I bound in the presence of 1.0 ng of standard A-I by the SPA dilutions described above. Binding is decreased in all but the highest SPA dilutions. Maxi-

mum binding is decreased by 1.0 ng of A-I, from 48.8 to 29.1%.

2. Mixing

Figure 14 shows the effect of not mixing with the rotary mixer on the standard curve. Maximum binding is decreased from 22 to 8.2%. The low standards show increased variability compared to the standard curve incubated with mixing.

3. Time and temperature

a. Rate of binding

The rates of binding of ^{125}I-A-I by SPA at 4°C and ambient temperature are shown in Figure 15. Binding was determined using SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a), and varying the incubation temperature. Binding increases at 4°C to a maximum of 45.6% at 23 hours. Ninety percent of maximum binding was attained after 10 hours at 4°C. Binding at ambient temperature increases to 24% at 8 hours and drops slightly to 23% at 23 hours. Ninety percent of maximum binding was attained by 1.5 hours at ambient temperature. Total binding was increased 95% by incubation at 4°C.

The rate of binding of ^{125}I-A-I by SPA for an extended period of time is shown in Figure 16. Binding continues to increase up to 50 hours and appears to level off up to 70 hours. Ninety percent of maximum binding was attained at 16 hours.

b. Standard curve

Standard curves prepared by incubation for 24 and 48 hours are shown in Figure 17. Binding at all points in the 48 hour standard curve are increased (8.3% at maximum binding). Both curves appear to be similar, other than for total binding.

4. Hydrogen ion concentration

Figures 18 and 19 show the degree of binding of ^{125}I-A-I by SPA in "assay buffer" of varying pH and constant ionic strength. Binding was determined using SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a) and varying the pH with saturated NaOH. Ionic strength was adjusted to a constant level by addition of 0.2M NaCl in varying amounts to the same ionic strength of the pH 10.3 buffer. In Figure 18, there is a broad peak beginning at pH 6.6 (18.5%), reaching an apex at pH 7.9 (35.0%) and decreasing to pH 10.3 (22.0%). In Figure 19, binding increases from pH 7.8 (25.0%) to pH 8.0-8.1 (29.5%).

5. Ionic strength

The effects of increasing the molarity of phosphate in the "assay buffer" on maximum binding of ^{125}I-A-I by SPA are shown in Figures 20 and 21. Binding was determined using SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a) and varying the molarity of phosphate in the "assay buffer." In Figure 20, there is a single peak beginning at 0.005M (15.5%), reaching its apex at 0.35M (44.0%) and dropping off gradually to 0.5M (39.5%). All tubes above 0.2M show crystallization. In Figure 21, the

the binding increases from 0.05M (37%) to 0.2M (42.5%).

6. Tris-HCl versus NaH_2PO_4

Table 8 shows the relative amounts of ^{125}I-A-I (14,000 CPM) bound by SPA (1:16) in either 0.1M Tris-HCl, pH 7.5 or 0.05M NaH_2PO_4, pH 7.5 based "assay buffer." Binding is decreased 75% in the Tris-HCl buffer.

7. Tween-20

The effects of increasing the concentration of Tween-20 in the "assay buffer" are shown in Figure 22. Binding decreased from 40.5 to 34.2% when the concentration of Tween-20 was increased from 0.5% (v/v) to 3.0% (v/v).

The effect of Tween-20 (0.5% v/v) on the standard curve is shown in Figure 23. Maximum binding was decreased 15.9%, and there appears to be increased variability in the low standards in comparison to a normal standard curve.

8. NaCl

The effects of increasing the concentration of NaCl in the "assay buffer" is shown in Figure 24. Binding decreased from 40.5 to 20.5% as the NaCl concentration was increased from 0 to 1.25M.

9. Lysozyme

The effect of lysozyme on the standard curve is shown in Figure 25. There appears to be some decrease in binding as the concentration of lysozyme is increased from 1 to 3 mg/ml ("zero" tubes).

10. NaN_3

The effect of NaN_3 on the standard curve is shown in Figure 26. Maximum binding was decreased 5%, and appears to be lower in the area of the standard curve below 108 pg/50 µl, in comparison to a normal standard curve.

11. Conditions for washing SPA-^{125}I-A-I complex after incubation in the SPARIA

a. Number and volume of washes

The effects of an increasing number of washes (5 x 4 ml) on a "zero" and high standard (1.0 µg) tube are shown in Figure 27. All tubes were washed by the usual washing procedure (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a) using 4.0 ml of buffer in place of 2.5 ml of buffer for each wash. Binding decreased in the "zero" tube with each wash. The total decrease was from 44-39%. Binding in the high standard tubes dropped until the third wash from 3.0-0.3% and then remained constant.

b. Delayed washing

The effects of delayed washing (2 x 5 minutes), after the addition of "wash buffer," on the standard curve are shown in Figure 28. Washing was delayed a total of 10 minutes by allowing the tubes to remain in the centrifuge an additional 5 minutes during each wash. The 5 minute delay occurred after the addition of "wash buffer" and before centrifugation. Delayed washing shifted the standard curve down an average

1.7%. This is equivalent to 20 pg at the level of 300 pg/50 µl on the standard curve.

c. Composition of "wash buffer"

The effects of washing the standard curve with "wash buffer" containing 0.5% (v/v) Tween-20, are shown in Figure 29. There do not appear to be any effects caused by washing with this buffer.

d. Temperature of "wash buffer"

The effects of washing the standard curve with "wash buffer" at ambient temperature are shown in Figure 30. Binding appears to be decreased throughout the standard curve.

12. "Low standard phenomenon"

a. Extended range standard curve

The "low standard phenomenon" is shown in an extended range standard curve in Figure 31. There are two peaks. The first peak rises from the "zero" tube, reaches its apex at 3 pg and drops to a nadir at 7 pg. The second peak begins at 7 pg, reaches its apex at 100 pg, and drops toward zero in the manner of a usual standard curve.

b. Washing SPA

It has been reported (20) for solid-phase intrinsic factor, that the "low standard phenomenon" could be removed by washing the SPA several times before using it for RIA. The effect of washing SPA on the "low standard phenomenon" is shown in Figure 32. Both curves have an apex at 5 pg which

was not affected by 3 x 4 ml "assay buffer" washes prior to RIA.

13. Washed "used" SPA

Figure 33 shows a standard curve run with SPA which had been used previously for RIA, and was washed with glycine-HCl, pH 2.0 (Methods, Solid-phase radioimmunoassay (SPARIA), 2) to remove ^{125}I-A-I. The curve appears to behave like a normal standard curve. The washing procedure reduced the original radioactivity of the "used" SPA by more than 95%.

14. Lyophilized SPA

a. Standard curve

Figure 34 shows a standard curve run with lyophilized SPA (100 mg in 100 ml). This curve appears not to be altered, compared to unlyophilized SPA (Fig. 52), by the lyophilization process.

b. Dilution curves

As shown in Figure 35, the dilution curves for lyophilized and unlyophilized SPA are nearly identical. Maximum binding was ca. 65% at a 1:10 dilution and dropped to ca. 3% at a 1:1,280 dilution. The lyophilized curve averages 1% lower than the unlyophilized curve. A small amount of SPA was lost in the lyophilizer jar, even when tissue paper was placed over the top of the tube containing the SPA.

15. Delayed addition of ^{125}I-A-I

Figure 36 shows the results of 24 hour late addition of ^{125}I-A-I to a standard curve incubated a total of 48 hours. Binding was decreased throughout the standard curve. Maximum binding was decreased from 25.8 to 21.5%. There appears to be greater variability in the standards in the low range. Between 6.7 and 100 pg/50 µl, the curve drops 6.5%, versus 4.6% for this same region of the normal standard curve. From 100 to 1,000 pg/50 µl, the curve drops 9.4%, versus 11.2% for the normal standard curve.

16. Delayed addition of A-I, reversibility

Figure 36 shows the results of 24 hour late addition of standards to a standard curve incubated for a total of 48 hours. Gradually increased, but irregular displacement of ^{125}I-A-I occurred, decreasing binding from 25-17.2%.

17. Specific activity of the ^{125}I-A-I preparation purified by Sephadex G-15-120 (Results, Preparation of ^{125}I-A-I, 6)

The specific activities and their locations in the chromatogram are represented in Figure 37. The self-displacement technique (29) consists of SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1) which is modified as follows: a 500 µl aliquot of every fifth fraction was tested and compared for "% bound" to a standard curve corrected for volume by addition of 500 µl of "assay buffer." Specific activity for a fraction was calculated from the activity per 500 µl (µCi) divided by the amount of standard A-I (µg) which produced an equivalent "% bound." There is one large peak beginning at 155 ml, reaching its apex at

230 ml, and dropping rapidly to the baseline at 350 ml. Specific activity at the apex is 1,750 μCi/μg. This peak corresponds to the third and largest peak on the ^{125}I-A-I purification chromatogram (Fig. 11).

18. Binding capacity of ^{125}I-A-I preparation

The binding properties of the ^{125}I-A-I preparation are shown in Figure 38. Binding capacity was determined using the SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a) with an excess (1:50 or 1:100 dilution) of SPA. Every fifth fraction of the ^{125}I-A-I preparation was diluted to give ca. 10,000 CPM/0.1 ml. Displacement was evaluated by adding 0.1 ml of a 1:10 dilution of A-I stock standard (10 ng) to a duplicate set of tubes. There are four peaks, one large and three small. The three small peaks, having apexes at 75, 110, and 400 ml, show nondisplaceable binding with A-I standard (10 ng). The largest peak begins at 140 ml, reaches its apex at 185-215 ml and drops rapidly to a nadir at 365 ml. This peak corresponds to the third and largest peak in the ^{125}I-A-I purification chromatogram (Fig. 11). Displaceable binding is observed throughout this peak. Table 9 shows the results of two commercial ^{125}I-A-I preparations which were run for comparison.

Determination of renin activity

1. Buffers

Table 10 gives the results of comparisons of buffers for generation of A-I in plasma. There appears to be little difference

between generation rates in the three phosphate buffers. Solubility limits the maximum achievable concentration of phosphate buffers, especially for KH_2PO_4. NaH_2PO_4 is the most soluble and 5M is attainable. A-I generation rates were at least 24% greater in maleic acid/ammonium maleate buffered plasma than in phosphate buffered plasma.

2. Hydrogen ion concentration

The results of variation of plasma pH on renin activity are shown in Figure 39. The pH of "control plasma" was adjusted to 6.0, as usual, with maleic acid inhibitor reagent and ammonium maleate buffer. Additional pH's were obtained by addition of up to ca. 30 µl of either 2M maleic acid or concentrated NH_4OH. There is a relatively symmetrical peak with its apex at pH 6.03. If this peak is taken as 100% activity, the activities at the other pH's tested are: 5.42 (63.3%), 5.74 (73.5%), 6.28 (77.0%), and 6.60 (65.0%). Precipitation was noted in the pH 5.74 (trace) and pH 5.42 (1+) plasmas; all of the other plasmas remained clear. Plasma pH's varied less than 0.05 during the 2 hours incubation. The error caused by dilution, in adjusting the plasma pH with up to 30 µl of either 2M maleic acid or concentrated NH_4OH, amounts to 3% or less.

3. Angiotensinase and converting enzyme inhibitors

Tables 11-13 show the results of comparisons of angiotensinase and converting enzyme inhibitors, by A-I generation rates, in plasma. Additional Na_2EDTA was added to plasma in a solution

of 1 mg/10 µl of distilled water. For plasma containing 1 mg/ml of either Na_2EDTA or K_2EDTA, the blood (5 ml) was collected in tubes containing 5 mg of either anticoagulant. BAL and 8-OH-quinoline were prepared as usual (Methods, Solid-phase radioimmunoassay (SPARIA), 1, a), in 2M maleic acid, and added to plasma (1 ml) in 10 µl volumes.

A-I generation rates vary no more than 0.4 ng/ml/h as the concentration of EDTA is increased from 1-3 mg/ml of plasma (Table 11). There was precipitation observed in the plasmas containing 2 and 3 mg/ml of EDTA. The rates were greatest (5.7 ng/ml/h) in plasmas containing 1 and 2 mg/ml of EDTA.

A-I generation rates differed by 0.2 ng/ml/h in Na_2EDTA versus K_2EDTA (Table 12). There does not appear to be any significant difference in the rates.

The rate of generation of A-I with BAL (2 µmol/ml) and 8-OH-quinoline (6.9 µmol/ml) was 1.5 ng/ml/h greater than for the same inhibitors at a concentration of 1.3 µmol/ml and 4.0 µmol/ml, respectively (Table 13).

4. Shaking during A-I generation

The rates of generation of A-I in "control plasma" with and without shaking, during incubation at 37°C in the waterbath, were 6.5 and 6.6 ng/ml/h, respectively.

5. Set-up temperature of RIA

Standard curves set up at 4°C and ambient temperature and allowed to sit for three hours with plasma added, are shown in Figure 40.

The shapes of the curves are different between 0 and 100 pg/50 µl, but above this concentration, they appear to be similar.

6. Thawing plasma

Renin activity determined with "control plasma" thawed overnight at 4°C, or at ambient temperature with mixing, gave 10.3 versus 9.5 ng/ml/h, respectively.

7. Centrifugation of plasma

Renin activity determined with "control plasma" centrifuged (2,000 RPM, 10 minutes, 4°C) after thawing gave 11.5 ng/ml/h, while uncentrifuged plasma gave 11.7 ng/ml/h. Small fibrin clots sometimes stick in the pipette tip. "Control plasma" had been frozen and thawed once before.

8. Incubation time

The renin activity of "control plasma" (Fig. 41), shows the effects of shortening the sampling intervals to 30, 60 and 120 minutes, from 60, 120 and 180 minutes, and fitting the curve to the two hour sample. The rate was increased from 6.7 ng/ml/h to 7.6 ng/ml/h in the same plasma sample. Precision appears to be decreased in the 2 and 3 hour samples compared to the 0, 30 and 60 minute samples. A different plasma sampled only at 0, 30, 60 and 120 minutes is also shown.

9. Reproducibility

The results of the reproducibility studies are shown in Tables 14 and 15. Within day precision of the RIA was determined using

a normal standard dilution, and replicate analyses, at three levels of A-I (54, 216 and 863 pg).

Precision of the renin activity determination was determined with "control plasma." For within day precision, 10 separate aliquots of a "control plasma" pool were run separately through the entire procedure, but were all read from the same standard curve. For between day precision, 12 separate weekly determinations of renin activity were made using "control plasma," over a period of 3 months.

10. Recovery

To measure recovery of A-I by SPARIA (Methods, Solid-phase radioimmunoassay (SPARIA), 1) 6.9 ng was added to 1.0 ml of plasma. The recovery after zero time incubation of plasma averaged 101.1% (Table 16).

The results of a similar recovery experiment which was carried through the 2 hour, 37°C incubation, are shown in Figure 42. Recovery is 110% at zero time, and drops to 69% after two hours. A renin activity curve run with "control plasma" containing only "assay buffer," remains parallel for 30 minutes and then the recovery curve appears to move closer to the buffer control curve. Both curves appear to be nonlinear after 1 hour.

11. Sensitivity

The sensitivity of the RIA for A-I at various levels, is given in Table 17. Using 2 standard deviations (based on data from Table 14), the sensitivity is expressed in pg, and ng/ml/h of A-I.

The sensitivity at "zero" (minimum detectable amount relative to "zero") was estimated from a number of standard curves. In the majority of curves a level of 20 pg/50 µl could be differentiated.

12. Addition of A-I to unknowns

If an unknown plasma contains a nearly equal amount of A-I as the plasma which is added to the standard curve, it is many times impossible to read the zero time sample, because of variability in the low standard range. Figure 43 shows the result of addition of ca. 100 pg of standard A-I to the RIA tubes of "control plasma." The renin activity curve is shifted upwards so that the zero tube may be read. The addition of 100 pg of A-I to the unknown plasma generally brings the zero tube into the linear portion of the standard curve.

Coupling of A-I to Sephadex

1. Buffer, pH and time

The results of coupling ^{125}I-A-I to 100 mg of activated Sephadex G-25-40, for various times and pH's, in either phosphate or carbonate-bicarbonate buffers, are shown in Figure 44. The amounts of ^{125}I-A-I bound to the tubes are shown in Figure 45. Maximum coupling of ^{125}I-A-I (41.5% of 2.5 ng) by activated Sephadex G-25-40 occurred after 42 hours in 0.1M NaH_2PO_4 at pH 9.0. Maximum coupling (5.2%) to polystyrene tubes (12 x 75 mm) occurred after 19 hours in 0.1M NaH_2PO_4 at pH 6.0. Coupling of ^{125}I-A-I to polystyrene tubes was (2.5%) for 0.1M NaH_2PO_4, pH 8.0 after 42 hours.

2. Coupling capacity

The coupling capacity of activated Sephadex G-25-40 for A-I, is shown in Figure 46. Varying amounts of A-I were incubated with 100 mg of activated Sephadex G-25-40 on the rotary mixer for 24 hours at ambient temperature. Increased binding was obtained as increasing amounts of A-I were present in the coupling mixture.

3. Characteristics of "purified" anti-A-I

a. Dilution curves

Figure 47 shows dilution curves prepared using Na_2SO_4 purified anti-A-I, and anti-A-I which was eluted from SPAg into 4 ml of glycine/HCl, pH 2.0. The overall dilution of "purified" anti-A-I was estimated and plotted for comparison, against the true dilution of Na_2SO_4 purified anti-A-I. Maximum binding of "purified" anti-A-I (1:400) was 8.6%, compared to 51.5% for a 1:400 dilution of Na_2SO_4 purified anti-A-I.

"Purified" anti-A-I was concentrated by cone ultramicrofiltration and used to run another dilution curve (Fig. 48). "Purified" anti-A-I was diluted in "assay buffer" to 7 ml, and concentrated by ultramicrofiltration using centrifugation. The dilution and concentration were repeated once more, and the anti-A-I was concentrated to ca. 1.25 ml. The concentrated "purified" anti-A-I was used to run a dilution curve. Maximum binding was 3.9%. Binding was linear versus the log of dilution, over the range of 1:62.5-1:600.

b. Standard curve

Figure 49 shows a standard curve prepared with "purified" anti-A-I which was concentrated by cone ultramicrofiltration. Maximum binding is 13.4%, and is displaced to 10.3% by standard A-I, over the range of 6.7-1,725 pg/50 µl. The points are erratic, especially in the low standard range.

SPA versus free antibody

1. Standard curves

Figure 50 shows a comparison of Na_2SO_4 purified anti-A-I diluted (1:5000), and SPA (1:200), standard curves. Both curves demonstrate parallelism throughout the standard range. The "zero" tube bindings are much different (SPA 25.1% versus Na_2SO_4 purified antibody 15.1%). The deviation of the free antibody system in the "zero" and 6.7 pg standard tubes is similar to the "low standard phenomenon" observed in the solid-phase system (Fig. 31).

2. Rates of binding

A comparison of rates of binding by Na_2SO_4 purified free antibody (1:1,225), and SPA (1:50) are shown in Figure 51. The initial rates for both systems were very similar, the free being slightly more rapid (25% vs. 30%) at 2 hours. The maximum binding was less for the free system (45% vs. 57%). The free system reached equilibrium (95% maximum binding) sooner than SPA (7.75 vs. 22 hours). Non-specific binding for the free system averaged 2.5%.

Suggested procedure for SPARIA of A-I for renin activity in plasma

1. Collection of specimens

   Plasma is collected without hemolysis, using 1 mg/ml disodium or dipotassium EDTA as the anticoagulant. Vacutainers are suitable for this purpose. The blood is mixed well with the EDTA and immediately chilled with ice. Plasma is separated as soon as possible by centrifugation at 2,000 RPM, at 4°C for 10 minutes. Maintain plasma at 4°C and store 1.3 ml aliquots at -20°C in 12 x 75 mm plastic tubes with caps.

2. Preparation of specimens

   Thaw plasma overnight at 4°C or at ambient temperature with frequent mixing. Centrifuge at 2,000 RPM for 10 minutes at 4°C.

3. Procedure for generation of A-I

   a. Place the following in 12 x 75 mm plastic tubes in an ice bath:

	Control and unknown (µl)
plasma	1,000
ammonium maleate buffer	10
maleic acid inhibitor reagent	10

   Mix well on the vortex mixer after the addition of each reagent; place in a 37°C waterbath and incubate for 2 hours. Withdraw 50 µl samples for SPARIA at 0, 0.5, 1 and 2 hours. Place a plastic cap on each tube, loosely.

   b. After incubation, check the pH of each plasma.

4. Procedure for SPARIA

a. Place the following in duplicate 12 x 75 mm plastic tubes in an ice bath:

	Standard (µl)	Control and Unknown (µl)
SPA in "assay buffer"	800	800
plasma from A-I generation step at 0, 0.5, 1 and 2 hours	---	50
"control plasma" at 0 hours	50	---
standard A-I dilutions	50	---
standard A-I (100 pg/50 µl)	---	50
^{125}I-A-I	100	100

Mix all tubes well by lateral shaking or vortex mixing after the addition of each reagent.

b. When the last plasma has been sampled, cap all tubes and place in the rotary mixer.

c. Mix overnight at 4°C.

d. Centrifuge all tubes for 3 minutes at 2,000 RPM and 4°C.

e. Remove caps and add 4.0 ml of "wash buffer" with a Cornwall syringe (5 ml) with automatic adapter. Wash tubes in the centrifuge using a strong stream of buffer directed against the upper side of the tubes.

f. Repeat step "d."

g. Aspirate the supernatant from all tubes while in the centrifuge. Remove all but ca. 0.3 ml. Aspirate into a 2 liter suction flask which has been set aside for radioactive material. Use a 16 gauge needle that has been cut to the proper length (ca. 6.5 cm), and has a large cork or

other retainer near the hub to prevent inserting the needle too deeply into the tube.

h. Repeat steps "e-g."

i. Count tubes in automatic gamma counter for one minute.

5. Standard curve

a. Plot the "% bound" or CPM bound versus standard A-I concentration (pg/50 µl) on 11 x 16 1/2 inch, 4 cycle, semi-log graph paper.

b. Draw a standard curve using a straightedge and French curve. See Figure 52 for an example.

6. Calculation of renin activity

a. Plot the A-I present at each time, on linear graph paper.

b. Draw a straight line to best fit the points.

c. Read the ng/ml/h from the curve. Fee Figure 43 for an example.

7. Normals (39)

Overnight recumbent	2.40 ± 2.28 ng/ml/h
Upright, 4 hour activity	5.38 ± <4.30 ng/ml/h

8. Notes

a. If the pH exceeds ca. 6.15 the sample should be rejected. The pH may be adjusted, if necessary, by the addition of 2M maleic acid at 4°C prior to incubation. The pre-incubation pH should be ca. 5.85-6.00.

b. If a straight line cannot be drawn for the 2 hour incubation, try to construct one for one hour. If this is not possible,

repeat the determination with a new sample.

c. High activity specimens should not be diluted. Shorten the sampling intervals as much as necessary and maintain accurate timing.

d. The actual normal range may be slightly higher for this procedure due to shortened incubation time.

e. Eppendorf pipettes may be used to add plasma, ammonium maleate buffer and maleic acid inhibitor reagent.

Cornwall syringes (1.0, 5.0 ml) with automatic adapters may be used to pipette "wash buffer," and SPA as long as the SPA is kept suspended by magnetic stirring.

Hamilton syringes (2.5, 5.0 ml), with repeating dispensers, may be used to add ^{125}I to all tubes, for addition of "control plasma" to standard tubes, and for adding standard A-I (ca. 100 pg/50 µl) to unknown tubes.

9. Reagents

a. 3M ammonium maleate buffer, pH 6.00: place 8.7 g of maleic acid in 5 ml of distilled water. Add 6-7 ml of concentrated NH_4OH with mixing and cooling. Adjust to pH 6.00 with concentrated NH_4OH and dilute to 25 ml with distilled water. Store at ambient temperature. Check the pH periodically.

b. 2M maleic acid inhibitor reagent: place 5.8 g of maleic acid and 2.5 g of 8-OH quinoline in 15-20 ml of distilled water. Warm in a boiling waterbath and dissolve with vortex mixing. Dilute to 25 ml with distilled water. Dispense

0.5 ml aliquots into 12 x 75 mm plastic tubes with caps, and store at ambient temperature. Protect from light. Before use place a 0.5 ml aliquot in the 37°C waterbath. Add 10 µl of BAL and mix with the vortex mixer. Replace in the 37°C waterbath and discard after use.

c. "Assay buffer": 0.2M NaH_2PO_4, adjusted to pH 8.0 with saturated NaOH. Add lysozyme to give a concentration of 1 mg/ml prior to use. Store at ambient temperature.

d. SPA (1:200): dilute 0.5 ml of stock preparation to 100 ml with "assay buffer."

e. "Control plasma": pooled, nonhemolyzed, nonicteric plasma, containing 1 mg/ml of disodium or dipotassium EDTA. Plasma collected in EDTA Vacutainers is suitable. Dispense 3.3 ml aliquots into 12 x 75 mm plastic tubes, cap and store at -20°C.

f. Standard A-I dilutions: 25 µl of standard A-I (10 µg/ml in "assay buffer") is diluted to 2.5 ml in a 12 x 75 mm plastic tube. Then dilute serially 1:2 from 1:100 to 1:1,024,000. See Methods, Solid-phase radioimmunoassay (SPARIA), 1, b for concentrations.

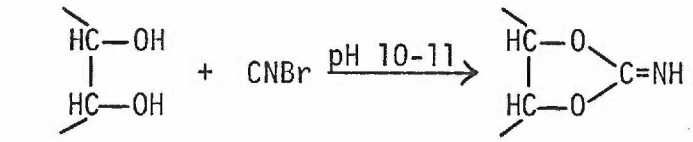
g. ^{125}I-A-I: dilute ^{125}I-A-I to 10,000-15,000 CPM/100 µl in "assay buffer."

h. "Wash buffer": 0.1M NaH_2PO_4 adjusted to pH 8.0 with saturated NaOH. Store at 4°C.

DISCUSSION

CNBr activation of Sephadex G-25-40

The activation of Sephadex G-25-40 with CNBr results in a marked weight gain (26.9% average). The reaction is thought to proceed as follows (62):



polysaccharide residue — activated polysaccharide

Presumably HBr is also a product of the reaction.

The properties of this reaction have been studied (6), and chemical and spectroscopic analysis data support the structure of the product as shown above. Marked reduction in the swelling properties of Sephadex were said to be consistent with increased cross-linking between polysaccharide chains. It was found to react with proteins or peptides with the formation of quanidino derivatives, and resulted in no net charge change. This factor may be of importance in the retention of immunologic activity of the bound material.

Purification of Anti-A-I with Na_2SO_4

Results of electrophoresis of the Na_2SO_4 precipitated fraction indicate that the major portion of the non-gamma globulin proteins have been removed from the antiserum.

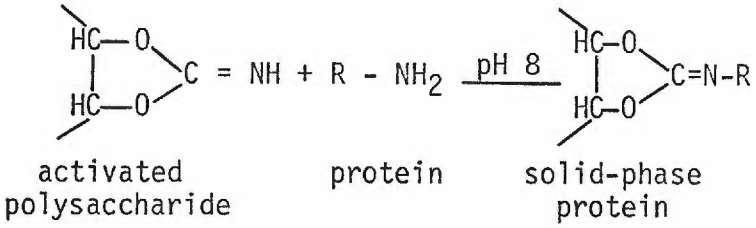
Loss of antibody activity using this procedure has been estimated at 2 to 15% (4). Whether this was due to poor recovery or altered antibody was not made clear.

Sodium sulfate purified antibody has been used for three months without observed loss of activity after coupling to Sephadex G-25-40 or by use in the free antibody assay.

Sephadex coupled antibody

1. Coupling

The coupling of CNBr activated Sephadex to the free amino groups of proteins has been described as follows (22):



activated polysaccharide — protein — solid-phase protein

Ammonia is also released as a product of the reaction (6).

In the preparation of SPA it was found that the binding capacity is decreased by the overactivation of Sephadex G-25-40 with CNBr (Table 5); but is relatively unaffected by variations in sonication time and method (Table 4) or by coupling in either sodium phosphate or sodium bicarbonate buffers at pH 8.

2. Stability

The binding capacities and standard curves prepared were unaffected by storage of SPA at -20°C over a period of at least 5.5 months (Table 5).

3. Lyophilized SPA

If the SPA could be lyophilized, stored frozen, and reused without altering its activity, then the dried preparation could be weighed

out, and more accurate activity comparisons could be made on the basis of weight rather than dilution.

Lyophilized SPA was found to give approximately the same dilution curve as similarly washed SPA which was not lyophilized (Fig. 35). A weighed amount of SPA (100 mg) was used to run a standard curve (Fig. 34). The curve did not appear to be altered by the lyophilization process. It looks as if this method would work well for making accurate comparisons between SPA preparations.

4. Washed "used" SPA

It would be an advantage if the SPA-^{125}I-A-I complex could be dissociated and the SPA reused for RIA. If so, it would also give some indication of the stability of the SPA and the antibody itself. ^{125}I-A-I was eluted over a period of 5 days to less than 5% of its original radioactivity by washing with glycine/HCl buffer, pH 2.0 (18). A standard curve (Fig. 33) run with washed "used" SPA appears to behave normally, indicating no gross reduction in the antibody binding constant. The loss of total antibody activity was not evaluated for lack of time, but an approximation on the basis of dilution suggested no greater than a two-fold change produced by washing. It seems that SPA may be washed and reused for RIA. This results in decreased utilization of antibody and could negate any losses of antibody from the coupling procedure. It is conceivable that SPA could be reused sufficiently to give more assays than the free antibody system.

Preparation of ^{125}I-A-I

1. Labeling of A-I

The labeling procedure (47) was adopted because it was claimed to reduce labeling damage through mild conditions of Chloramine-T oxidation. An additional advantage was that compared to several other procedures (12, 33, 55, 56) 5-10 times less Na^{125}I was required (ca. 200 µCi).

2. Purification of ^{125}I-A-I

Sephadex G-15-120 appears to be well suited to the purification of ^{125}I-A-I iodination mixtures. It has been demonstrated that ^{125}I-A-I, and A-I may be separated by this type of column. The iodide peak (Fig. 8) is well removed from the other peaks. There is some overlap of the ^{125}I-A-I (Fig. 10) and A-I peaks (Fig. 9), but this is probably minimal as only 10 µg or less of A-I are used in the preparation.

The order of appearance of the ^{125}I-A-I and A-I peaks is the reverse of that expected using Sephadex gel chromatography, as larger molecules normally appear first. Since the exclusion limit of Sephadex G-15-120 is around 1,500 mol. wt., it is possible that the separation of ^{125}I-A-I and A-I observed is due to a chromatographic effect. The pK of tyrosine (10.07) is reduced to 6.48 by diiodination. The pK of monoiodotyrosine is thought to be between 6.48 and 10.07 (33). Ionization of iodotyrosine is a likely explanation for the observed chromatographic effect. Sephadex has been shown before to give separation of certain aromatic

compounds, and small peptides on other than a size or molecular weight basis (23). The mechanism by which this occurs is not known.

The identity of the second minor peak (Fig. 11) following the void volume is undetermined. Several possibilities exist, but all are largely speculative. A relatively small amount of non-displaceable binding (Fig. 38) appeared in this peak and thus may consist of higher molecular weight aggregates of iodoangiotensin-I of low specific activity. Diiodoangiotensin-I might also appear here due to exclusion of this higher molecular weight species from the gel.

Separation of ^{125}I-A-I and A-I has been shown previously using DEAE-Sephadex A25 (47). This procedure also removes iodide. The order of appearance from the column is the same as for the present method. With 190-210 μCi of Na^{125}I used for labeling, the ^{125}I-A-I appeared in ca. 30 ml, beginning at 60 ml, and containing 52-77% of the original radioactivity. The position of the iodide peak was not shown.

By comparison, the present method gave 71.7% of 182 μCi (Table 7), appearing in 213 ml (Fig. 11).

Small columns of Sephadex G-15 have been used before, for the purification of iodination mixtures (56). Its use, however, was confined to the removal of unreacted iodide, and other small molecular weight reactants, rather than for fractionation of the iodinated products.

Stockigt et al. (55) used Sephadex G-15 to purify ^{125}I-A-I after preliminary purification on a small column of AG1-X2 resin. ^{125}I-A-I was developed on the 1.5 x 90 cm Sephadex G-15 column with 0.05M acetic acid containing bovine serum albumin, neomycin and thimersol. Two peaks showed binding with excess anti-A-I. A first narrow peak gave up to 96% binding to anti-A-I and 99% precipitation with dextran-coated charcoal. The second broad peak would not bind more than 80% with anti-A-I but was almost completely precipitated by dextran-coated charcoal. The first peak was used for RIA and had to be repurified every 2-3 weeks on Sephadex G-15. Deterioration of ^{125}I-A-I was defined as non-parallelism of plasma generated A-I dilutions compared to the standard curve.

From the information given it appears that Stockigt et al. chose the peak which corresponds to the second minor peak (Fig. 11) of the present method. This is a peak showing some binding (Fig. 38) but little or no displacement by standard A-I. This peak was not further evaluated and the second broad peak was used for all RIA without difficulty and without the need for periodic repurification for a period of 4 months.

3. Specific activity

The determination of specific activity by the self-displacement technique (29) has been used widely for evaluation of labeled preparations (10, 32, 36). There are, however, certain limitations to this method. The labeled hormone is assumed to react

with antibody in a manner identical to unlabeled hormone. The limits of RIA sensitivity and precision make measurement of small amounts of hormone, such as are found in labeled preparations, subject to error. The limitations of precision and sensitivity were overcome in the present method by assaying larger volumes (500 µl) of labeled material, and correcting the standard tubes by addition of an equal amount of "assay buffer." The problem of the relationship between antibody and labeled hormone versus unlabeled hormone was not determined for this system.

Dietrich (25) found that in the case of anti-A-II, the mono-iodinated A-II reacted with greater affinity than the unlabeled A-II. However, for diiodinated A-II, binding was decreased. Goodfriend, Ball and Farley (30) found iodo-A-II to be less immuno-reactive (66%) than A-II, but did not specify the iodinated species. Thus the specific activities determined by the self-displacement method, may be either overestimated or underestimated depending upon the actual relationship which exists with this particular anti-A-I.

The highest specific activity estimated (1,750 µCi/µg) corresponds to 1.0 atoms of iodine per molecule of A-I. Maximum reported specific activities for ^{125}I-A-I preparations have not exceeded 720 µCi/µg (47) although Cambridge Nuclear claims 1,050 µCi/µg for their preparations. Most methods determine specific activity on pooled products, and are not directly comparable. A potential advantage of the present method of purification of ^{125}I-A-I lies in the broad peak of variable

specific activities obtained (Fig. 38), which allows the selection of various portions of the peak having different specific activities. By making smaller pools of lower specific activity after iodination the preparation may be used over a longer period of time and more efficiently.

The maximum theoretical specific activity for ^{125}I-A-I may be calculated as follows (49):

$A = N\lambda$ where A = activity in Ci x 86,400

N = number of atoms or molecules

λ = specific decay factor for ^{125}I

(0.693/57.4d)

Assuming that no more than two atoms of ^{125}I may be substituted into each molecule of A-I, the maximum theoretical specific activity is 3.5 mCi/μg. The determination of specific activities in the present method falls within this theoretical limit.

4. Binding capacity

Another measure of the quality of labeled preparations is their ability to bind to excess antibody (10, 32, 36). A preparation which will bind label in excess of 90% with excess antibody is considered to be suitable for RIA. Binding with excess antibody does not necessarily mean that the label will bind to the same degree in the RIA with less antibody (32). The present preparation of ^{125}I-A-I approaches this goal (maximum 80% binding) and gives good results with low non-specific binding in both the solid-phase and free antibody systems. Displacement of label by

standard (Fig. 38) seems also to be a good measure of immunological competence. A fundamental principle of RIA is that the label be uniform from tube to tube, and react identically from tube to tube. A maximum binding of 80% or 90% should not be necessary. There must be sufficient labeled immunoreactive material present, and the system must be able to select this material. Thus an immunospecific method such as SPA may have the advantage of being able to use relatively impure labeled preparations, and specifically react, only with undamaged or immunoreactive label.

General method of RIA

In setting up a method certain criteria are followed in order that a reasonable degree of accuracy and precision can be expected (32). The amount of ^{125}I-A-I is kept to a minimum, but enough must be added to insure adequate counting accuracy. A count rate of 10,000 to 15,000 CPM was initially chosen rather arbitrarily. As it turns out, 10,000 to 20,000 CPM are generally accepted as adequate if enough counts are bound to give adequate precision.

1. Dilution

The dilution of the SPA in part determines the sensitivity of the assay system, the amount of ^{125}I-A-I that will be bound, and thus whether or not the specific activity of the label is sufficient. The particular dilution which is finally used will be ultimately dependent upon the binding constant of the antibody, and the requirements of the assay system in terms of sensitivity and precision.

2. Displacement

Competitive binding of ^{125}I-A-I with A-I is a basic requirement of the RIA and indicates the useability of the labeled preparation (32).

3. Mixing

It is necessary in the case of the Sephadex G-25-40 to physically mix the system (62) especially when the 10-40 μ rather than the 1-10 μ particle size is used. Apparently, without mixing, the antigen-antibody reaction does not occur probably because of reduced collision frequency and physical obstruction. This is a disadvantage in that special equipment is required (rotary mixer), but this has been solved by building the apparatus out of available materials for less than $10. The present rotary mixer has a larger tube capacity than other commercially available types (Heto Rotamix, Labquake), and is less expensive. Only one mixing speed has been examined, and it may be than another speed would give better results.

4. Time and temperature

Most RIA's are incubated at ca. 4°C overnight to obtain maximum binding. Increased binding at 4°C seems to be a common property of antibodies used for this purpose.

If the amount of bound label were sufficient for accurate and precise counting, it would make little difference if the incubation temperature were 4°C or ambient temperature for up to 4 hours (Fig. 15). Beyond this period, 4°C is definitely

superior, and at 23 hours total binding is 1.9 times greater than at ambient temperature.

5. Hydrogen ion concentration

   The hydrogen ion concentration is known to be a most important factor in antigen-antibody binding (18). Previously the RIA was run at pH 7.6 (39). However, binding at pH 7.6 is about 97% of maximum (Fig. 19) so pH 8.0 was chosen.

6. Ionic strength

   Wide (62) recommends the use of 0.05M phosphate buffer, pH 7.5 for RIA. By the present method, it was found that maximum binding increased 17.6% by raising the molarity from 0.05 to 0.2M (Fig. 21). Above this molarity it was felt that crystallization might interfere with the washing procedure, even though binding did not begin to decrease until after 0.35M was reached (Fig. 20). Decreasing the molarity below 0.05M continued to decrease the total binding.

7. Tris/HCl versus NaH_2PO_4 RIA buffers

   Tris/HCl buffer (pH 7.2-9.0) should be better than NaH_2PO_4 (pH 5.0-8.0) for buffering at pH 8.0. If was found, however, that maximum binding was decreased 75% in the Tris/HCl based assay buffer (Table 8).

8. Tween-20

   Tween-20 (0.5% v/v) was recommended by Wide (62) for use in RIA buffer to decrease non-specific adsorption. Tween-20 has

been reported to increase binding up to 3 fold for the solid-phase RIA of insulin (19).

Figure 23 shows that the addition of Tween-20 to the assay buffer decreases maximum binding. Figure 22 demonstrates increased variability in the standard curve when Tween-20 is added to the assay buffer. For these reasons, Tween-20 was removed from the assay buffer.

9. NaCl

Wide (62) recommends the use of 0.9% (w/v) NaCl in the RIA buffer. However, in these experiments, maximum binding was decreased 12.3% when the concentration of NaCl in the "assay buffer" was increased from 0 to 0.9% (w/v) (Fig. 24). Solutions of NaCl are known to increase the degree of antigen-antibody dissociation (18).

10. Lysozyme

Some protein is added to all RIA buffers to act as a radioactive scavenger and prevent the physical adsorption of peptides to container surfaces such as glass and plastic (10, 32). Wide (62) recommends the use of bovine serum albumin (BSA) (2 mg/ml) in the RIA buffer. It has been observed in this laboratory that even the best grades of BSA are subject to changes in appearance from lot to lot which makes one question its suitability. Lysozyme is another protein which is used quite widely in RIA for the same purpose as BSA. Lysozyme has the additional property of being able to hydrolyze the β-1,4 glucosidic linkages which

are found in the cell walls of a number of microorganisms (64). For these reasons lysozyme was chosen to replace BSA in the RIA buffer.

Figure 25 shows that an increase of lysozyme from 1-3 mg/ml causes a small but detectable down-shift in the standard curve. For this reason it was decided to use 1 mg/ml of lysozyme in the RIA buffer. It is also more economical to use 1 mg/ml.

11. NaN_3

Wide (62) recommended the addition of sodium azide 0.05% (w/v) to the RIA buffer as an inhibitor. Figure 26 shows that sodium azide causes a small but detectable decrease in binding in the standard curve. It is felt that if the RIA buffer were to be kept for an extended period of time it might be helpful to add the NaN_3 as an inhibitor.

12. Buffer used for washing the ^{125}I-A-I-anti-A-I complex

a. Composition and volume

Wide (62) recommended 3 x 2.5 ml saline-Tween-20 washes. It was noted that the 2.5 ml wash did not fill the 12 x 75 mm tubes sufficiently to wash down a small amount of SPA which had collected around the cap. A 4 ml wash was found to be more suitable for this purpose. Both saline and Tween-20 were later removed from the wash buffer and 0.1M NaH_2PO_4, pH 8.0 was substituted. Saline and Tween-20 were shown to adversely affect binding, and phosphate buffer was closer in composition to the RIA buffer. A 0.1M NaH_2PO_4, pH 8.0 buffer was found not to crystallize during prolonged storage at 4°C.

It is seen in Figure 27, that after 2 x 4 ml washes the binding in the "zero" and high standard tubes do not decrease significantly. This reduces the washing procedure by one wash so that the time for washing 96 tubes and placing them into the automatic gamma counter is about 45 minutes. Separation of "bound" and "free" with dextran-coated charcoal in the free antibody system (39) requires 1 hour or more, including reagent preparation.

b. Non-specific binding

The amount of binding remaining in the high standard (1.0 μg) tube, by SPARIA, is a measure of the amount of non-specific binding due to coprecipitation and trapping (40). Two washes reduced non-specific binding to 0.5%, and three washes reduced it to 0.3% for SPA diluted 1:100 (Fig. 27). This is less than most other RIA's report for non-specific binding, and using SPA diluted 1:200 for routine RIA should cause an even further decrease.

c. Ambient temperature versus 4°C wash buffer

The 1.4% decrease in the standard curve produced by washing with ambient temperature wash buffer (Fig. 30) was felt to be great enough to keep the wash buffer at 4°C.

d. Delayed washing

The 1.7% decrease in the standard curve produced by 2 x 5 minute delays in the washing procedure resulted in a 6.7% error in reading the value of the points at a level of 300 pg (Fig. 28). Thus the washing procedure should not be

excessively delayed if more than 96 tubes are to be washed and compared to the same standard curve. A 10% error has been reported in the case of delayed separation (5 minutes) of "bound" and "free" using dextran-coated charcoal (39).

13. "Low standard phenomenon"

The so-called "low standard phenomenon" (Fig. 31) has not been widely reported and was thought at first to be a characteristic of the solid-phase system. A similar phenomenon has been reported by Ceska and Lundkvist (20) in the RIA of vitamin B_{12} with solid-phase (Sephadex) coupled intrinsic factor. They showed that the increased binding they observed could be produced by the addition of small amounts of free intrinsic factor, and could be reduced by prewashing of the solid-phase intrinsic factor before RIA. Washing was said to remove non-covalently bound intrinsic factor from the solid phase. They did not attempt to demonstrate the absence of this phenomenon with the free intrinsic factor RIA.

In the present study, the "low standard phenomenon" was found to remain after 3 x 4 ml RIA buffer washes prior to RIA (Fig. 32). In addition, a similar effect was found during a comparison of SPA and free antibody standard curves (Fig. 50).

The nature of the observed increase in binding caused by the addition of small amounts of standard A-I is unknown. A possible explanation might be that the addition of one molecule of A-I to a multivalent antibody molecule increases the antibody affinity for a second molecule of A-I. In this case, either one or both

A-I molecules might be labeled with ^{125}I producing the observed increase in binding in the low standard portion of the curve.

The presence of such a phenomenon could cause large errors in the reading of unknowns, if the hormone level were to fall in that portion of the standard curve. It is observed in Figure 31 that if a reading is made at 28% bound, results of 1, 10 and 160 pg/50 µl could be read. This would result in calculated values for A-I generation rates of 0.02, 0.2 or 3.2 ng/ml/h, respectively.

14. Delayed addition of ^{125}I-A-I

The late addition of label has been said to increase the sensitivity of certain RIA's (51, 62). Figure 36 shows a standard curve incubated for a total of 48 hours but with label added 24 hours before washing. An increased slope (6.5 vs. 4.6%) between 6.7 and 100 pg/50 µl indicates increased sensitivity in this area of the standard curve. Above this level the normal standard curve is more sensitive. By late addition of ^{125}I-A-I approximately 41.3% greater sensitivity is obtained in the area of 6.7-100 pg/50 µl. The late addition of label requires more work and a longer incubation period. However, if the sensitivity of the assay proved to be critical in this range, the delayed addition of label might be worthwhile.

15. Delayed addition of A-I, reversibility

Certain solid-phase systems have demonstrated relatively irreversible binding of the antibody-label complexes, even in the presence of excess antigen (15, 19, 52). Figure 36 shows that

at least 42% of bound ^{125}I-A-I activity was displaceable by 6.9 ng of standard A-I. A larger amount of standard A-I might cause greater displacement. The reversibility of solid-phase antibody-antigen complexes, compared to free antigen-antibody complexes, may indicate an alteration of the antibody binding affinity, caused by coupling to the solid phase.

Determination of renin activity

1. Buffer for A-I generation

   Lehfeldt and Hutchens (39) stated that the rates of plasma A-I generation were lower in sodium buffers, than in ammonium or potassium buffers. In order to match the buffer used for A-I generation, with the phosphate RIA buffer, sodium, potassium and ammonium phosphate buffers were compared with maleic acid/ammonium maleate buffer for the determination of renin activity (Table 10). Compared to maleic acid/ammonium maleate buffer, A-I generation rates were decreased, 19.6% or more, by the above phosphate buffers.

2. Plasma pH

   Maximum rates of angiotensin generation have been reported in the range of pH 5.5-6.0 for RIA and bioassay methods (38, 39, 53). Approximately three fold reductions in activity are reported for angiotensin generation rates at pH 7.0-7.5. In this procedure maximum activity was found at pH 6.0 (Fig. 39) in agreement with other reports (39, 53). At pH 5.42 the rate was only 63.3%

of maximum, and there was precipitation observed in the plasma. As a general rule, it is felt that if the pH is maintained between 5.88-6.16 (90% activity range from Fig. 39) acceptable results should be obtained. Better regulation of pH is desirable for future development of this assay.

3. Angiotensinase and converting enzyme inhibitors

A-I is protected from conversion to A-II by converting enzyme and from destruction from peptidases, by chelation of divalent metal ions. Na_2EDTA, 8-hydroxyquinoline and 2,3-dimercapto-1-propanol (BAL) are added to plasma as inhibitors. In 1971, Page et al. (48) reported a study in which 8-hydroxyquinoline sulfate (1.3 μmol/ml) and BAL (4.0 μmol/ml) were found to give optimal inhibition of angiotensinases and converting enzyme. In the present study it was found that, using the above inhibitor concentrations, the rate of generation of A-I was decreased (Table 13) relative to the inhibitor concentrations used by Lehfeldt and Hutchens (39). Therefore, the 8-hydroxyquinoline was left at 6.9 μmol/ml and the BAL at 2.0 μmol/ml.

Three concentrations of EDTA were tested to evaluate this anticoagulant/inhibitor. The rates of A-I generation did not differ significantly (Table 11) but it was found that increasing the EDTA concentration from 1, to 2 or 3 mg/ml of plasma caused precipitation to occur at 37°C during incubation.

It has been reported (39) that sodium ion caused inhibition of renin and therefore dipotassium EDTA should be used as an

anticoagulant/inhibitor rather than the disodium salt. To date only five renin inhibitors have been identified (26,37). They are, renin preinhibitor (lysophospholipid), certain synthetic tetrapeptides and a pentapeptide, heparin, and taurodeoxycholic acid. In this study no effect could be observed when either disodium or dipotassium EDTA were used as the anticoagulant/inhibitor (Table 12).

4. Shaking during A-I generation

Lehfeldt and Hutchens (39) stipulate the use of a shaking water bath during the generation of A-I. Since the generation of A-I is on a molecular level the effect of shaking was questioned. It could not be demonstrated that the rate of generation of A-I in "control plasma" was any different with or without shaking during incubation at 37°C. This step was left out of the proposed procedure for renin activity.

5. Set-up temperature of RIA

It is not known whether any other study of the actual effect of the set-up temperature has been made. Since it was observed that the initial binding rates are almost equal for the first four hours at 4°C and ambient temperature, little difference was expected. It was found that definite fluctuation occurred in the low standards when the standard curves were allowed to incubate at ambient temperature, with plasma added (Fig. 40). While appearing to have no effect above 100 pg/50 µl, it is possible that the fluctuation could be misread as an increased concentration

of A-I, resulting in an error of 100 pg/50 µl or more.

6. Thawing plasma

Thawing plasma at 4°C requires several hours, and thawing at higher temperatures, while more rapid, may cause renin substrate depletion. It could not be shown that any significant effect on A-I generation rates in "control plasma" occurred, by varying the temperature and time of thawing.

7. Centrifugation of plasma

The thawing of frozen plasma results in the formation of fibrin clots which may plug the tips of pipettes, or displace plasma in the sampling. No effect on A-I generation rates could be observed, but it is felt that routine centrifugation after thawing will alleviate this potential source of error.

8. Incubation time

It has been observed in this laboratory, and throughout this study, that the rate of A-I generation drops off quite often from 2 to 3 hours. Previous reports have stated that renin substrate is not rate limiting, and that renin activity follows zero order kinetics during the 3 hour incubation (31). It has recently been demonstrated that the renin substrate concentration in the normotensive population (<1 nmol/ml) is suboptimal and that renin activity reaches zero order kinetics at around 3 nmol/ml (31). Therefore, it appears that in order to have optimal activity in the assay system additional substrate must be added.

Until a suitable preparation is available it was decided to reduce the incubation time by 1 hour, and change the sampling intervals to 30, 60 and 120 minutes. It may be seen in Figure 41 that the graphic calculation of renin activity, in the same sample, was increased 13.4% by this method. The decrease in A-I generation rate with time, has been observed often in this laboratory and has been reported before (39). The increased rates of A-I generation in plasma buffered at pH 6.0 with maleic acid/ammonium maleate (39) increases the rate of substrate depletion and would tend to intensify any such effects.

9. Reproducibility

The results of the reproducibility studies are given in Tables 14 and 15. It is seen that the addition of plasma to the standard curve increases the variability. Reproducibility was expressed at several levels of A-I and it was noted that the variability was greater at the high end of the standard curve. This may be due to the decreased count rates in this area, and to the fact that the assay system was adjusted to assay of normal levels of A-I which are read on the lower portion of the standard curve.

Table 18 attempts to compare the reproducibility of several other RIA's of A-I for renin activity (27, 33, 34, 39, 55, 56). There is little statistical uniformity, but it is felt the present assay is comparable to most.

10. Recovery

The results of the recovery experiment (Table 16) suggest that all added A-I is recovered from plasma at zero time. In Figure 42 the recovery rate appears to drop off after 1 hour. This may be a loss of A-I or an artifact due to poor precision in this concentration range.

Table 19 compares the recovery of several other RIA's of A-I for renin activity to the present method (12, 27, 34, 55, 56). Only one other method (56) gives comparable recovery.

11. Sensitivity

The sensitivity of a RIA is a poorly defined concept (32). There are two definitions of sensitivity, both of which are used to describe a particular system. The most common definition is: that amount of a substance being measured which can be differentiated from zero on a statistically significant basis. A second, and perhaps more useful definition is: the amount of substance which can be differentiated at any given point on the standard curve with statistical significance. The latter is used routinely in the RIA.

The level of significance chosen for this study was 95% (2s) based on the precision at three different levels on the standard curve. Due to the "low standard phenomenon" no statistical conclusions could be drawn at the "zero" level and the minimum detectable limit was estimated on the basis of no overlap of standard points in a large number of standard curves. The ng/ml

estimates based on precision (Table 17) give a practical limit imposed by the RIA for the sensitivity of various levels of renin activity. In Table 20 several RIA's of A-I are compared to the present study on the basis of sensitivity. The sensitivity of the present method is as good or better than those reported.

12. Addition of A-I to unknowns

It has been demonstrated (Fig. 43) that due to the "low standard phenomenon" a plasma sample may be misread by as much as two orders of magnitude. For this reason it was felt that by addition of a known amount of A-I to each unknown plasma a baseline could be established where the concentration was known. An additional benefit was that the concentration of A-I standard added may be adjusted to shift the A-I generation curve to the most sensitive portion of the standard curve (the range of linearity). Since a volume of buffer has to be added to the unknown tubes to compensate for standard added to the standard curve, there is no additional pipetting involved.

Coupling of A-I to activated Sephadex G-25-40

It has been demonstrated that A-I may be covalently coupled to activated Sephadex G-25-40 by using ^{125}I-A-I to follow the reaction (Figs. 44-46). The amino acid sequence of human A-I is as follows (3, 35):

Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu
1	2	3	4	5	6	7	8	9	10

There are only two free amino groups available for coupling with Sephadex; the N-terminal amino group on the aspartate residue, and the amino groups of the guanidino group on the arginine residue. It is felt that the coupling may occur to either group (6).

The C-terminal is probably most important immunologically (25). Thus, by coupling the A-I molecule to Sephadex near the N-terminal it was felt that a most suitable SPAg would be produced.

1. Binding capacity of SPAg

   The anti-A-I binding capacity of A-I coupled to Sephadex may be estimated from the amount of A-I bound to Sephadex. The molecular weight of A-I is 1,297. So if the molecular weight of anti-A-I is assumed to be 200,000, the theoretical maximum of anti-A-I binding capacity is 154 times the weight of A-I coupled to Sephadex. Based on electrophoresis (Fig. 5) and total protein, the gamma region of anti-A-I antiserum contains 191 µg of protein per 10 µl. Since some protein is probably lost by Na_2SO_4 precipitation, and anti-A-I makes up only part of the total gamma globulin, the actual amount of anti-A-I incubated with SPAg is less than 191 µg. SPAg (100 mg), containing 39 µg of A-I, has a potential binding capacity of 6 mg for anti-A-I, assuming a molecular weight of 200,000.

2. Purified anti-A-I

   A small amount of anti-A-I was isolated from the SPAg as shown by Figures 47-49. Since 10 µl of anti-A-I can be used for around 1,000 assay tubes when diluted 1:5,000, the amount of anti-A-I

recovered is very small (equivalent to less than 2 ml of a 1:120,000 dilution) compared to the direct Na_2SO_4 purified anti-A-I dilution curve (Fig. 47). The estimated amount of anti-A-I (10 µl originally) remaining is around 1.7×10^{-3} µl, or 0.017%. A loss of this order is unacceptable for purposes of routine RIA where a valuable reagent (anti-A-I) should be conserved.

By comparing the standard curves prepared with "purified" anti-A-I and Na_2SO_4 purified anti-A-I (Fig. 49), no apparent difference was seen in the position of the displacement curve. If a shift to the left (lower concentration of A-I) had been observed, it might have suggested the isolation of anti-A-I having higher binding affinity. A higher binding affinity antibody could be used to produce a more sensitive assay. However, the appearance is that which would be produced by anti-A-I dilution alone. It is also possible that anti-A-I was damaged by the purification procedure.

It is thought that the low yield of this experiment may be due to a SPAg in which the antigenic sites, although undamaged, are unavailable to anti-A-I due to their proximity on the surface of the Sephadex. In other words, a SPAg containing less A-I, spaced farther apart, might be more successful in isolating the anti-A-I in higher yields. Another possibility is that the antigen could be made more available to the antibody by extension from the surface of the solid phase. This could be accomplished by coupling A-I to a larger molecule and, in turn, coupling the

complex to the solid phase (21).

SPA versus free antibody

Important considerations concerning the adoption of any solid-phase system are its utilization of antibody, and whether or not the binding affinity has been altered by attachment to the solid phase.

One ml of anti-A-I antiserum may be used for 100,000 assay tubes in the free antibody system (using 50 µl of a 1:5,000 dilution). In the present solid-phase system SPA (100 g) equivalent to 1 ml of anti-serum, may be used for 25,000 assay tubes (using 0.8 ml of a 1:200 dilution). This assumes, in both cases, that the diluted antibody is used completely. In the case of SPA, the dilution may be reconcentrated by centrifugation, stored at -20°C and rediluted in fresh buffer at a later time. It would be much more difficult to do this with the free antibody dilution. With 100% utilization in both systems the solid phase requires 4 times as much antibody in RIA of A-I for renin activity.

Based on a comparison of SPA and free-anti-A-I standard curves, no differences, other than might be produced by dilution, were observed (Fig. 50). If an alteration of antibody binding affinity were to have occurred, the result would have been a shift to the right (higher A-I concentration) for a decreased binding constant, and left for an increased binding constant. Since both curves remain parallel and are nearly superimposable, it is unlikely that the binding affinity has been altered significantly, if at all. The difference seen in the "zero" tubes may be due to small fluctuations

in the low standard region as already mentioned.

Another factor which might reflect an altered binding constant is the binding rate (Fig. 51). The initial binding rates reflect the antibody association constants more closely, and later reflect the overall rate due to both association and dissociation. It appears that the free antibody is somewhat more rapid initially, but this may be due to the limited mobility of the SPA, reducing the collision frequency. The differences observed in maximum binding at the equilibrium state are probably due to different total antibody concentrations. It cannot be stated that there is a decreased rate of binding for SPA since the effects of these factors are unknown.

SUMMARY AND CONCLUSIONS

The SPARIA method of Wide (62) has been applied to the measurement of A-I, for the determination of renin activity (39), using ^{125}I-A-I. Optimal conditions for the RIA were found to be overnight incubation at 4°C, in 0.2M NaH_2PO_4, pH 8.0 containing 1 mg/ml lysozyme. Mixing of the SPA incubation mixture was accomplished with a rotary mixer built from a barbecue spit. A-I was generated in maleic acid/ammonium maleate buffered plasma at pH 6.0, with Na_2EDTA (1 mg/ml), BAL (2.0 µmol/ml), and 8-hydroxyquinoline (6.9 µmol/ml) added to inhibit angiotensinases and converting enzyme.

Compared to optimal conditions, antigen-antibody binding was decreased by the following: pH greater than 8.1 or less than 8.0, incubation at ambient temperature, the presence of Tween-20, NaN_3, Tris/HCl buffer, increased concentrations of lysozyme, or decreased concentrations of phosphate buffer. A-I generation rates were decreased by: Na_2EDTA (2 and 3 mg/ml), BAL (4.0 µmol/ml), and 8-hydroxyquinoline (1.3 µmol/ml).

^{125}I-A-I was prepared by a modified method (47) using Chloramine-T iodination, and purified on Sephadex G-15-120. It was stable for at least four months stored at -20°C. Specific activity, determined by self-displacement, is higher than any yet reported. Binding with excess SPA was 80%.

SPA was stable for at least six months at -20°C. It can be lyophilized with little or no loss of activity. The SPA-^{125}I-A-I complex may be dissociated with glycine/HCl buffer, pH 2.0 and reused

for RIA. Diluted SPA may be reconcentrated by centrifugation and stored at -20°C for later use. Increased sensitivity was obtained by 24 hour late addition of ^{125}I-A-I.

The antibody binding constant is probably not affected by coupling to the Sephadex G-25-40, as evidenced by unaltered standard curves, and binding rate, relative to a free-antibody system.

Increased binding of ^{125}I-A-I with the addition of small amounts of A-I (low standard phenomenon) were found for both the solid-phase and free-antibody systems.

Sensitivity, accuracy and reproducibility are comparable to other RIA systems of A-I for renin activity.

The solid-phase system has several advantages. There is antibody-specific separation of "bound" and "free." The RIA procedure is simplified. Compared to other solid-phase systems, SPA is stable and less antibody is required. SPA is more versatile than free-antibodies or other solid phases for antibody handling and utilization.

The disadvantages of the SPARIA are: that is uses 4 times more antibody than the free system; requires a special reagent preparation one or two times a year; and requires specially capped tubes and equipment for rotary mixing.

Anti-A-I has been isolated using A-I coupled to Sephadex G-25-40 (SPAg), and demonstrated by dilution and standard curves with ^{125}I-A-I. Low recovery makes the current procedure of little value for isolating anti-A-I for use in RIA. No increase in binding constant was demonstrated for anti-A-I isolated with this method.

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Table 1

Radioimmunoassays

Adrenalin
Adrenocorticotrophin
Albumin
Aldosterone
Androstenediol
Androstenedione
Angiotensin I and II

Barbiturates
Bile acids
Bradykinin

Calcitonin
Carbonic anhydrase
Carcinoembryonic antigen
C-1-esterase
Cholecystokinin-pancreozymin
Cholesterol
Chorionic gonadotrophin
Corticosterone
Cortisone
C peptide
3',5'-Cyclic adenosine monophosphate

Dehydroepiandrosterone
Deoxycorticosterone
20α-Dihydroprogesterone
20β-Dihydroprogesterone
Digitoxin
Digoxin

Eosinophil peroxidases
17α-Estradiol
17β-Estradiol
Estriol
Estrone
2-Hydroxyestrone

Fibrinogen
Folic acid
Follicle stimulating hormone
Fructose-1,6-diphosphate

Gastrin
Glucagon, enteric and pancreatic
Growth hormone
Guanosine-3',5'-monophosphate

Hepatitis associated antigen
Histocompatability antigens
Hydrocortisone

IgG,A,M,E
Insulin, α and β chains

Luteinizing hormone

α and β Melanocyte stimulating hormone
Morphine
Myelin

Oxytocin

Parathormone
Pregnanediol
Pregnenolone
Progesterone
17α-Hydroxyprogesterone
11-Hydroxyprogesterone
Proinsulin
Prolactin
Prostaglandins

Retinal binding protein
Rheumatoid factor

Secretin
Schistosomiasis
Staphylococcus toxin

Testosterone
Tetanus toxin
Tetraiiodothyronine
Thyrotropin
Triiodothyronine

Vasopressin
Virus, mouse tumor

Table 2

Methods for separating "bound" and "free"

Electrophoresis
Chromatoelectrophoresis
Gel filtration
Chromatography
Ion exchange resin
Activated charcoal adsorption
Silica adsorption
Talc adsorption
Cellulose powder adsorption
QuSO adsorption
Antibody precipitation
Polyethylene glycol precipitation
Salt precipitation
Organic solvent precipitation
Salt and alcohol precipitation
Enzyme proteolysis of "free"
Solid-phase antibody (SPA)
Solid-phase antigen (SPAg)
Filtration

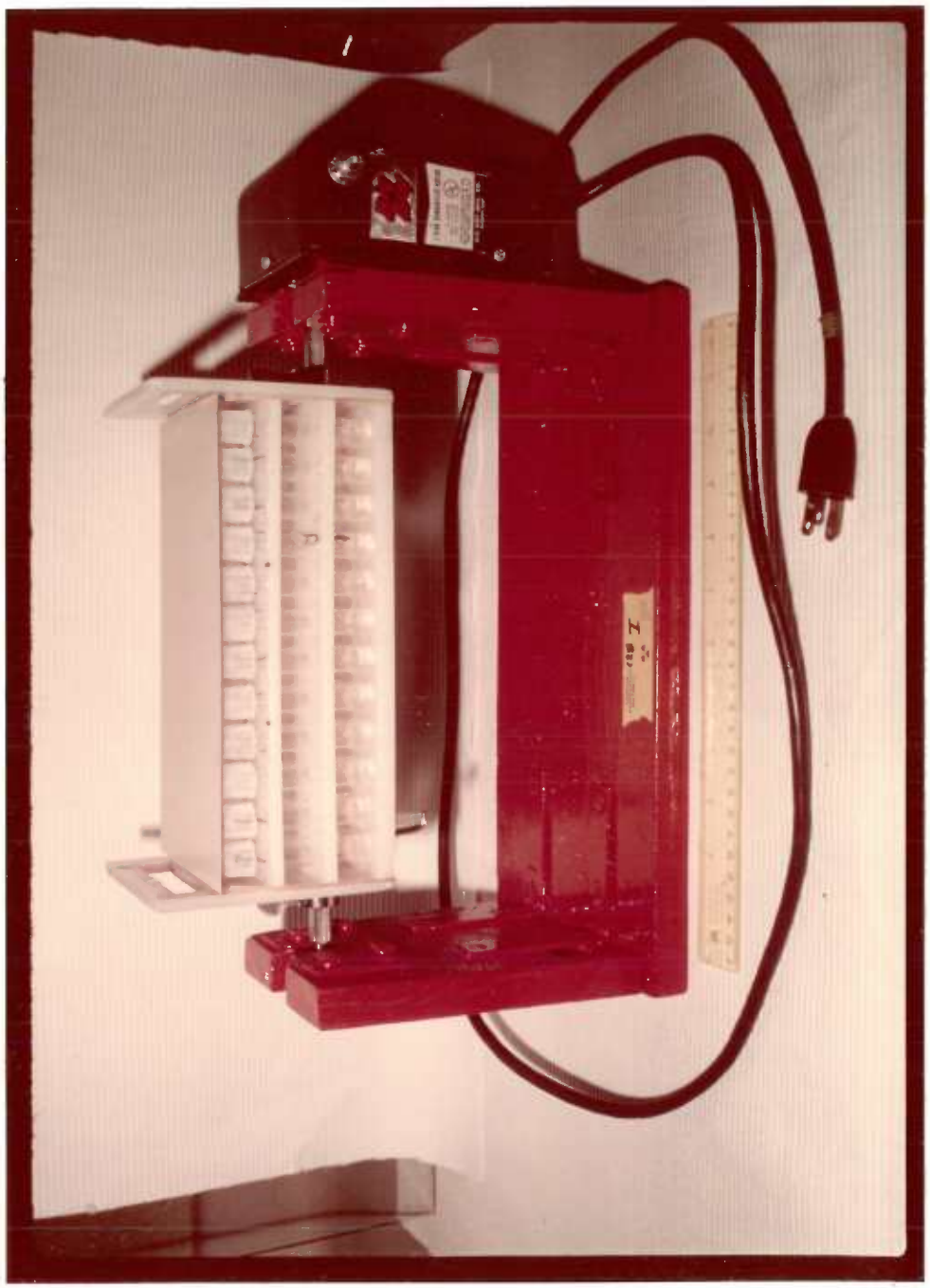

Figure 1. Rotary mixer set up to mix up to 72, 12 x 75 mm tubes.

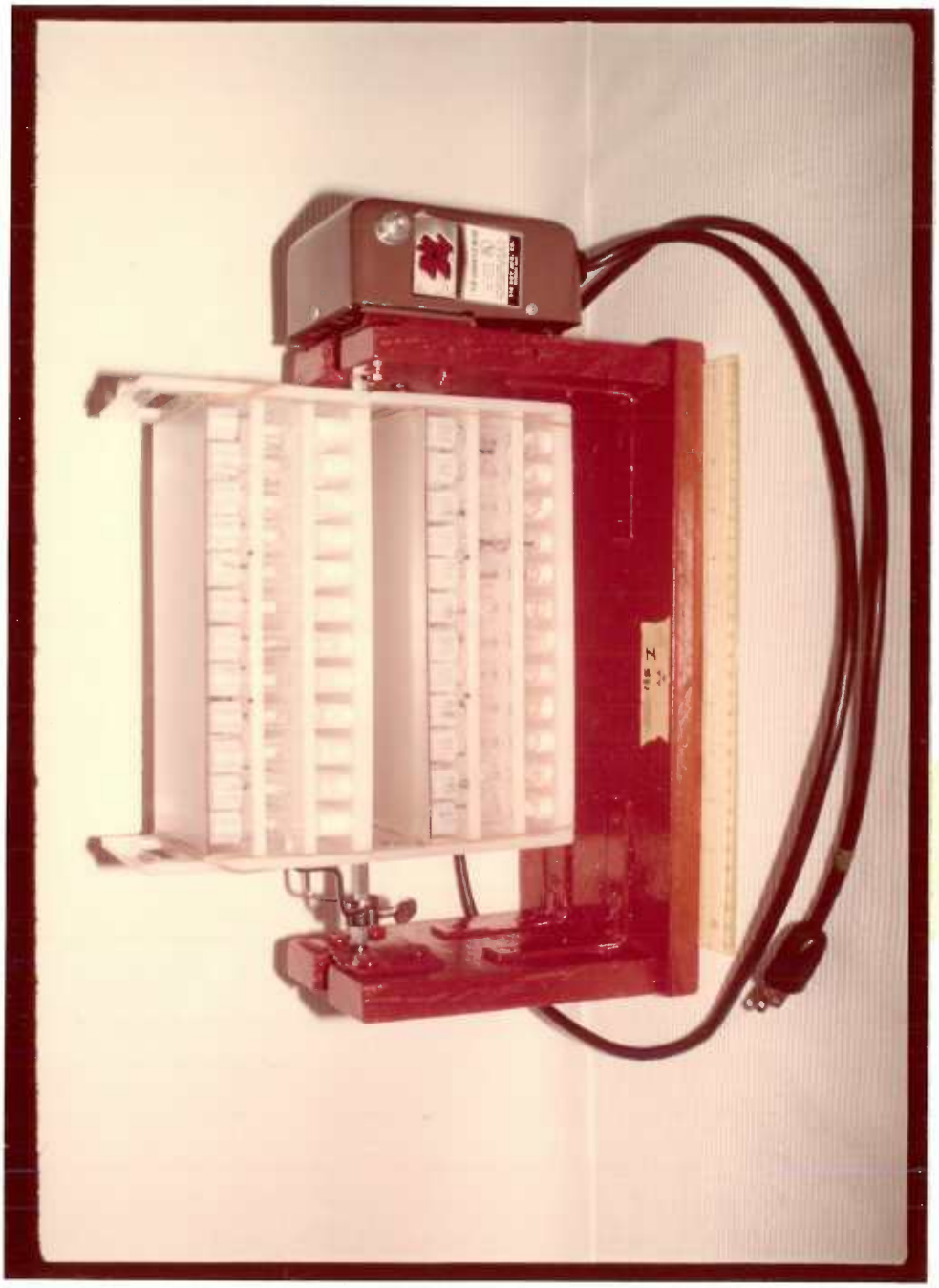

Figure 2. Rotary mixer set up to mix up to 144, 12 x 75 mm tubes.

Table 3

Sephadex G-25-40 weight gain due to activation

Weight g		
Before activation	After activation	% Increase
5.00	6.00	20.0
5.00	6.20	24.0
5.00	6.23	24.6
5.00	5.80	16.0
5.00	7.50	49.8

Figure 3. Cellulose acetate electrophoresis of "normal" rabbit serum

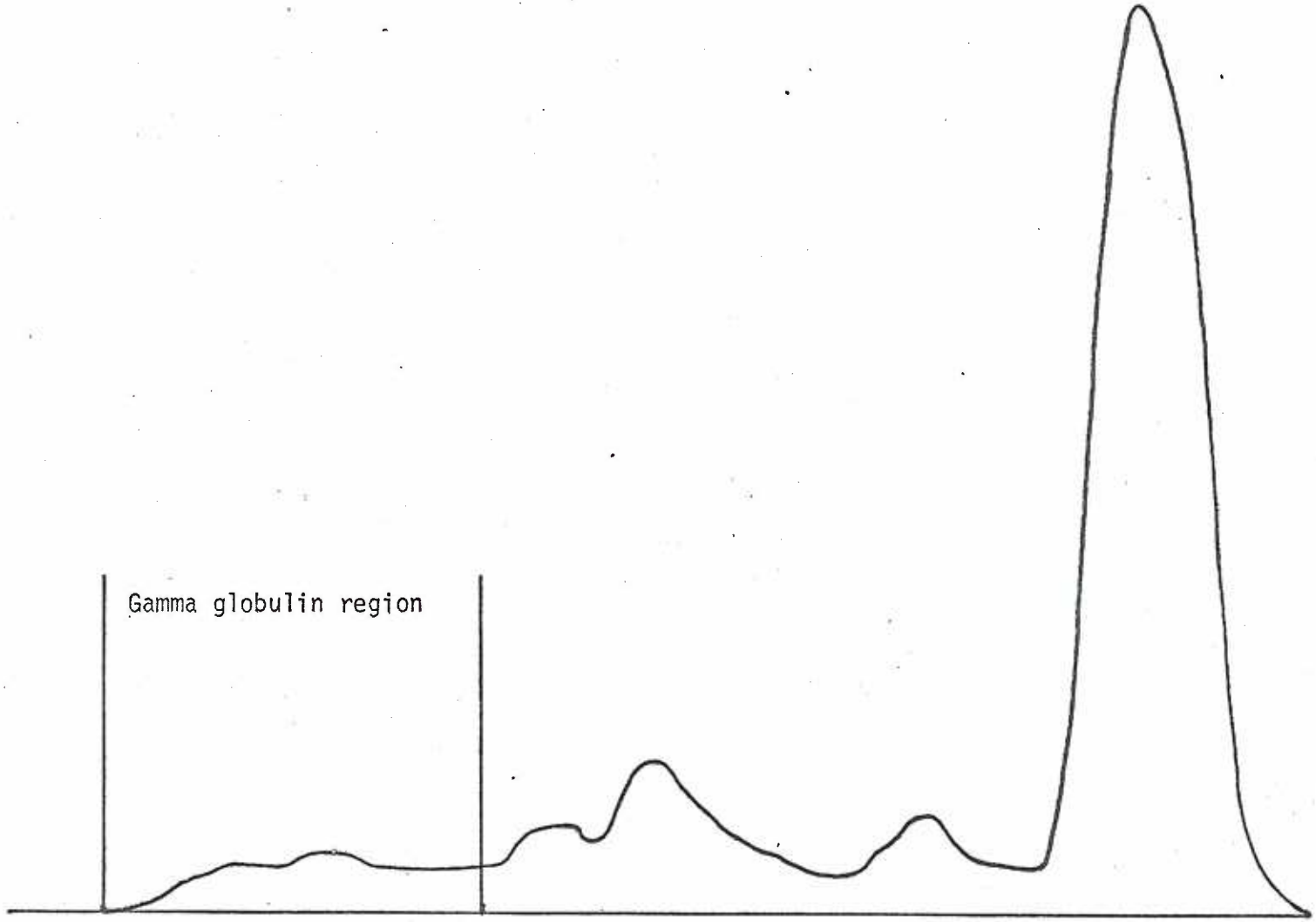

Figure 4. Cellulose acetate electrophoresis of "normal" rabbit serum after Na_2SO_4 precipitation

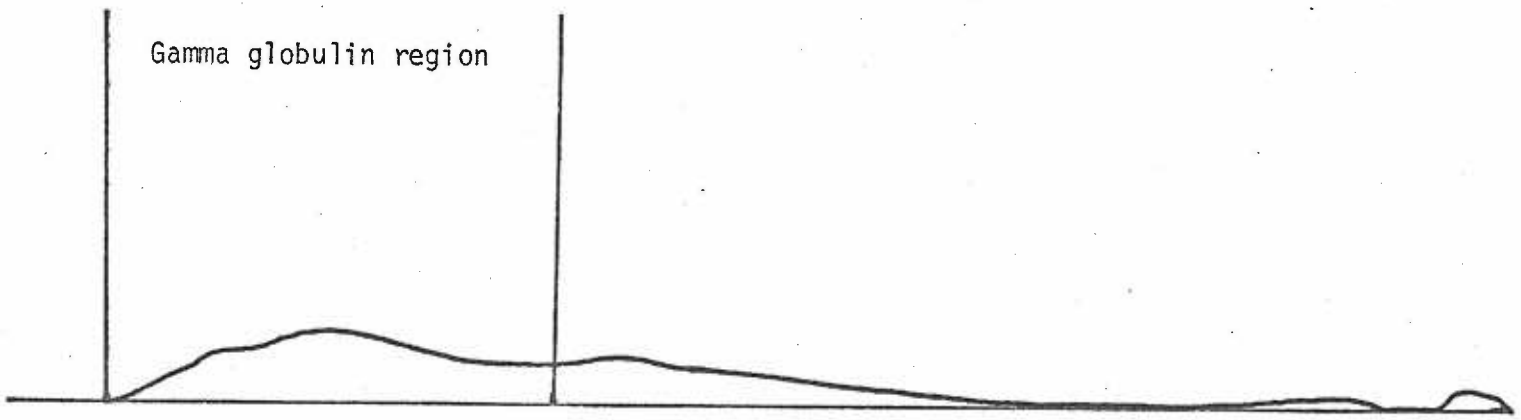

Figure 5. Cellulose acetate electrophoresis of anti-A-I antiserum. Rabbit III, lot 3

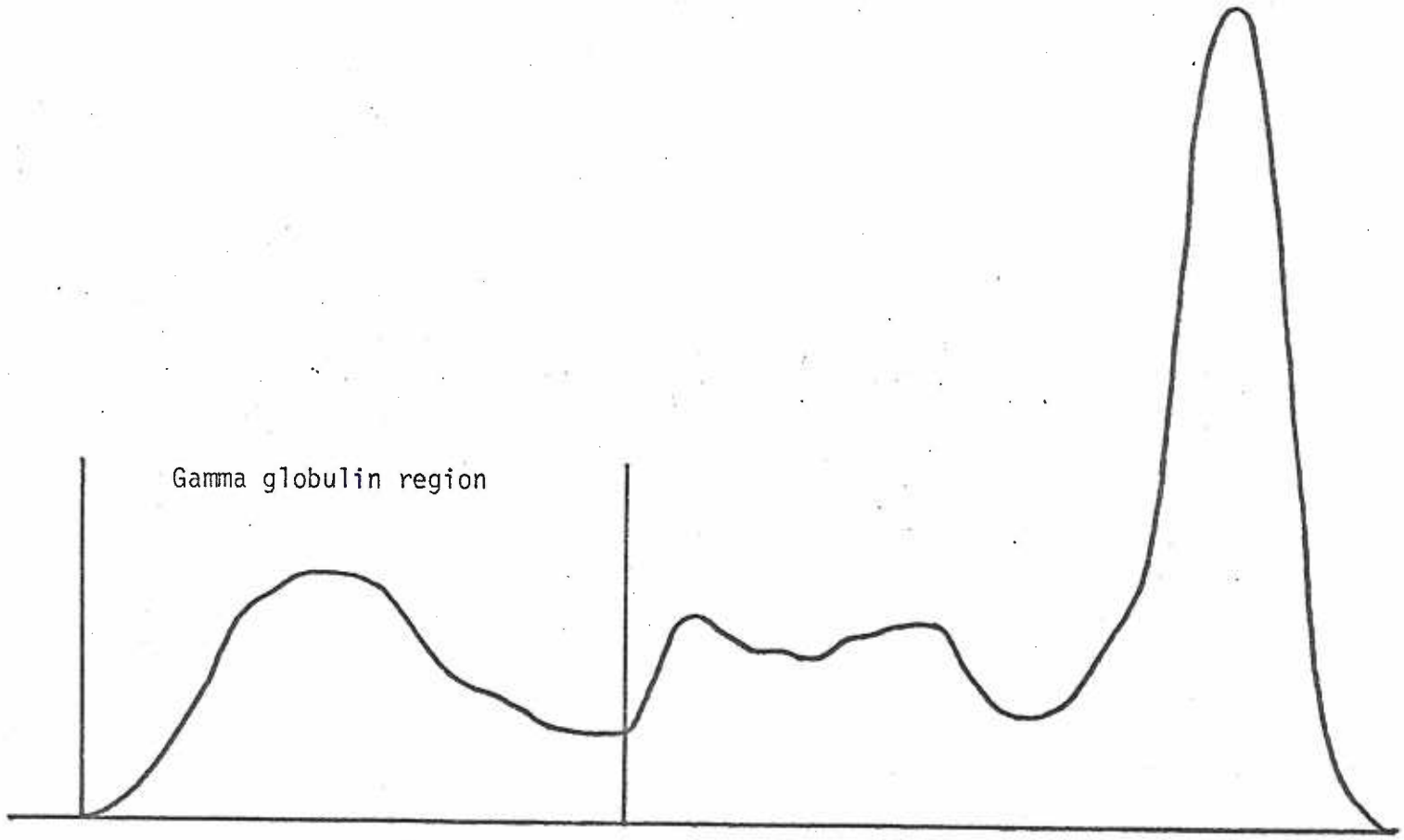

Figure 6. Cellulose acetate electrophoresis of anti-A-I antiserum after Na_2SO_4 precipitation. Rabbit III, lot 3

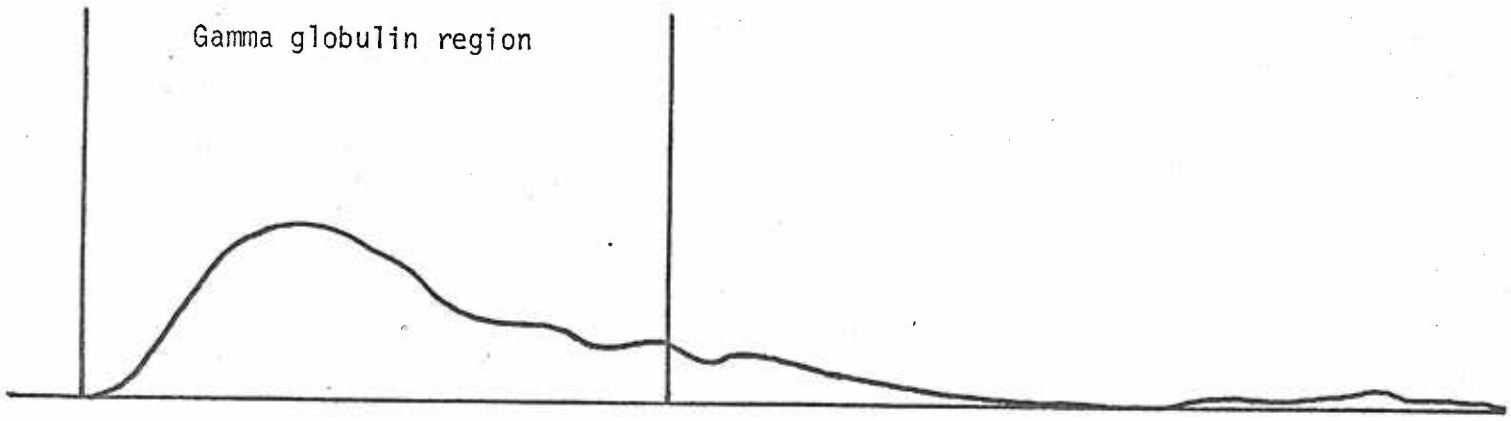

Table 4

Effect of sonication time on binding capacity

SPA Dilution	Sonication Time (min.)	% Bound
1. 1:4	0.1	63.8
		64.7
2. 1:4	0.25	62.8
		64.0

Table 5

Binding capacities of various SPA preparations

Age of preparation (months)	CNBr used for activation (mg/%)	Temperature of coupling (°C)	Time of coupling (d)	Sonicator	Maximum binding (%)
1. 8	25*	ca. 25	1	Probe	6.4
2. 5.5	2.5	ca. 25	1	Probe	27.0
3. 2.5	2.5	4	3	Bath	25.7

* saturated

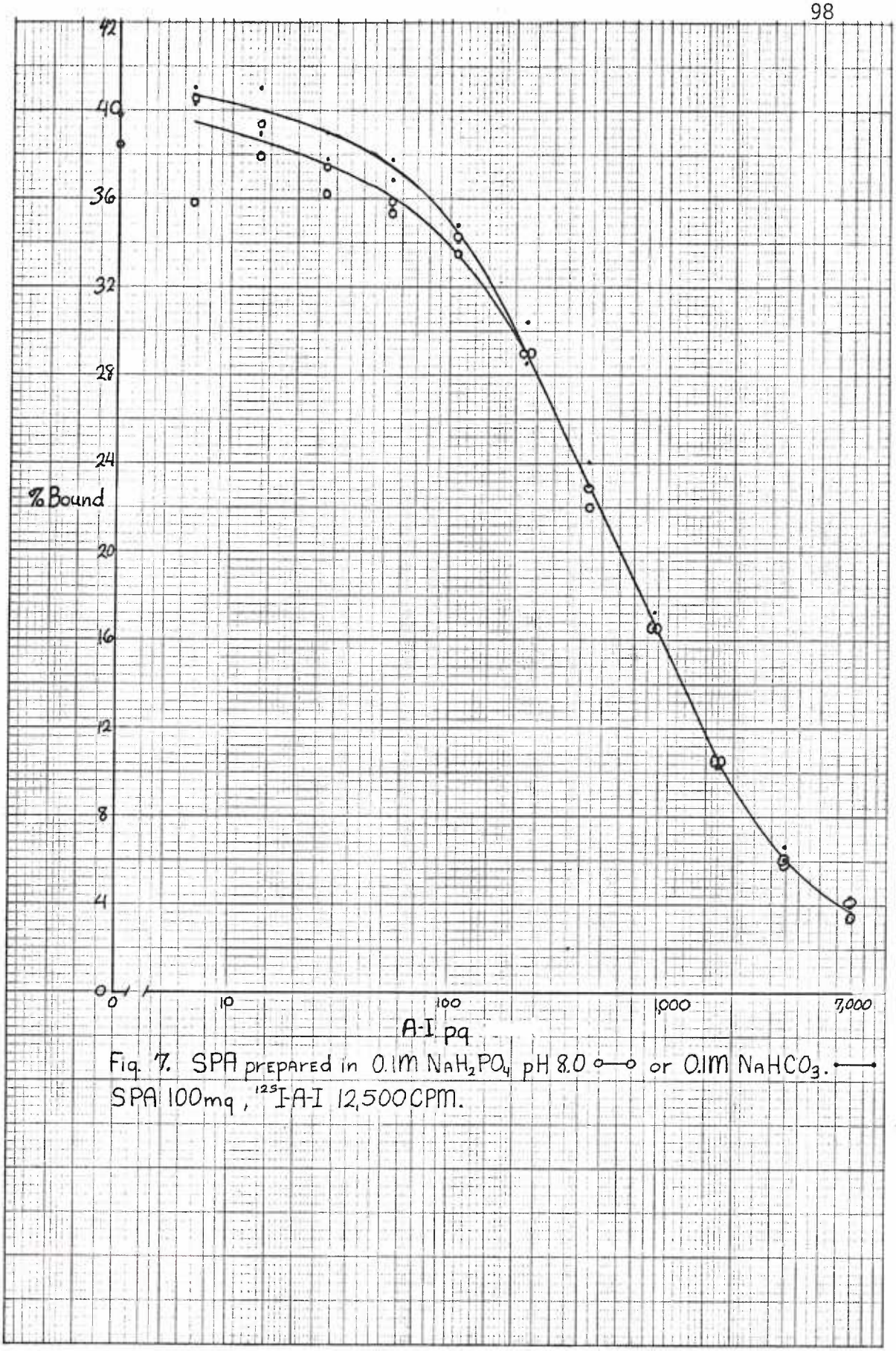

Fig. 7. SPA prepared in 0.1M NaH_2PO_4 pH 8.0 o—o or 0.1M $NaHCO_3$. •—•
SPA 100mg, ^{125}I-A-I 12,500CPM.

Table 6

Properties of the Sephadex G-15-120 column

column volume	220 ml
gel bed height	37.8 cm
gel bed volume	187 ml
gel dry weight	61.3 g
H_2O in gel bed	165 ml
temperature (water jacket, 80 ml/min)	14°C
flow rate (working)	20 drops/min

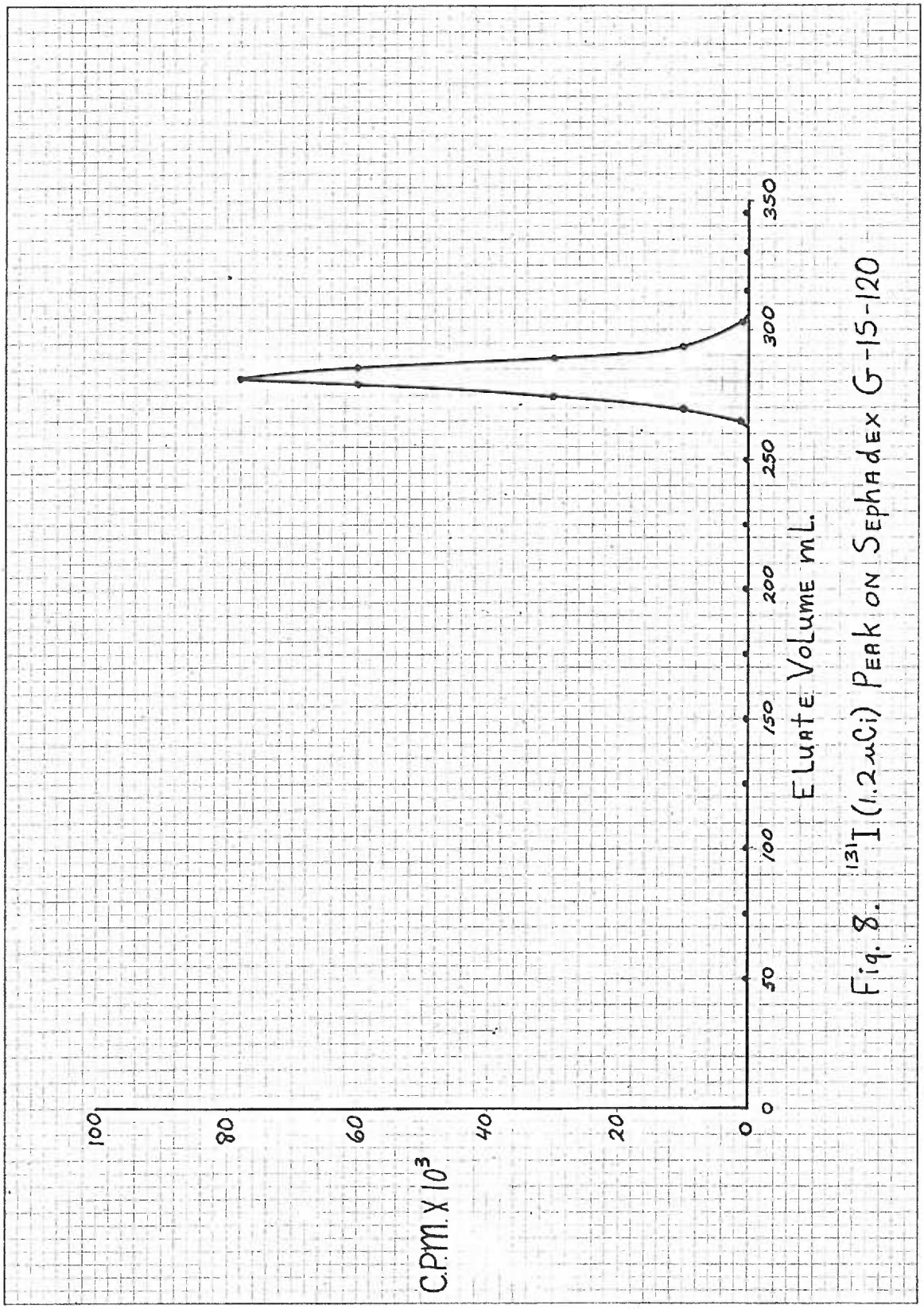

Fig. 8. ^{131}I (1.2 uCi) Peak on Sephadex G-15-120

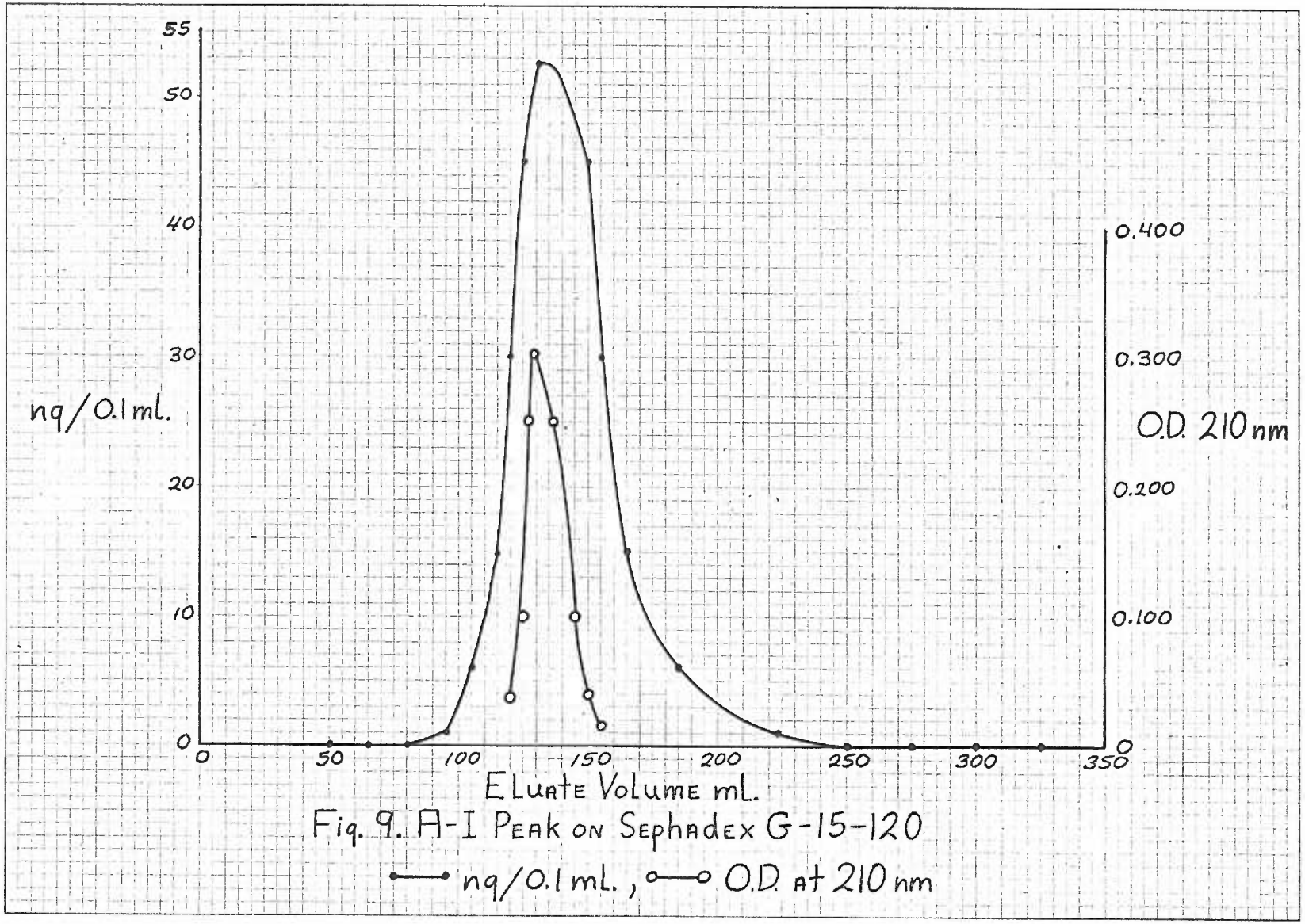

Fig. 9. A-I Peak on Sephadex G-15-120

—● nq/0.1 ml., o—o O.D. at 210 nm

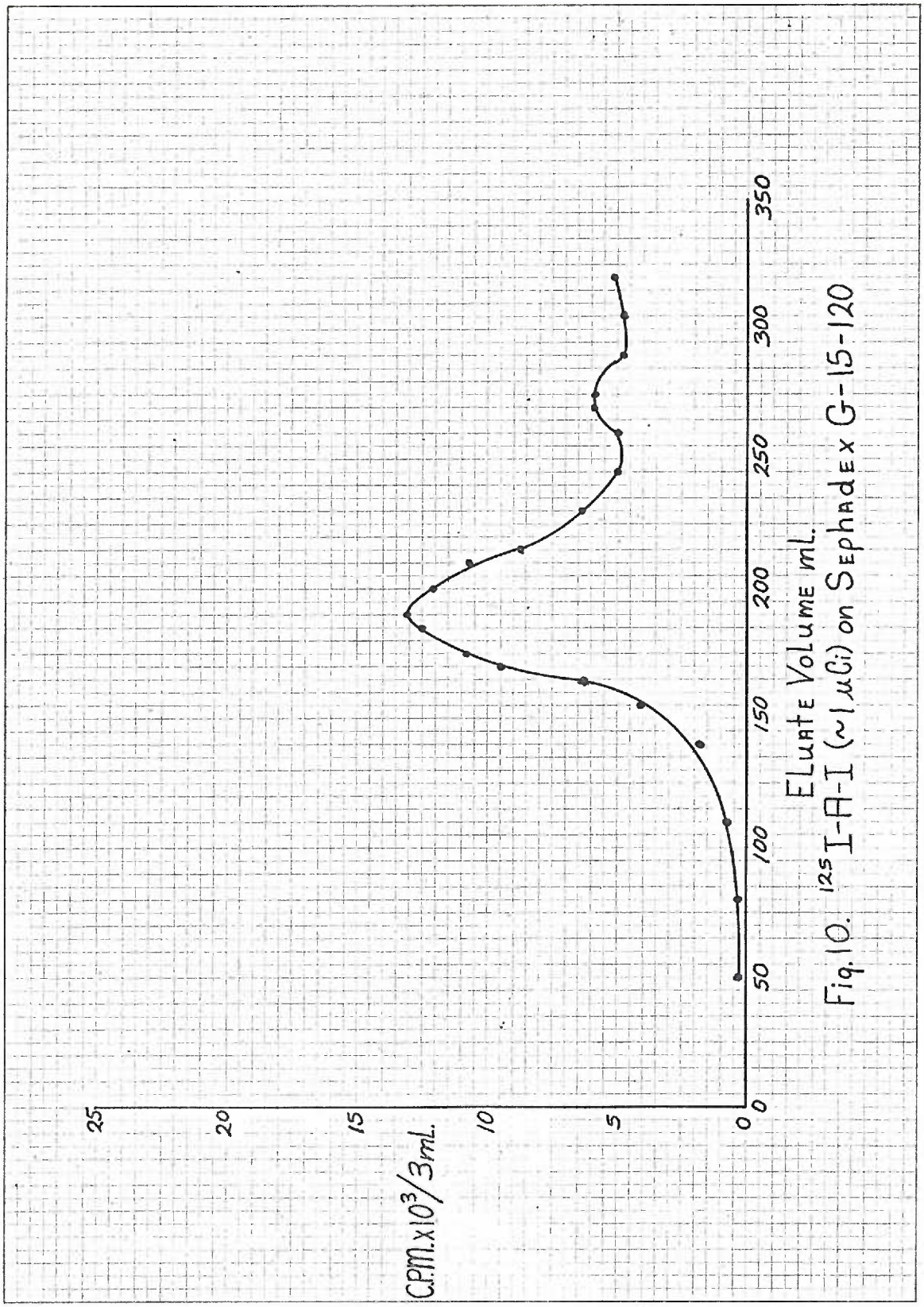

Fig. 10. ^{125}I-A-I (~1 μCi) on Sephadex G-15-120

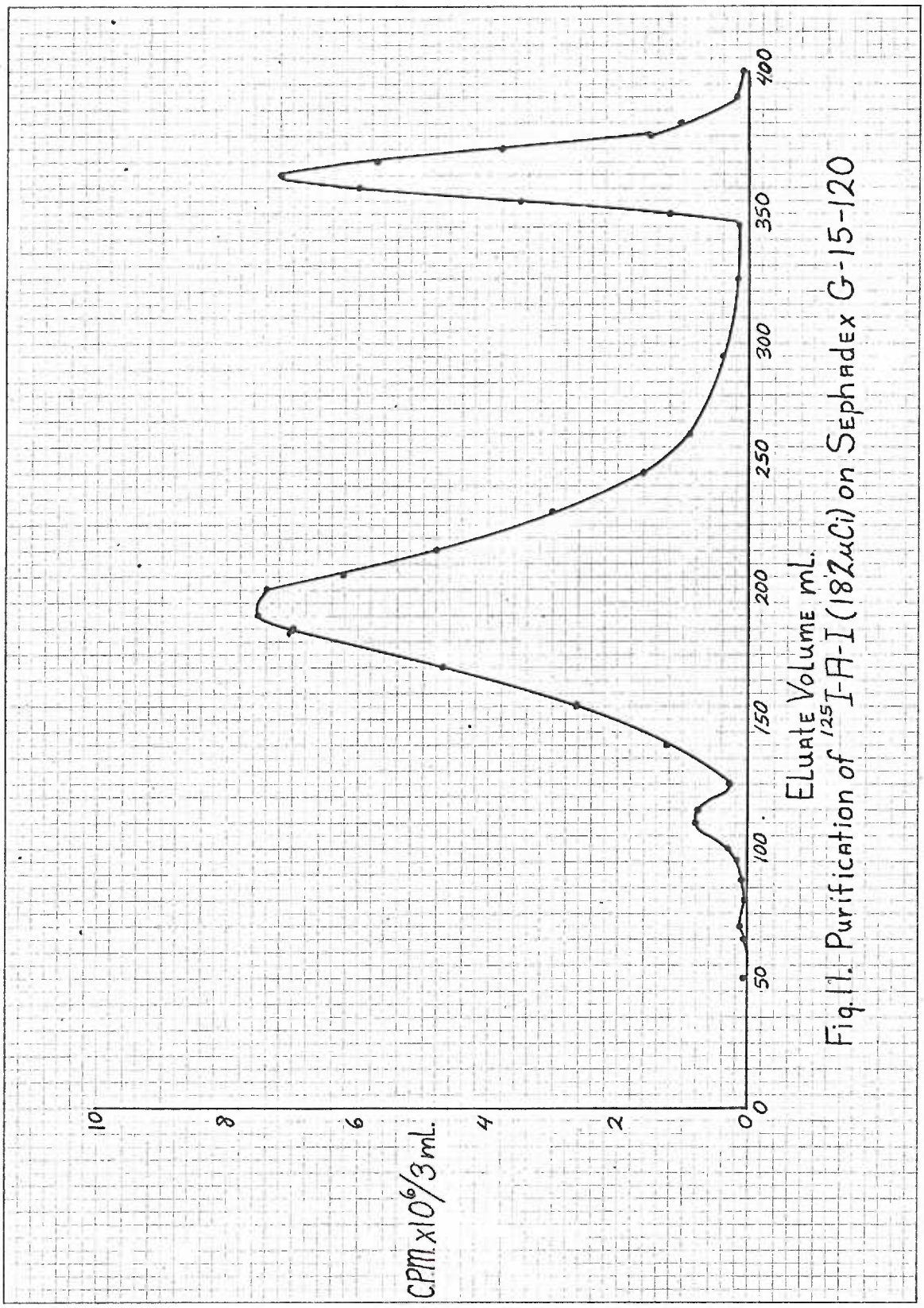

Fig. 11. Purification of ^{125}I-A-I (182uCi) on Sephadex G-15-120

Table 7

Activity recovered in the preparation of ^{125}I-A-I

Peak	Activity(μCi)	% of Total Activity (182 μCi)
1	0.3	0.2
2	3.2	1.7
3	130.6	71.7
4	35.7	19.6
Total	169.8	93.3

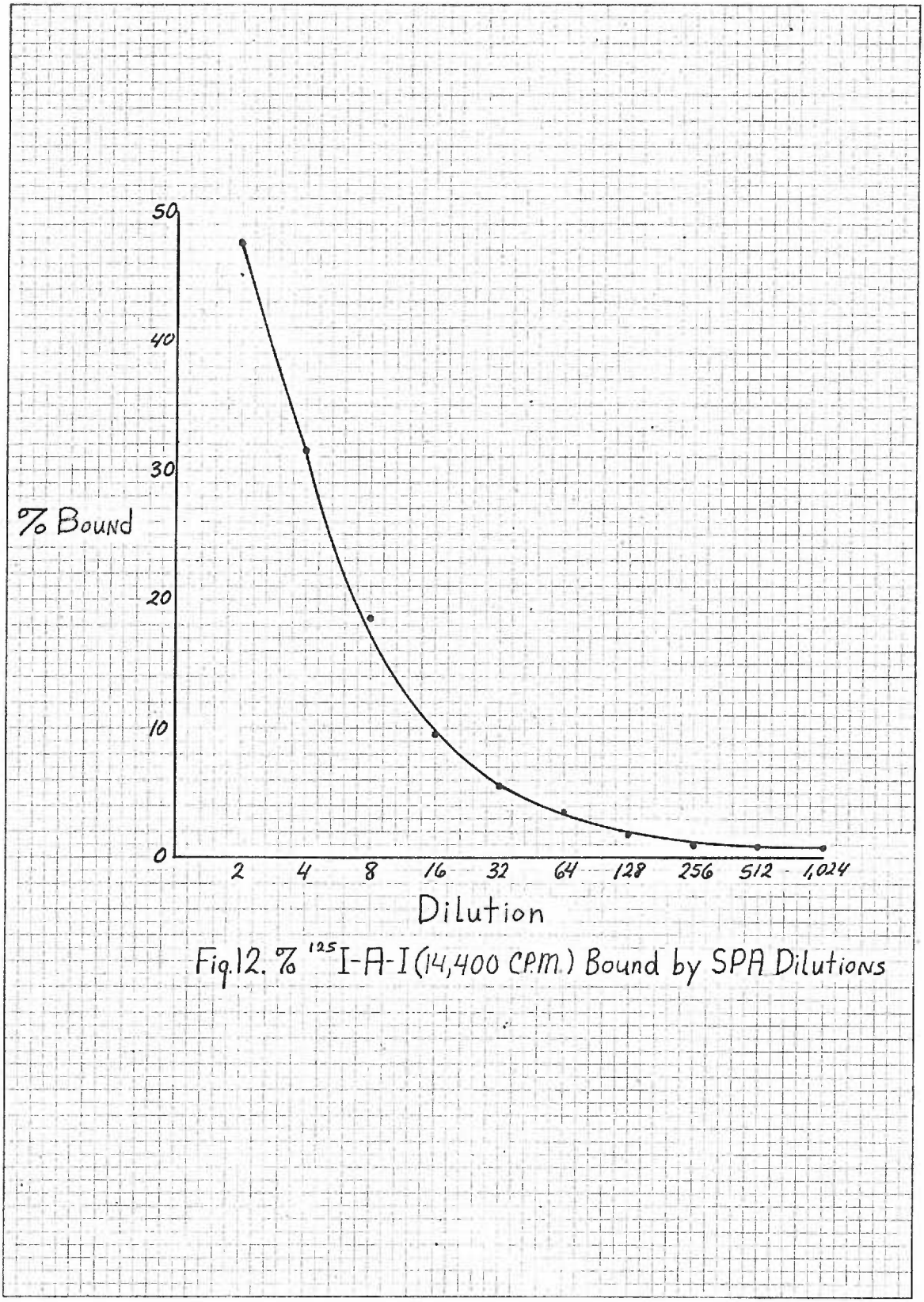

Fig. 12. % ^{125}I-A-I (14,400 CPM) Bound by SPA Dilutions

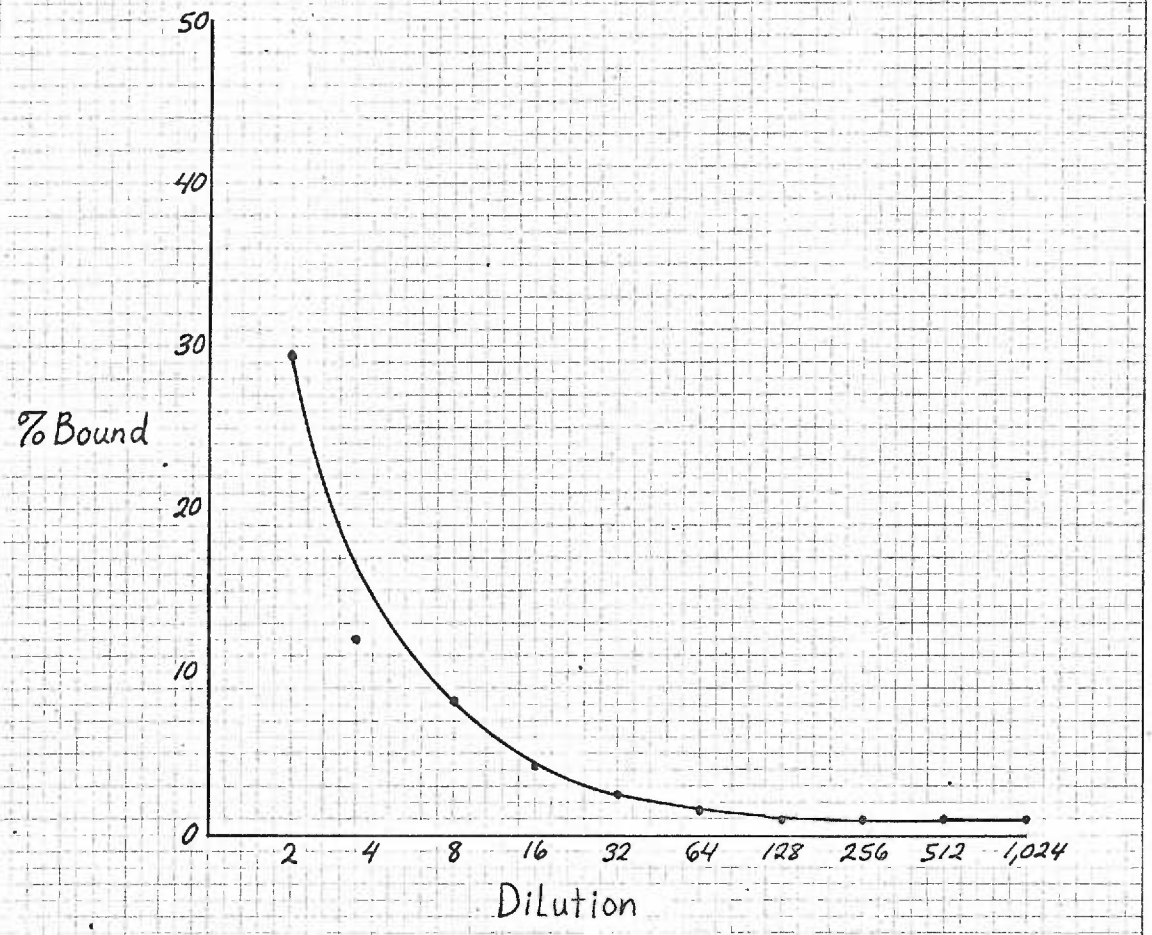

Fig. 13. % ^{125}I-A-I (14,400 CPM) Bound by SPA Dilutions with A-I Standard (1 ng)

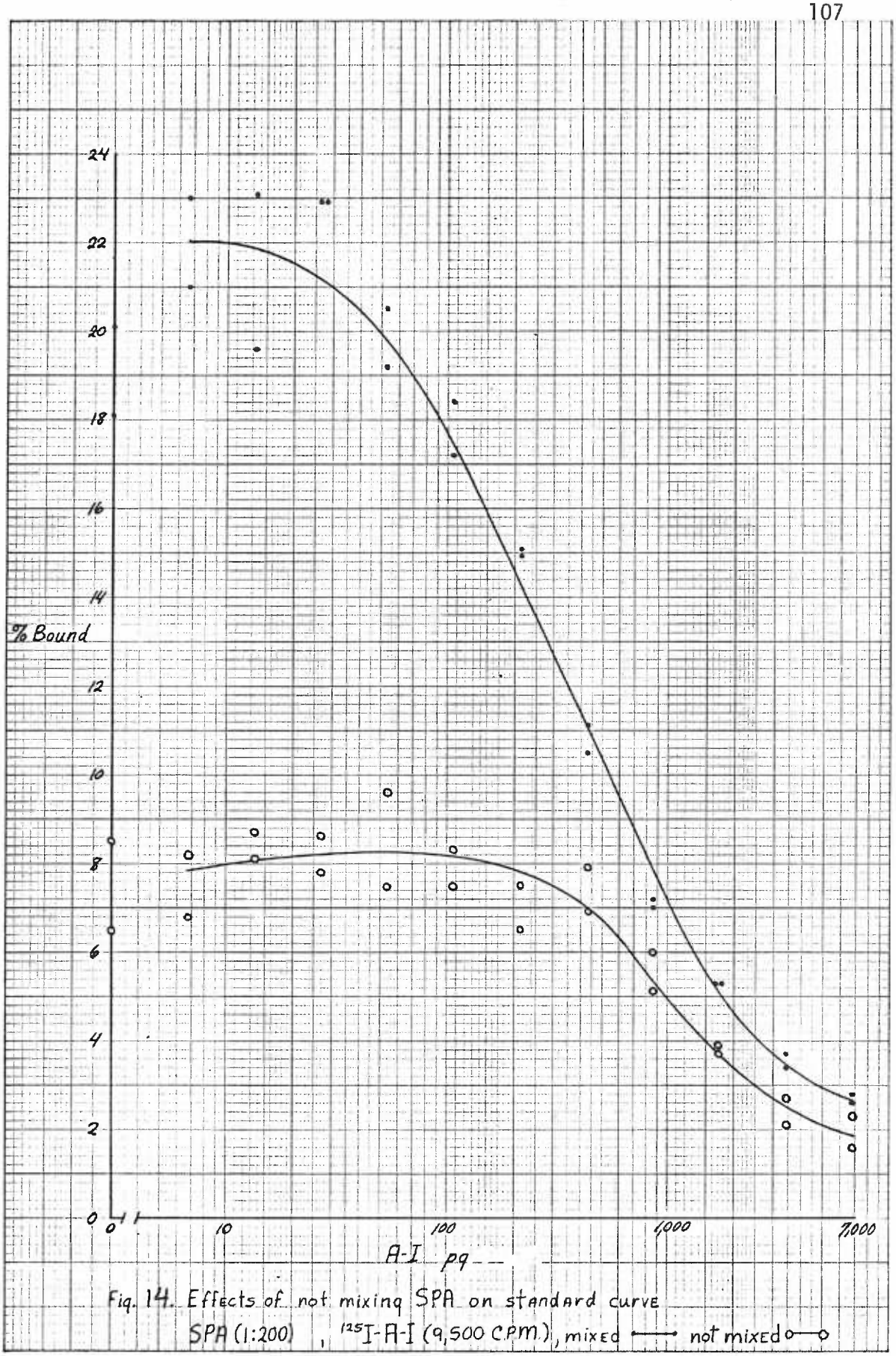

Fig. 14. Effects of not mixing SPA on standard curve
SPA (1:200), ^{125}I-A-I (9,500 CPM), mixed ——— not mixed o——o

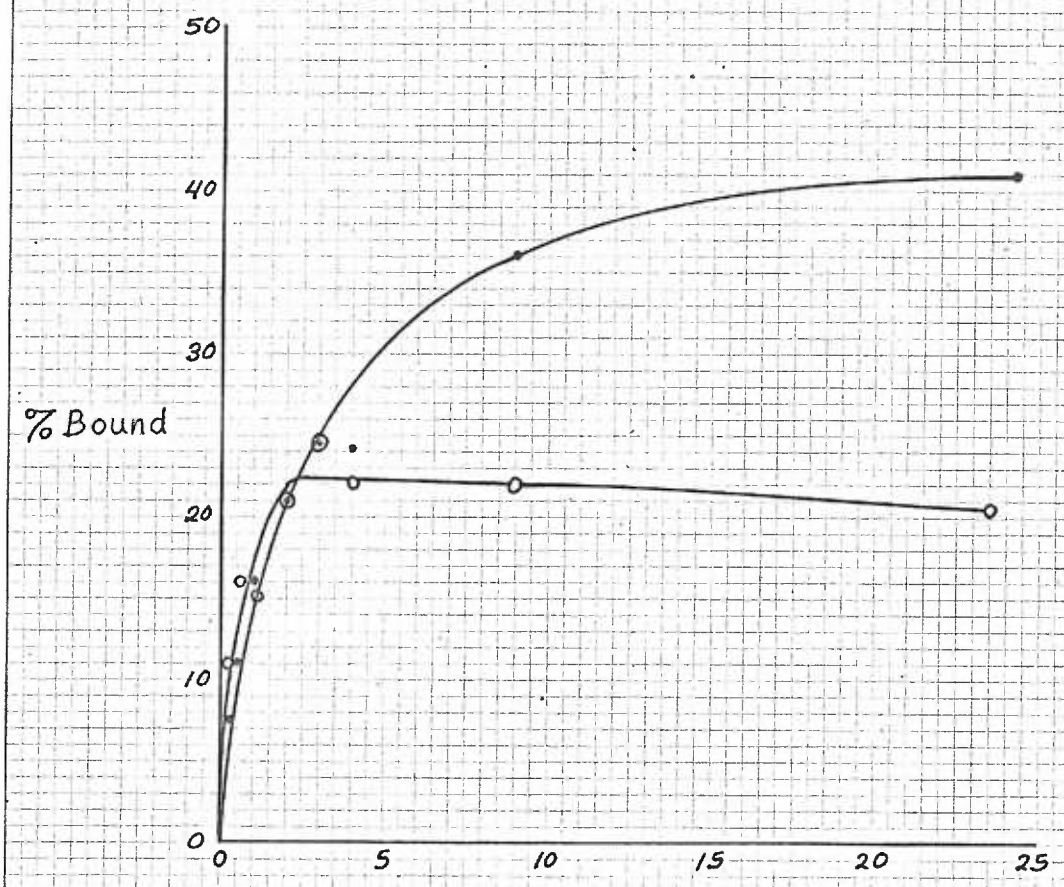

Fig. 15. Rates of binding of ^{125}I-A-I by SPA

SPA (1:8), ^{125}I-A-I (14,000 CPM)

—● 4°C, o—o ambient temperature

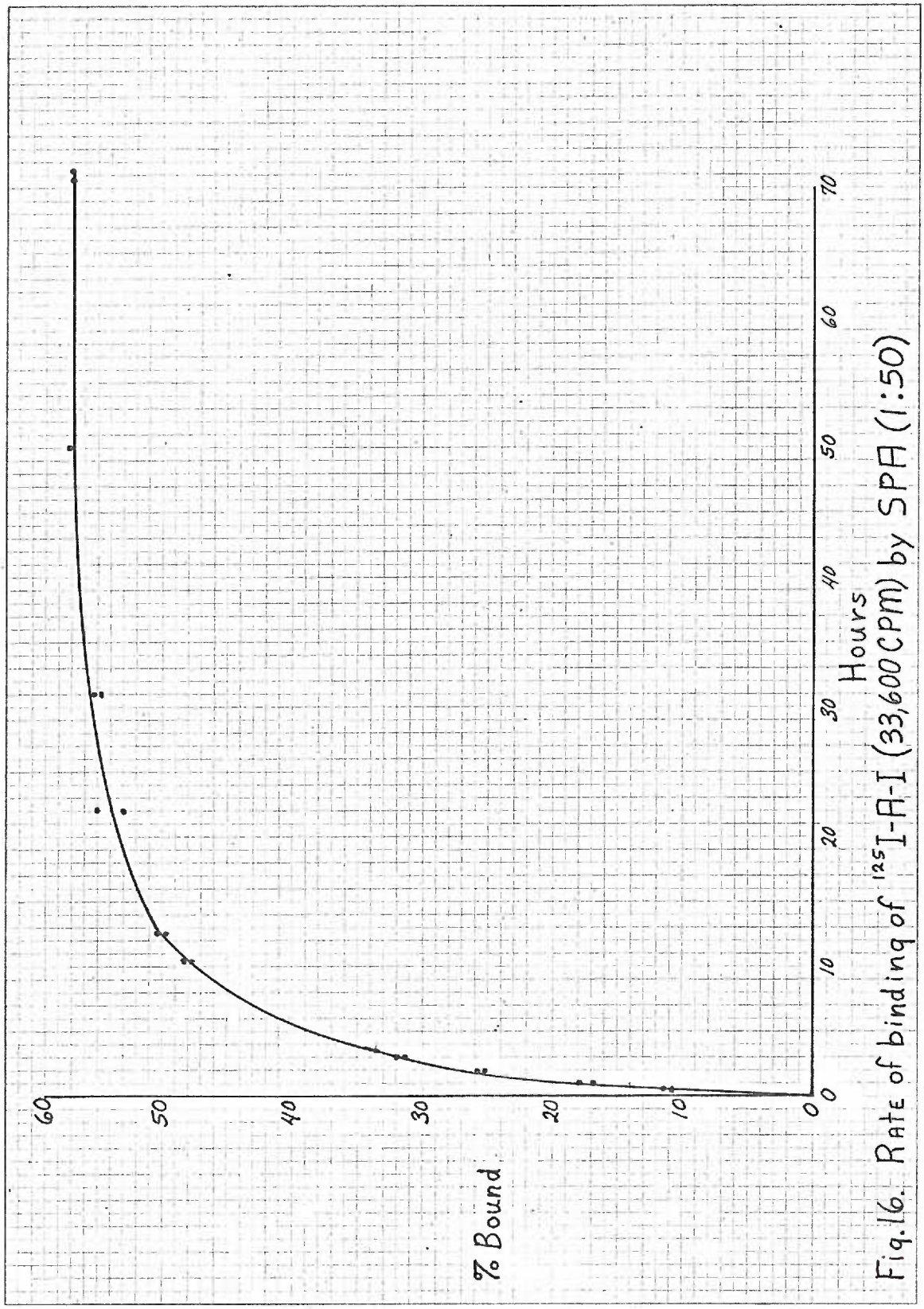

Fig. 16. Rate of binding of ^{125}I-A-I (33,600 CPM) by SPA (1:50)

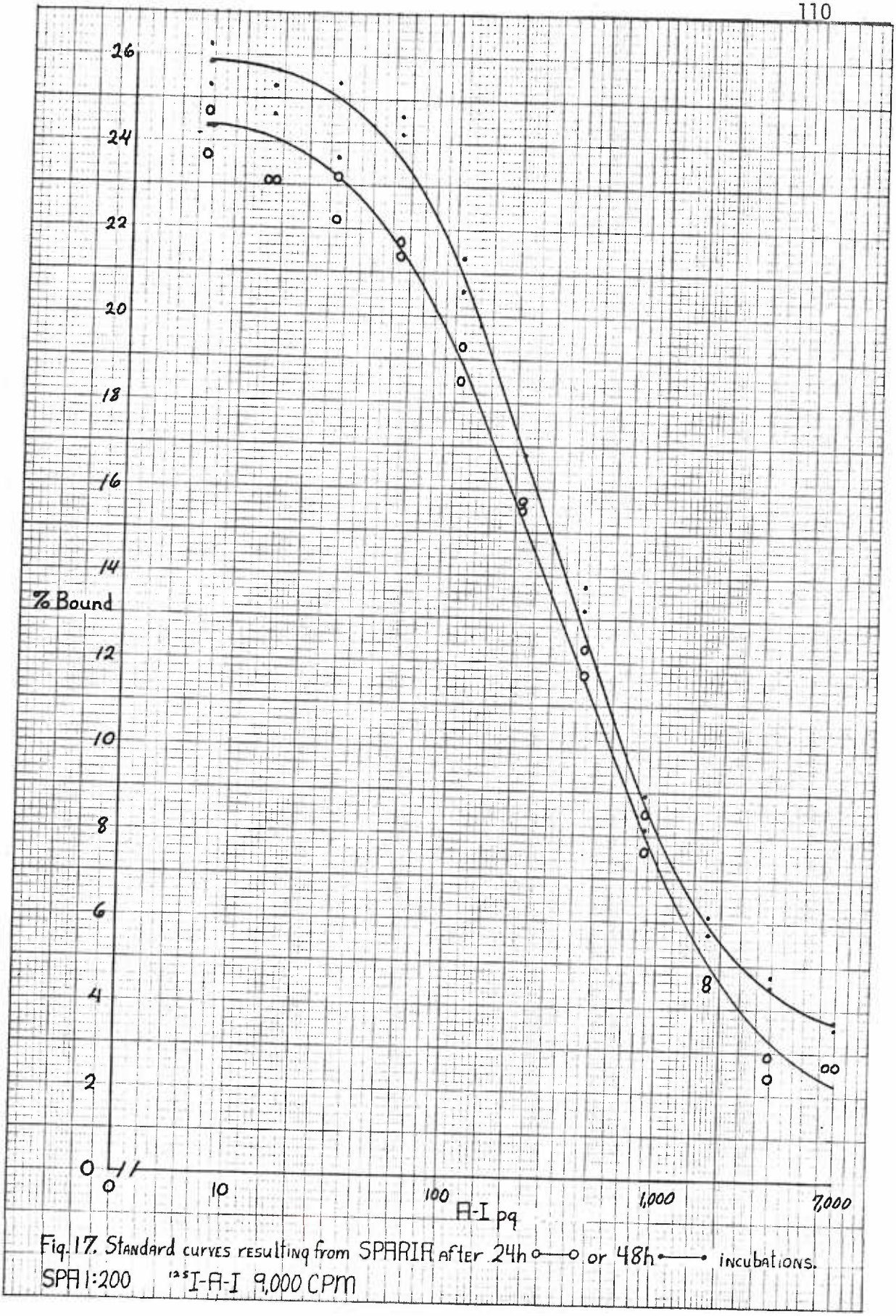

Fig. 17. Standard curves resulting from SPARIA after 24h o—o or 48h •—• incubations.
SPA 1:200 ^{125}I-A-I 9,000 CPM

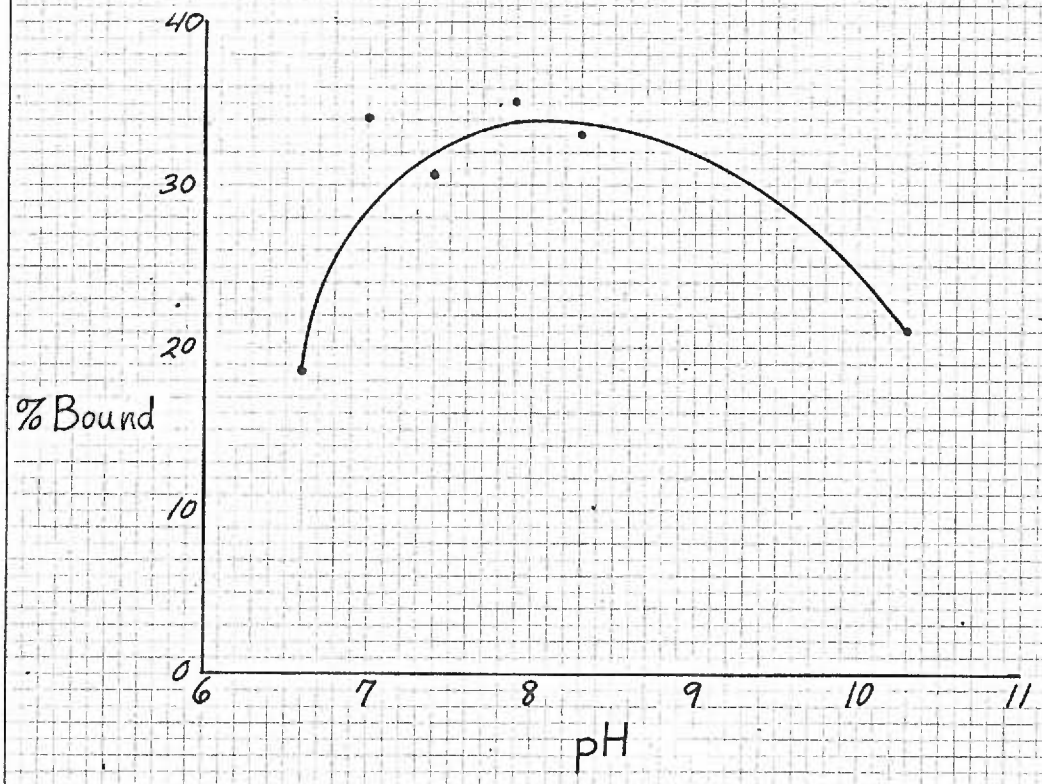

Fig. 18. Degree of binding of ^{125}I-A-I (14,000 CPM) by SPA (1:16) in "assay buffers" of varying pH and constant ionic strength

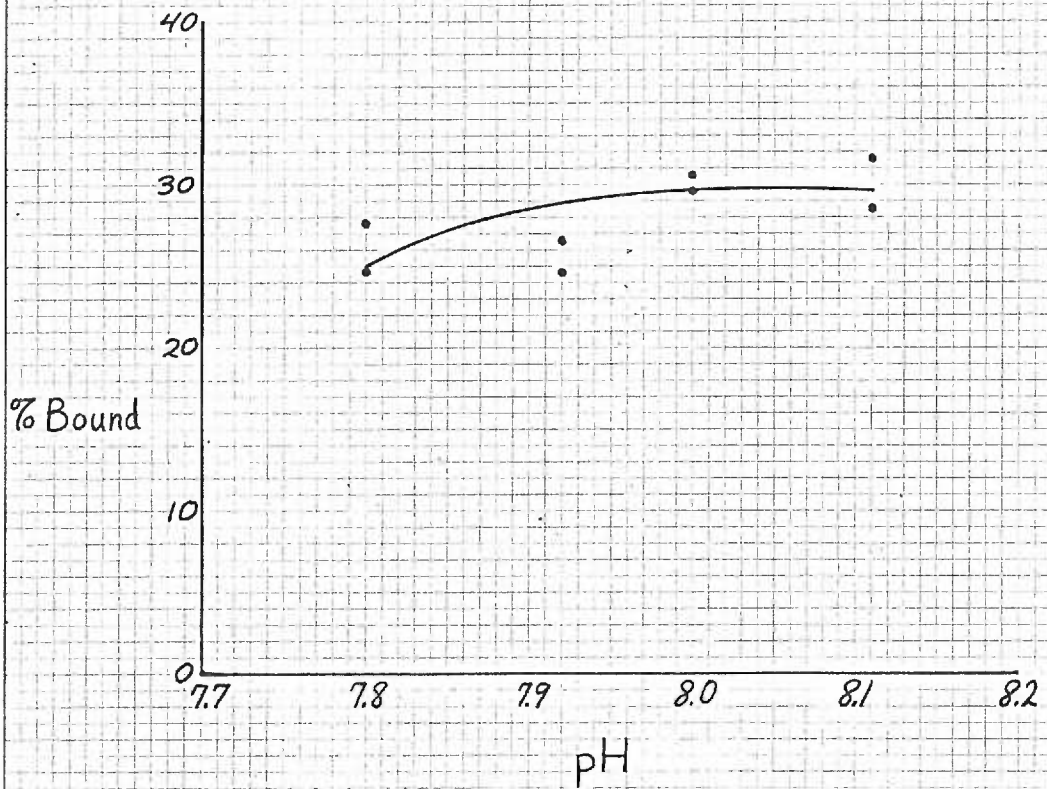

Fig. 19. Degree of binding of ^{125}I-A-I (14,000 CPM) by SPA (1:16) in "assay buffers" of varying pH and constant ionic strength

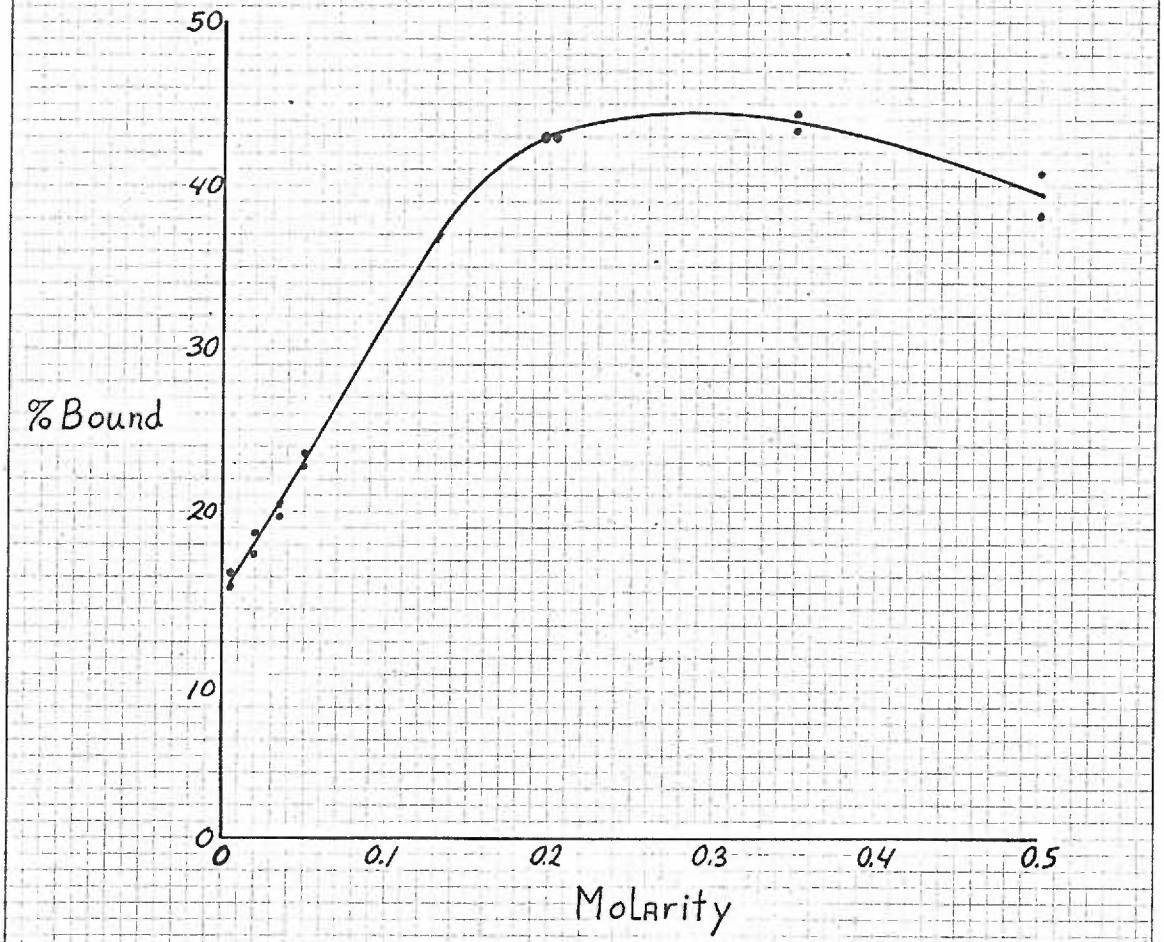

Fig. 20. Effect of increasing Molarity of NaH_2PO_4 pH 8.0 on binding of ^{125}I-A-I (14,000 CPM) by SPA (1:32)

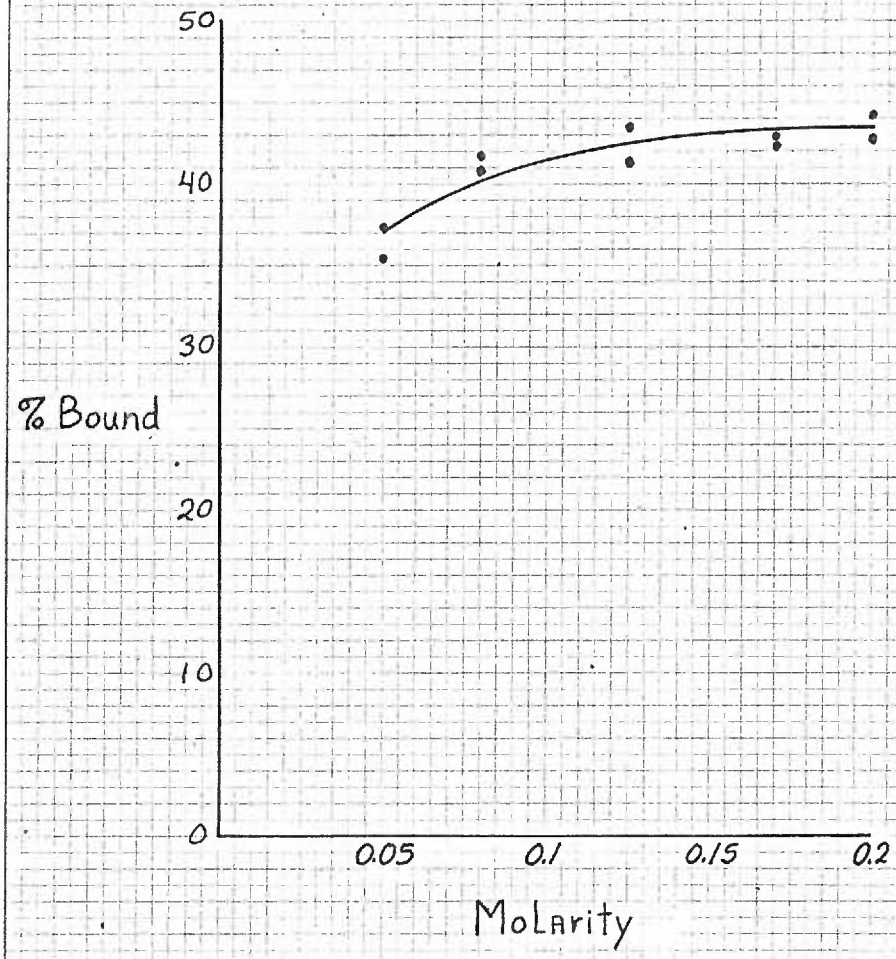

Fig. 21. Effect of increasing Molarity of NaH_2PO_4 pH 8.0 on binding of ^{125}I-A-I (14,000 CPM) by SPA (1:32)

Table 8

Tris-HCl versus NaH_2PO_4"assay buffer"

Buffer	% Bound
0.1M Tris-HCl pH 7.5	8.5
0.05M NaH_2PO_4 pH 7.5	32.5

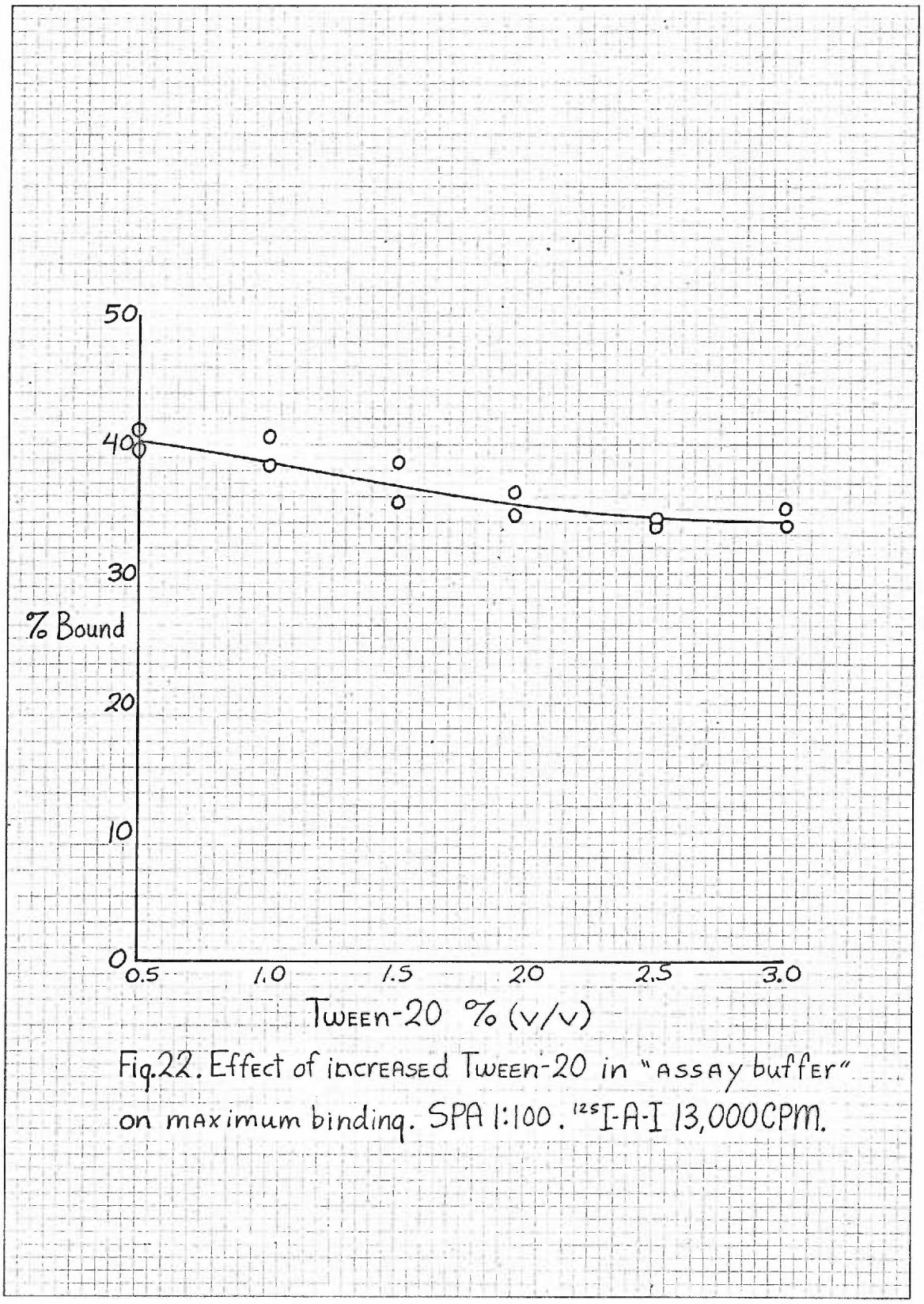

Fig.22. Effect of increased Tween-20 in "assay buffer" on maximum binding. SPA 1:100. ^{125}I-A-I 13,000CPM.

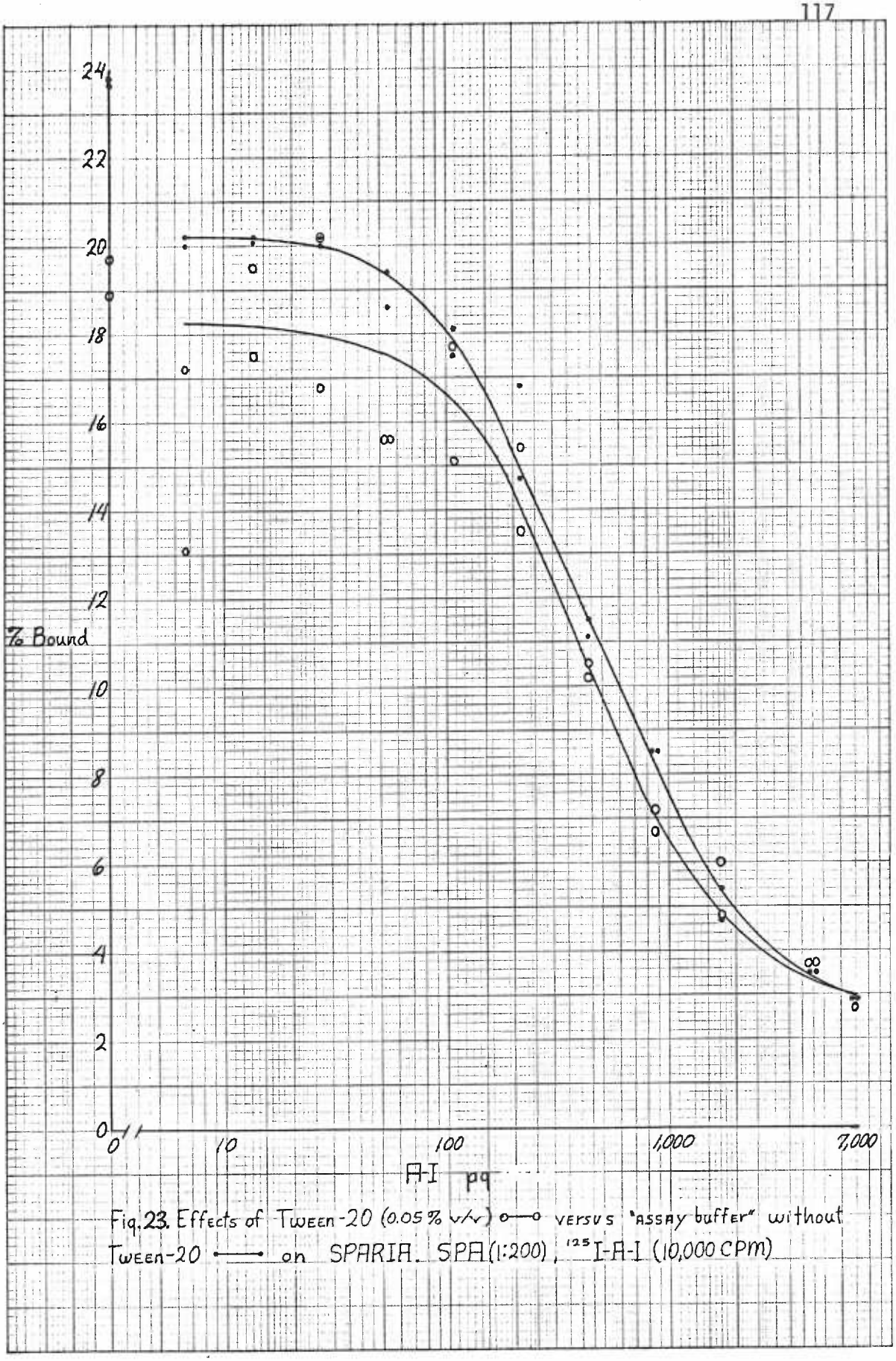

Fig. 23. Effects of Tween-20 (0.05% v/v) o—o versus "assay buffer" without Tween-20 •—• on SPARIA. SPA(1:200), ^{125}I-A-I (10,000 CPM)

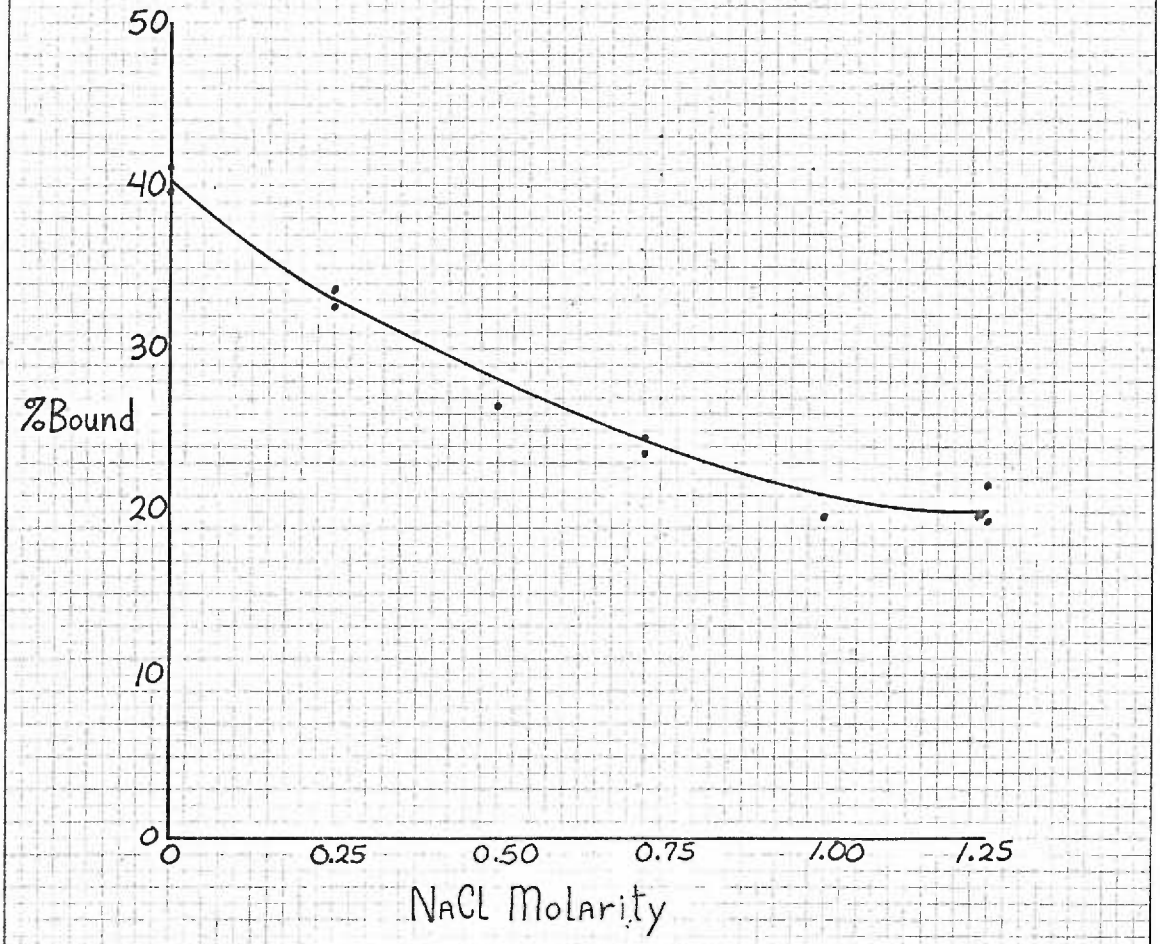

Fig. 24. Effect of increased NaCl in "assay buffer" on maximum binding. SPA 1:100. ^{125}I-A-I 13,000 CPM.

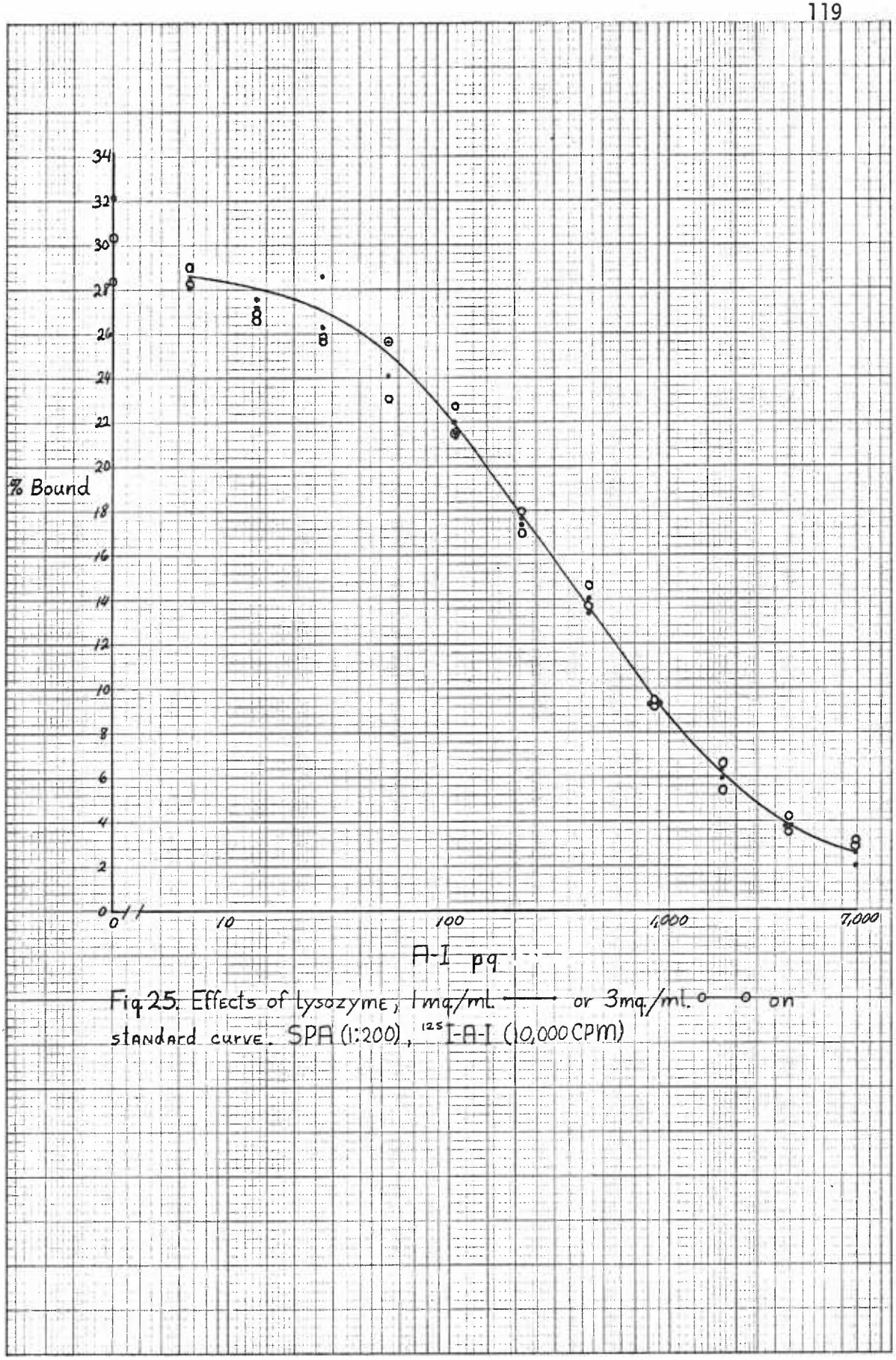

Fig 25. Effects of lysozyme, 1mg./ml. —— or 3mg./ml. o—o on standard curve. SPA (1:200), ^{125}I-A-I (10,000 CPM)

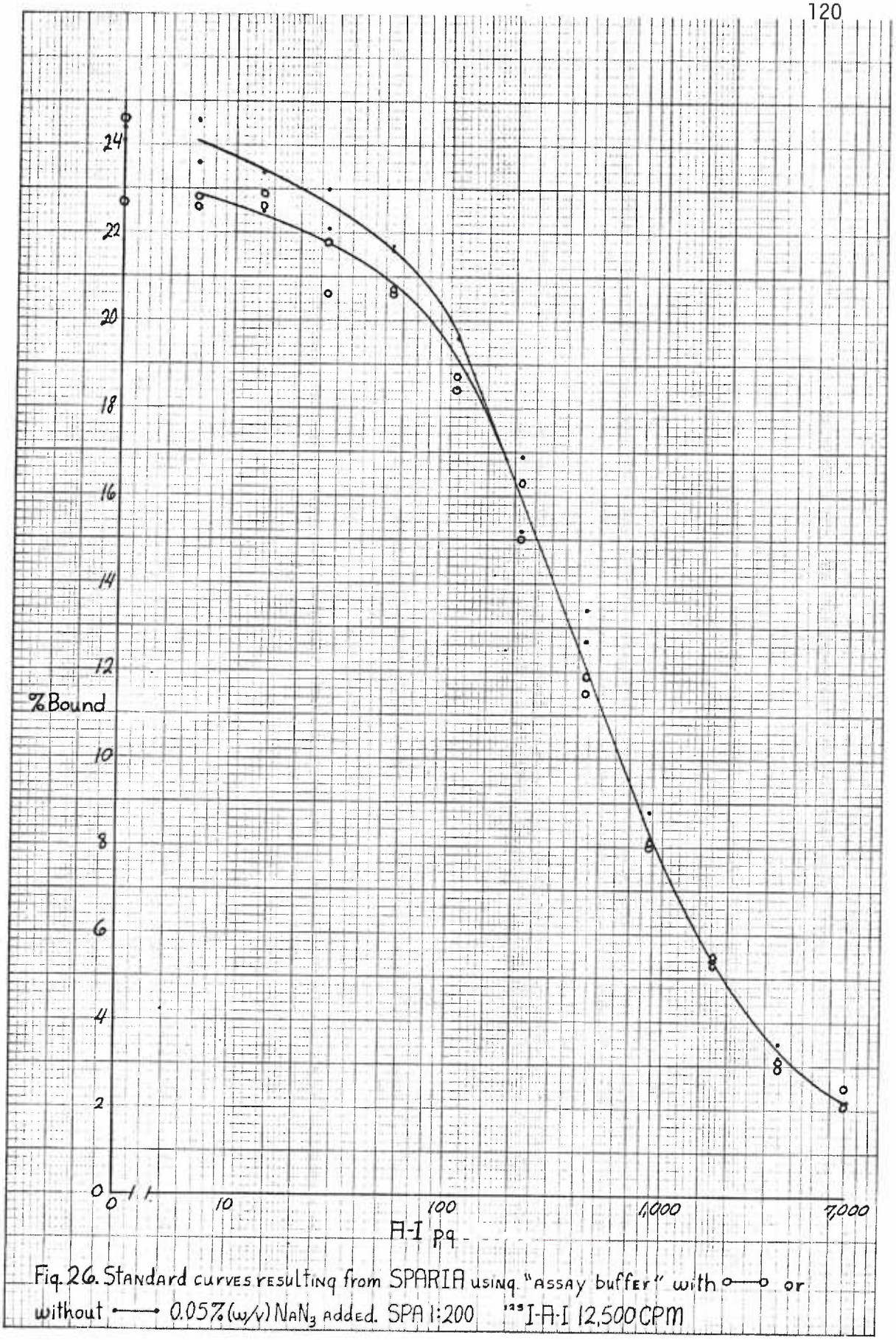

Fig. 26. Standard curves resulting from SPARIA using "assay buffer" with o—o or without ——— 0.05% (w/v) NaN_3 added. SPA 1:200 ^{125}I-A-I 12,500 CPM

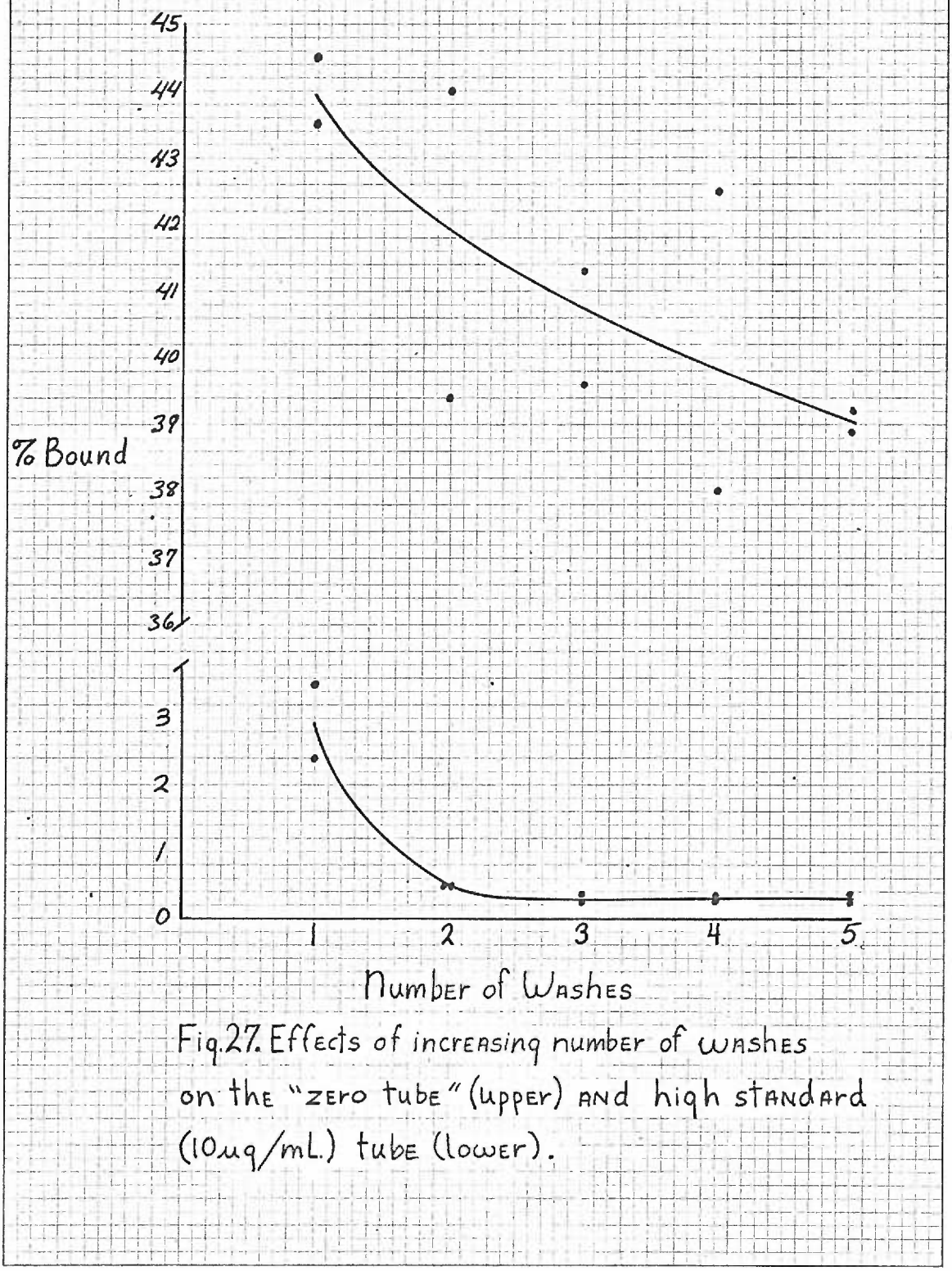

Fig. 27. Effects of increasing number of washes on the "zero tube" (upper) and high standard (10 µg/mL.) tube (lower).

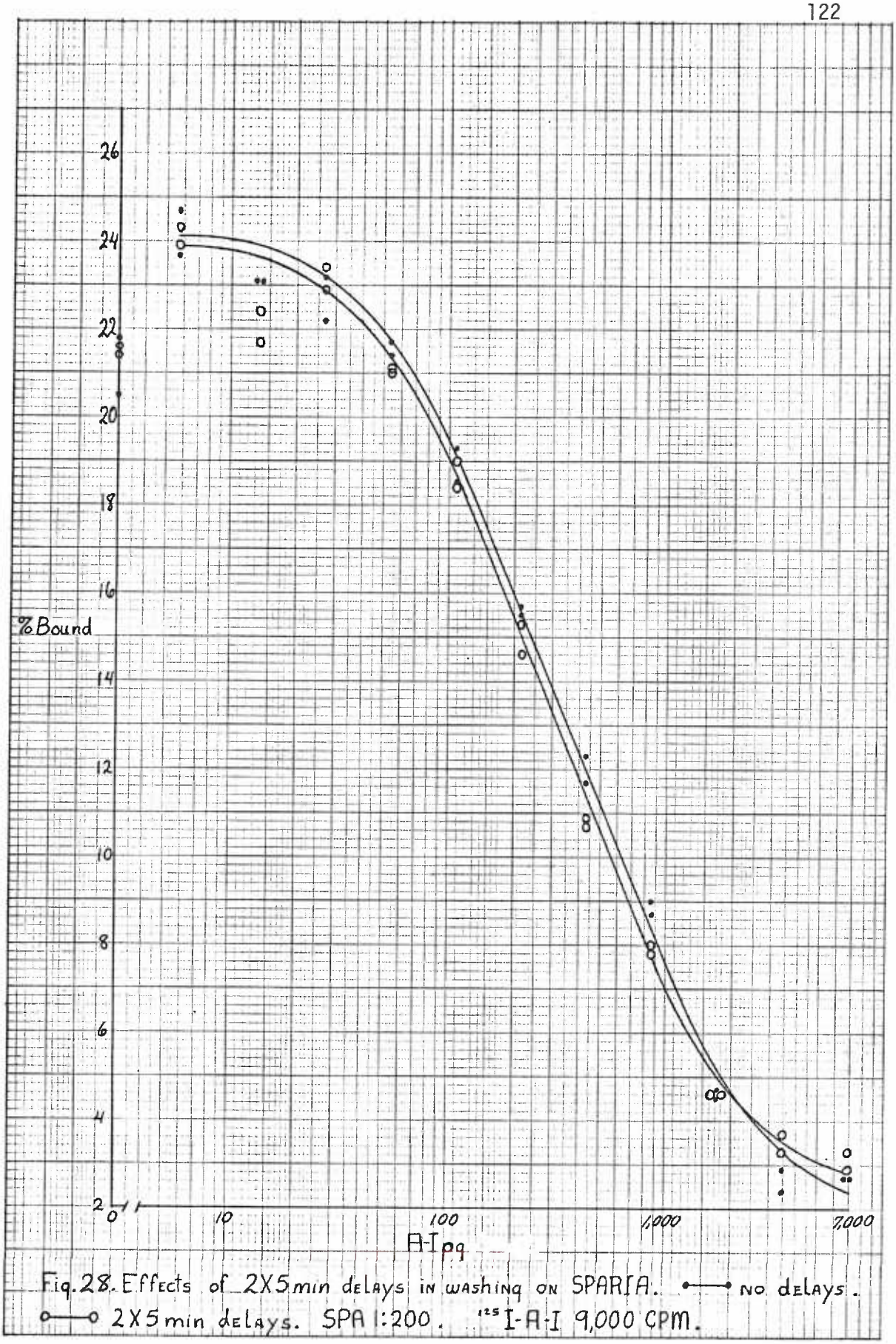

Fig. 28. Effects of 2X5 min delays in washing on SPARIA. ●—● no delays. o—o 2X5 min delays. SPA 1:200. ^{125}I-A-I 9,000 CPM.

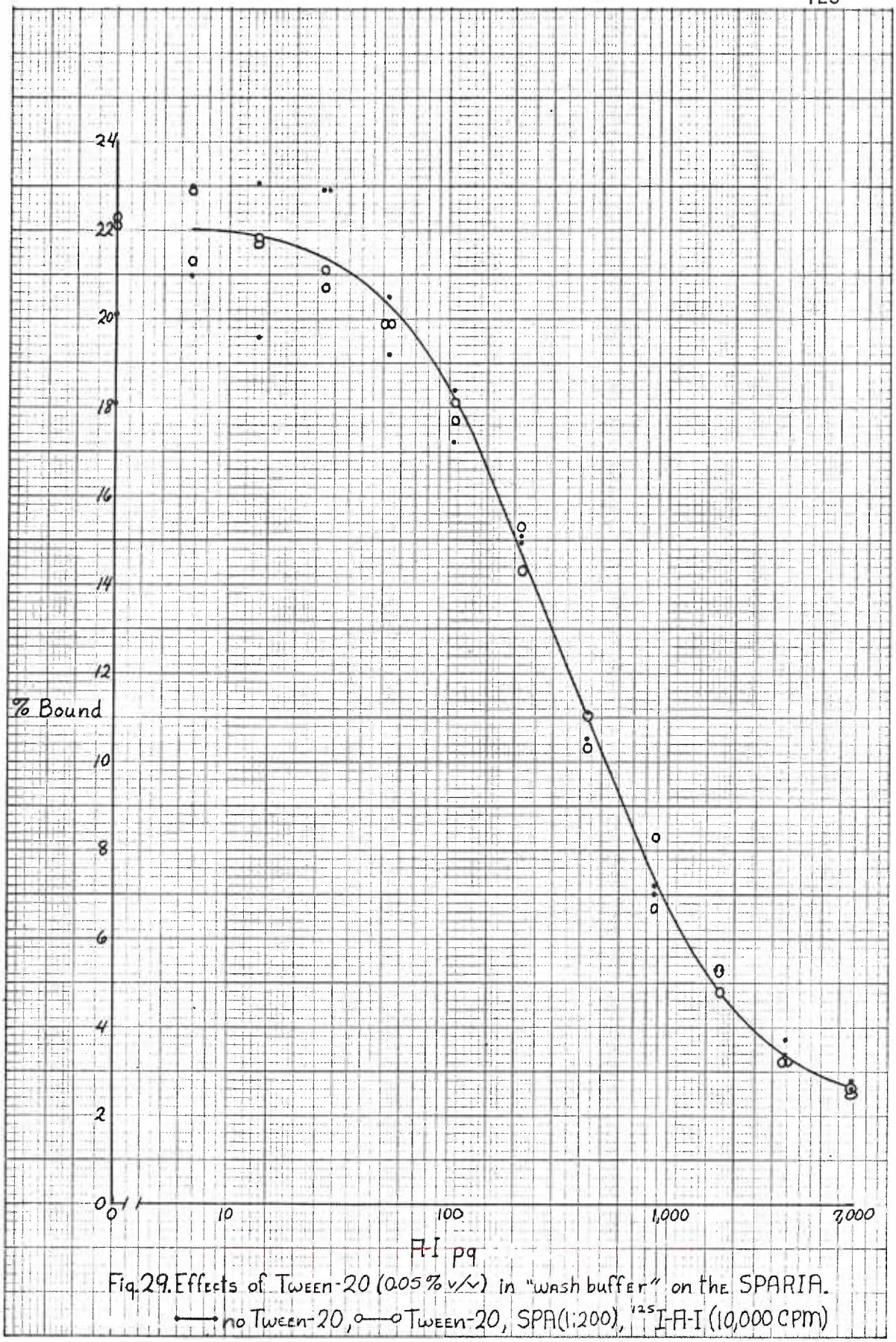

Fig. 29. Effects of Tween-20 (0.05% v/v) in "wash buffer" on the SPARIA.
—●— no Tween-20, o—o Tween-20, SPA(1:200), ^{125}I-FI (10,000 CPM)

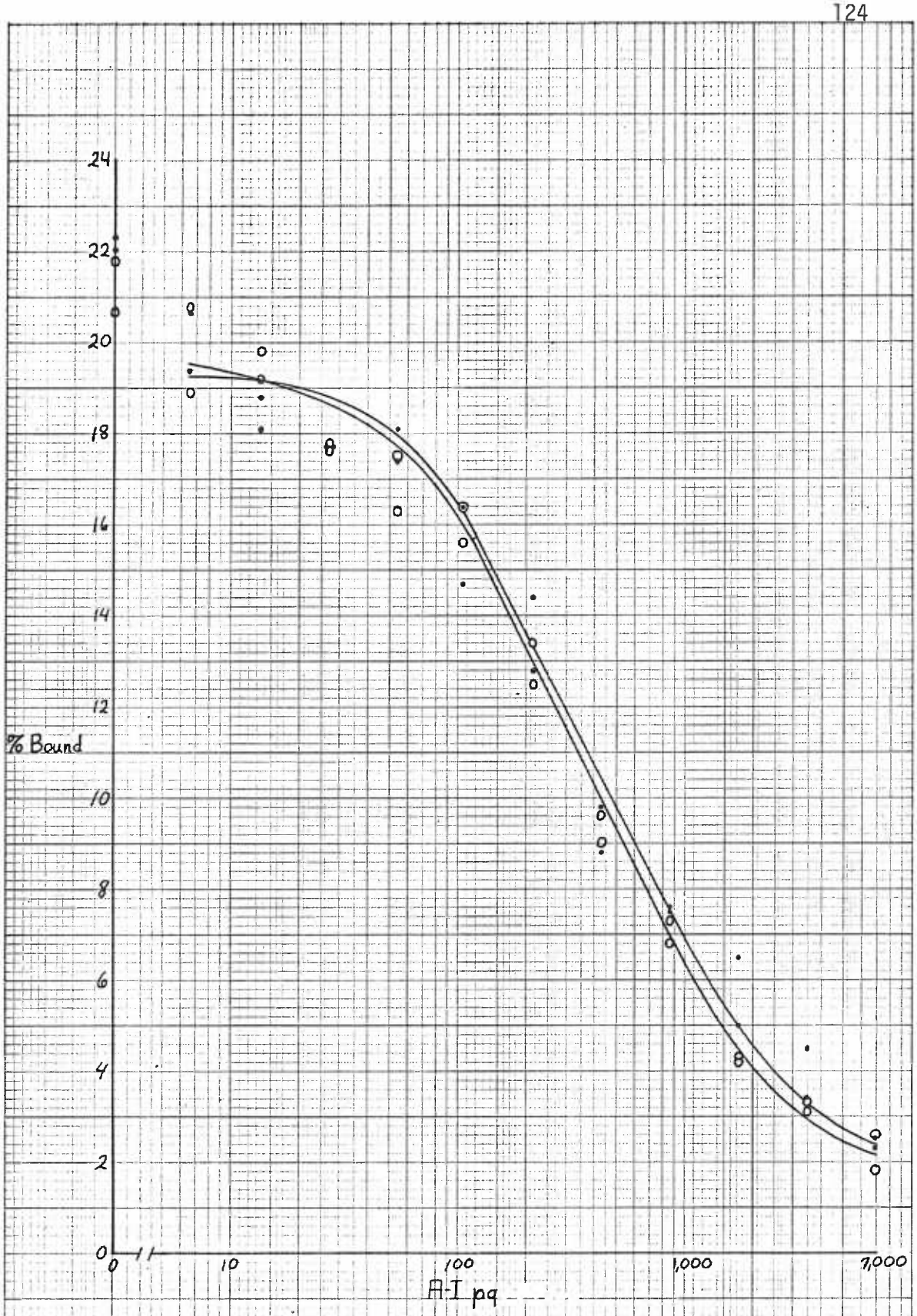

Fig. 30. Effects of 4°C ——•—— and ambient temperature o——o wash buffer on SPARIA.

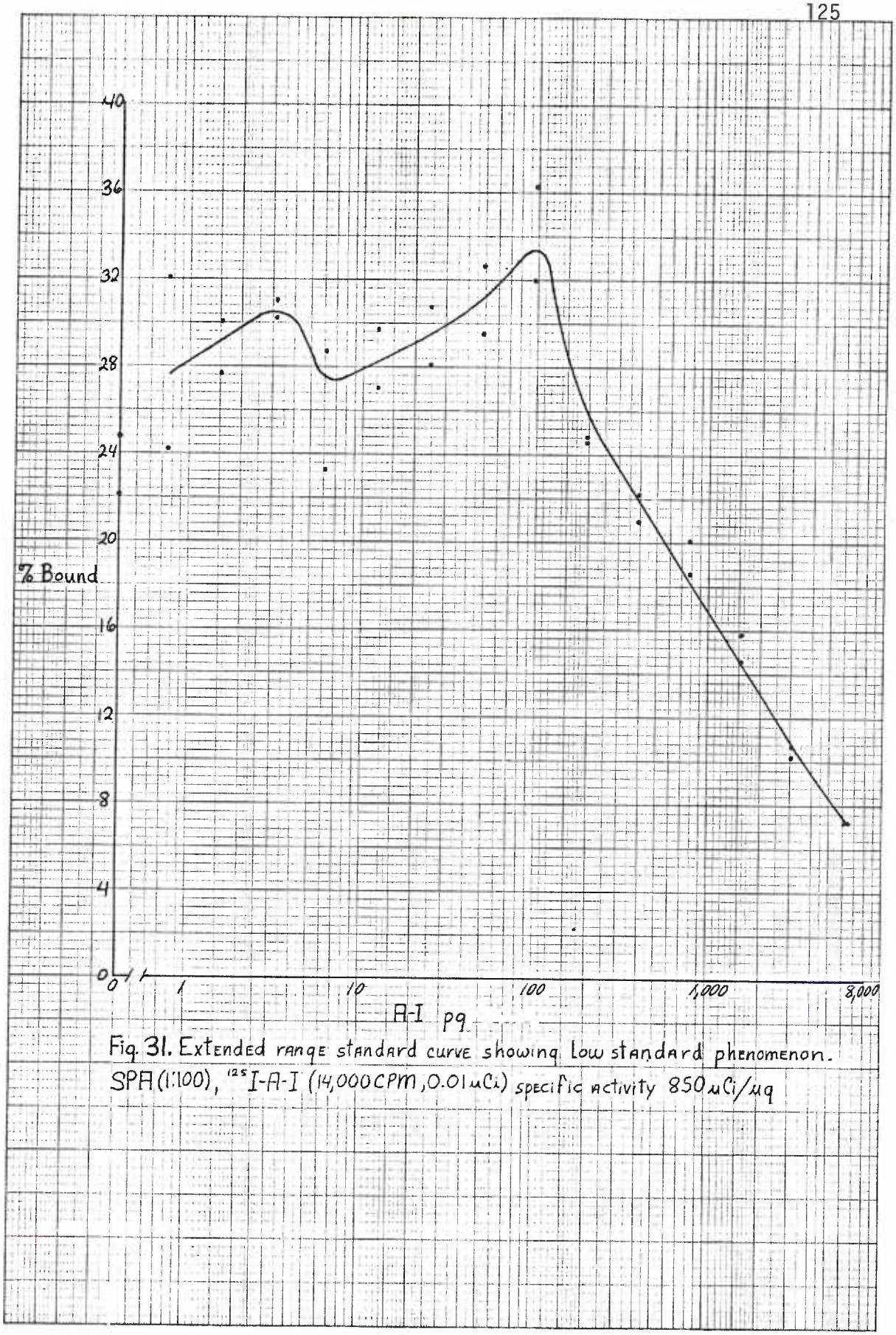

Fig. 31. Extended range standard curve showing low standard phenomenon. SPA (1:100), ^{125}I-A-I (14,000 CPM, 0.01 uCi) specific activity 850 uCi/ug

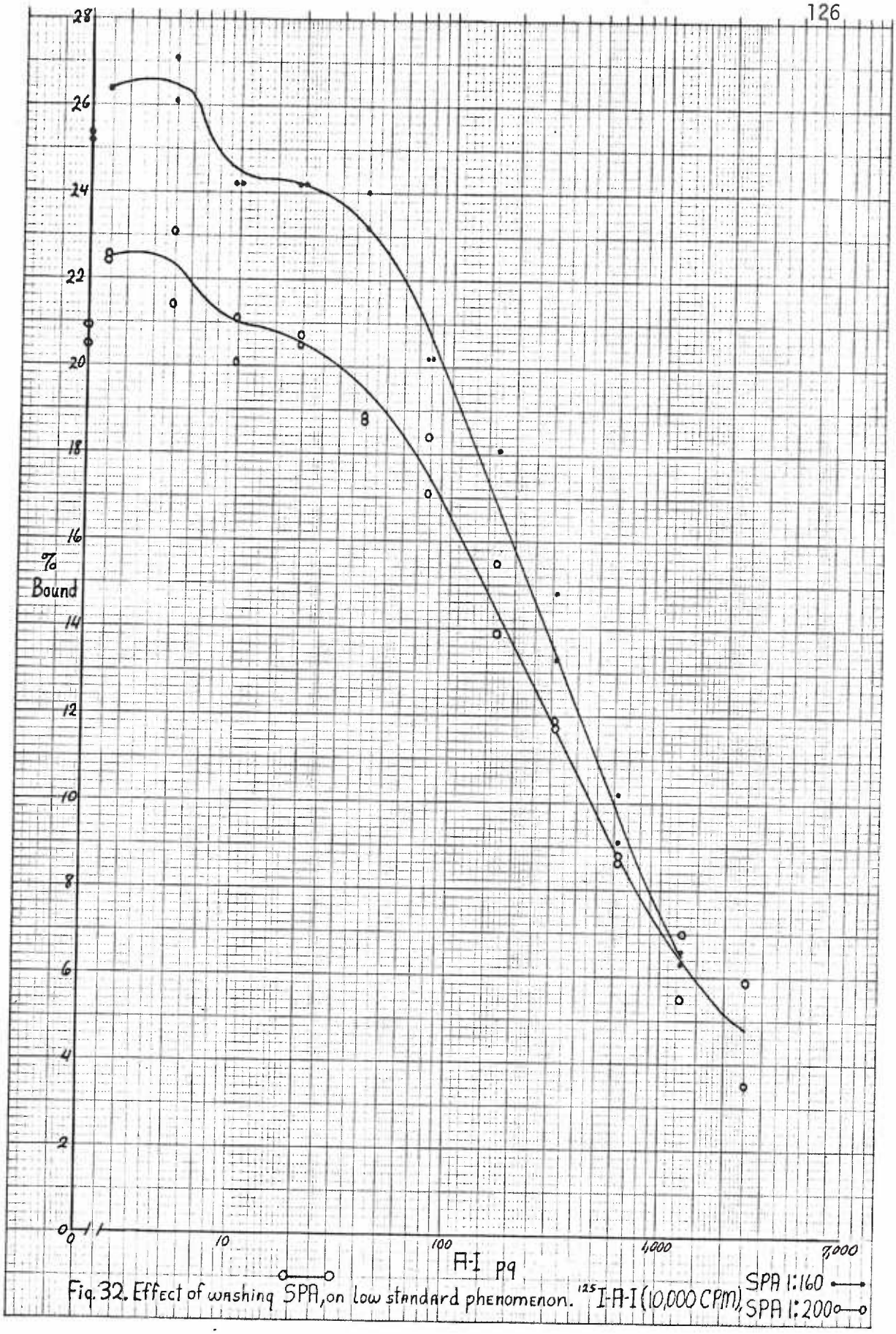

Fig. 32. Effect of washing SPA, on low standard phenomenon. ^{125}I-A-I (10,000 CPM), SPA 1:160 ●—● SPA 1:200 ○—○

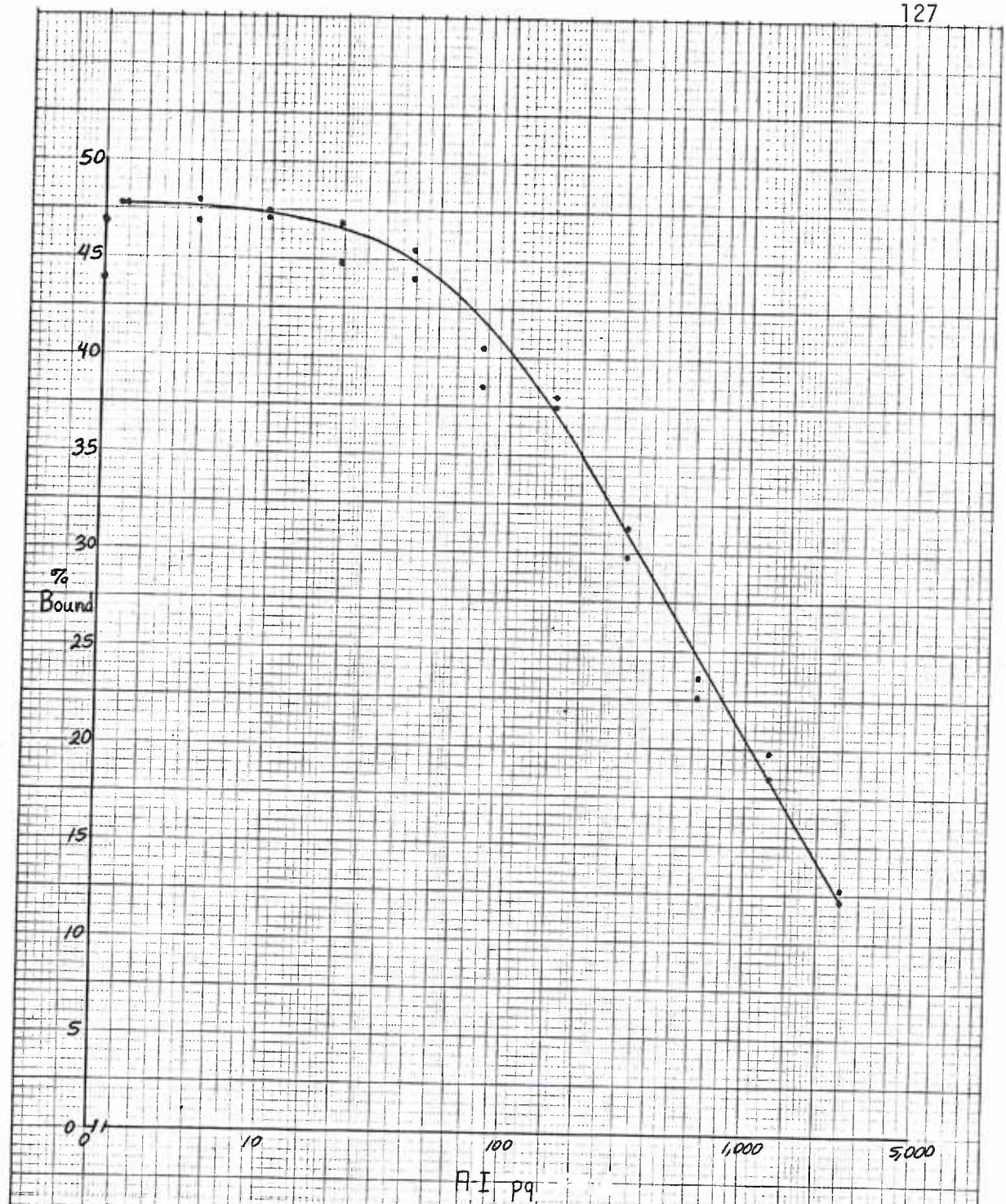

Fig. 33. Standard curve resulting from SPARIA using SPA (1:80) used previously for RIA and washed with glycine–HCl pH 2.0. ^{125}I-A-I 10,000 CPM.

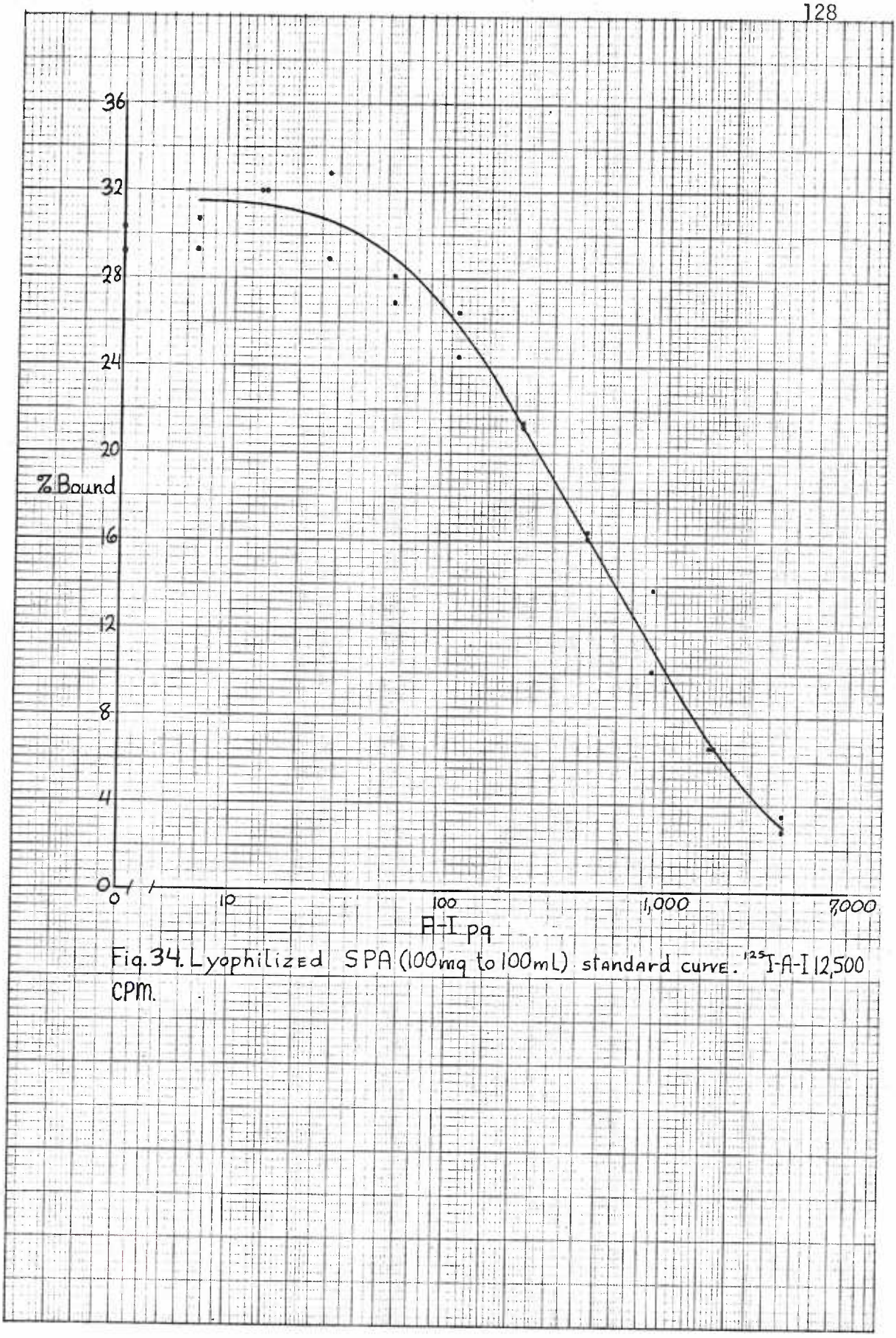

Fig. 34. Lyophilized SPA (100mg to 100mL) standard curve. ^{125}I-A-I 12,500 CPM.

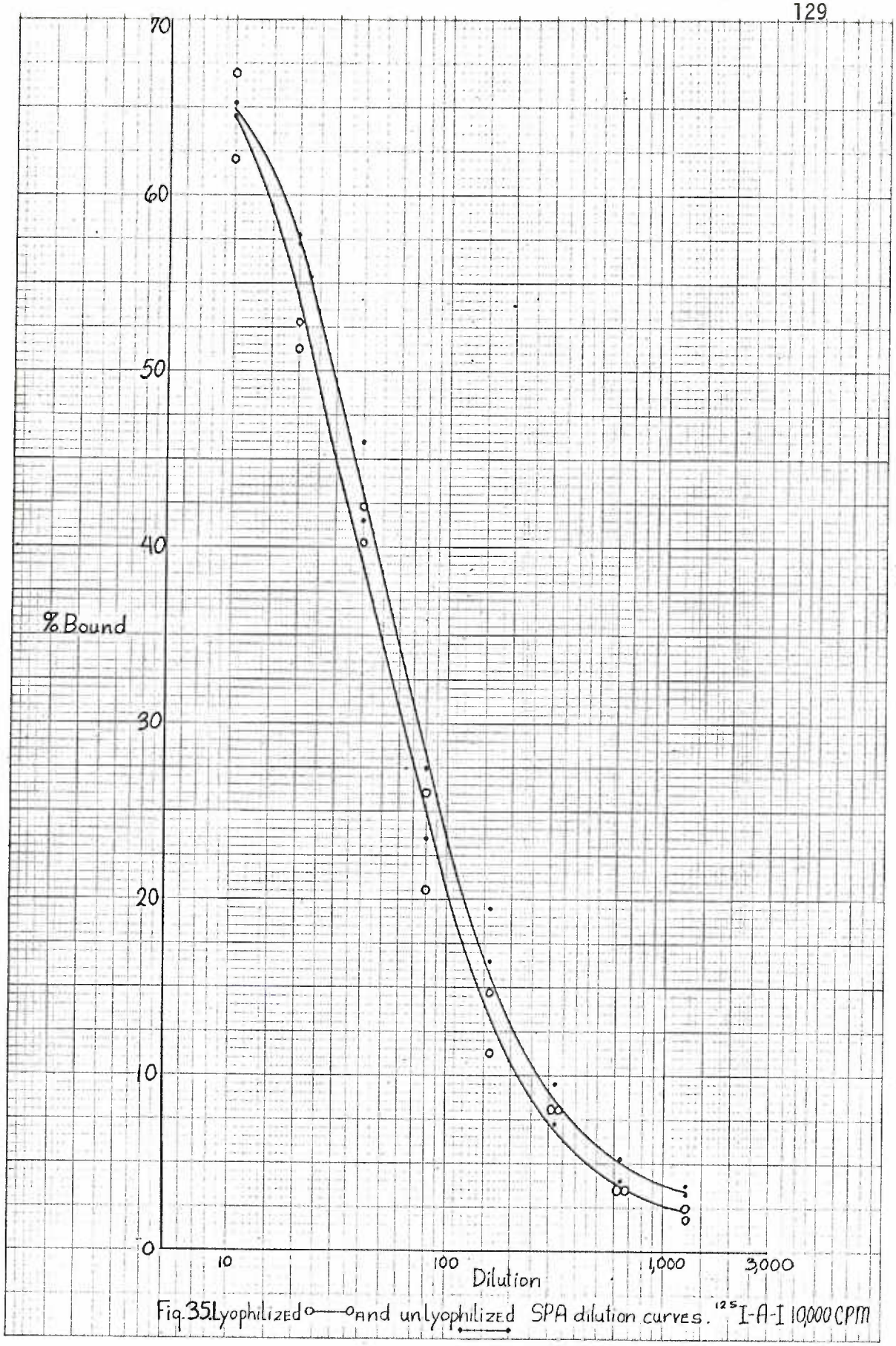

Fig. 35 Lyophilized o—o and unlyophilized SPA dilution curves. ^{125}I-A-I 10,000 CPM

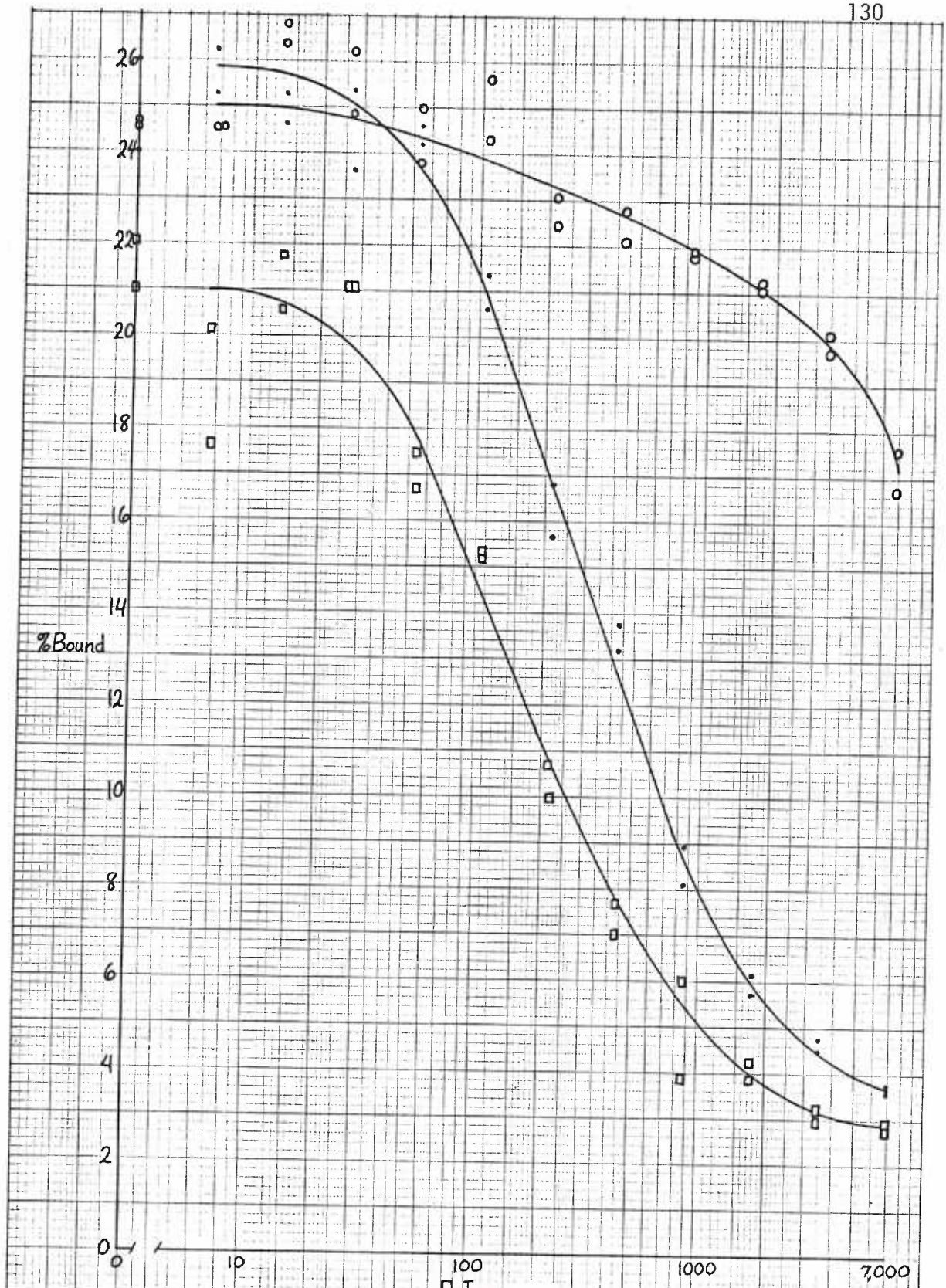

Fig. 36. 48h standard curves. Normal ——, 24h late addition of ^{125}I-A-I □—□, 24h late addition of standards o—o. SPA 1:200, ^{125}I-A-I 9,000CPM.

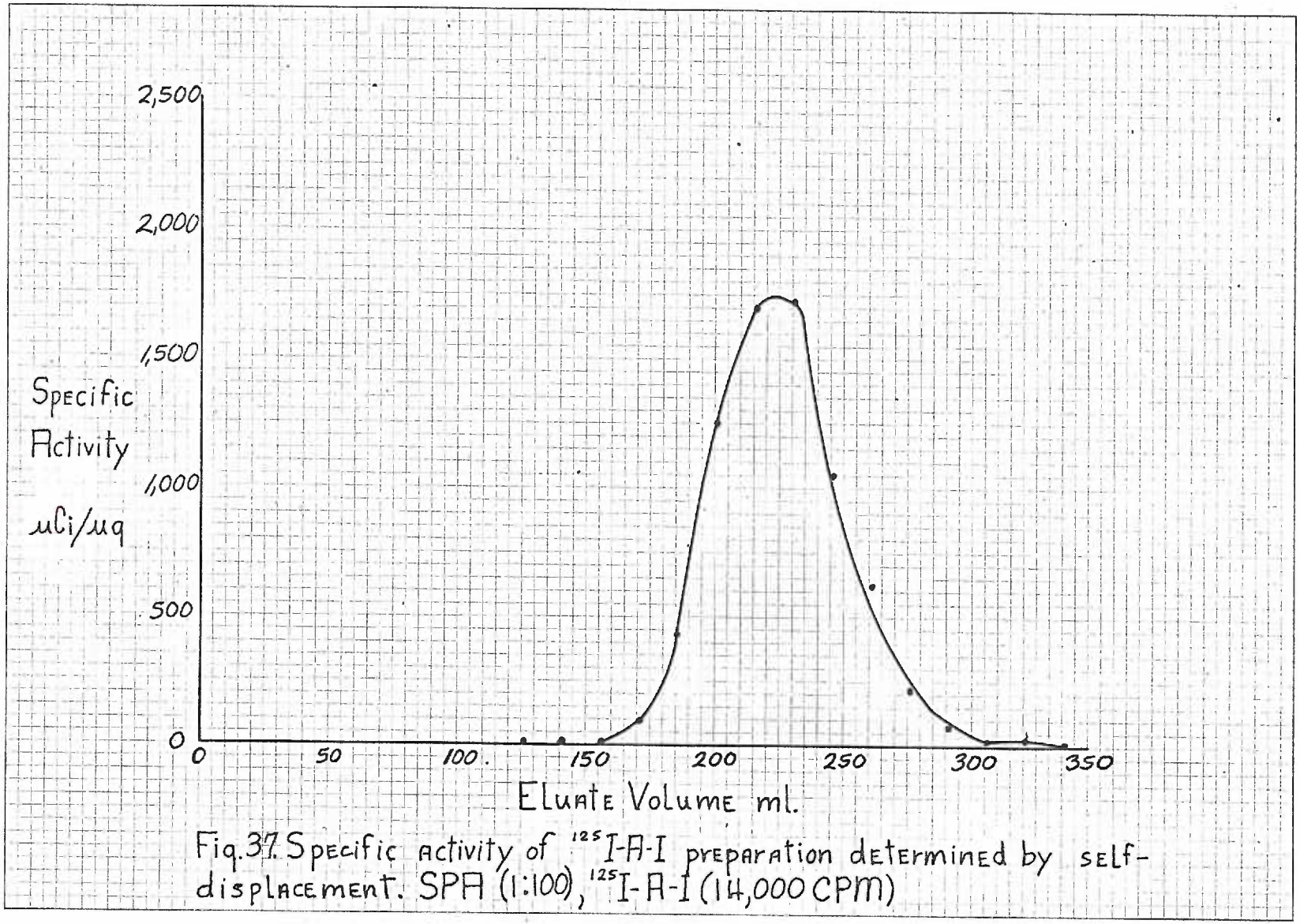

Fig. 37 Specific activity of ^{125}I-A-I preparation determined by self-displacement. SPA (1:100), ^{125}I-A-I (14,000 CPM)

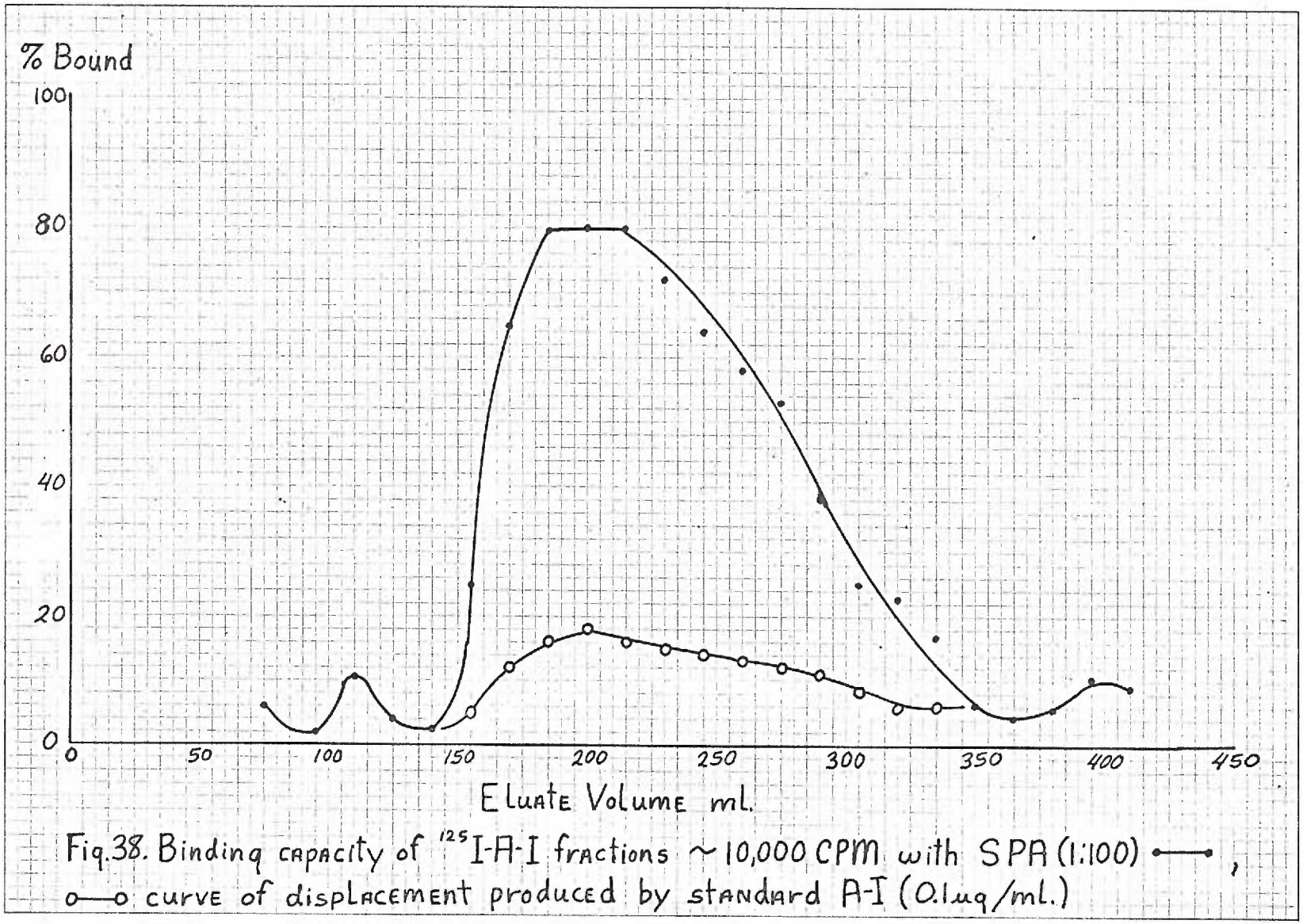

Fig. 38. Binding capacity of ^{125}I-A-I fractions ~10,000 CPM with SPA (1:100) ——, o—o curve of displacement produced by standard A-I (0.1μg/ml.)

Table 9

Comparison of binding capacities of three ^{125}I-A-I preparations

Source of ^{125}I-A-I	Sp. act.	Maximum binding %	Binding displaced %
Cambridge Nuclear	ca. 1,050 µCi/µg	52	12.5
Schwarz-Mann	ca. 400 µCi/µg	37	9.3
Present preparation	ca. 1,750 µCi/µg	80	16.0

Table 10

Comparison of buffers used for generation of A-I

Buffer		H_3PO_4 0.28M (µl)	Maleic acid 2M (µl)	A-I (ng/ml/h)
Composition	Volume (µl)			
3M ammonium maleate, pH 6.0	10	--	10	4.6
2M NaH_2PO_4, pH 5.4	50	50	--	3.7
2M $NH_4H_2PO_4$, pH 5.4	50	50	--	3.6
1.7M KH_2PO_4, pH 4.2	150	--	--	3.3

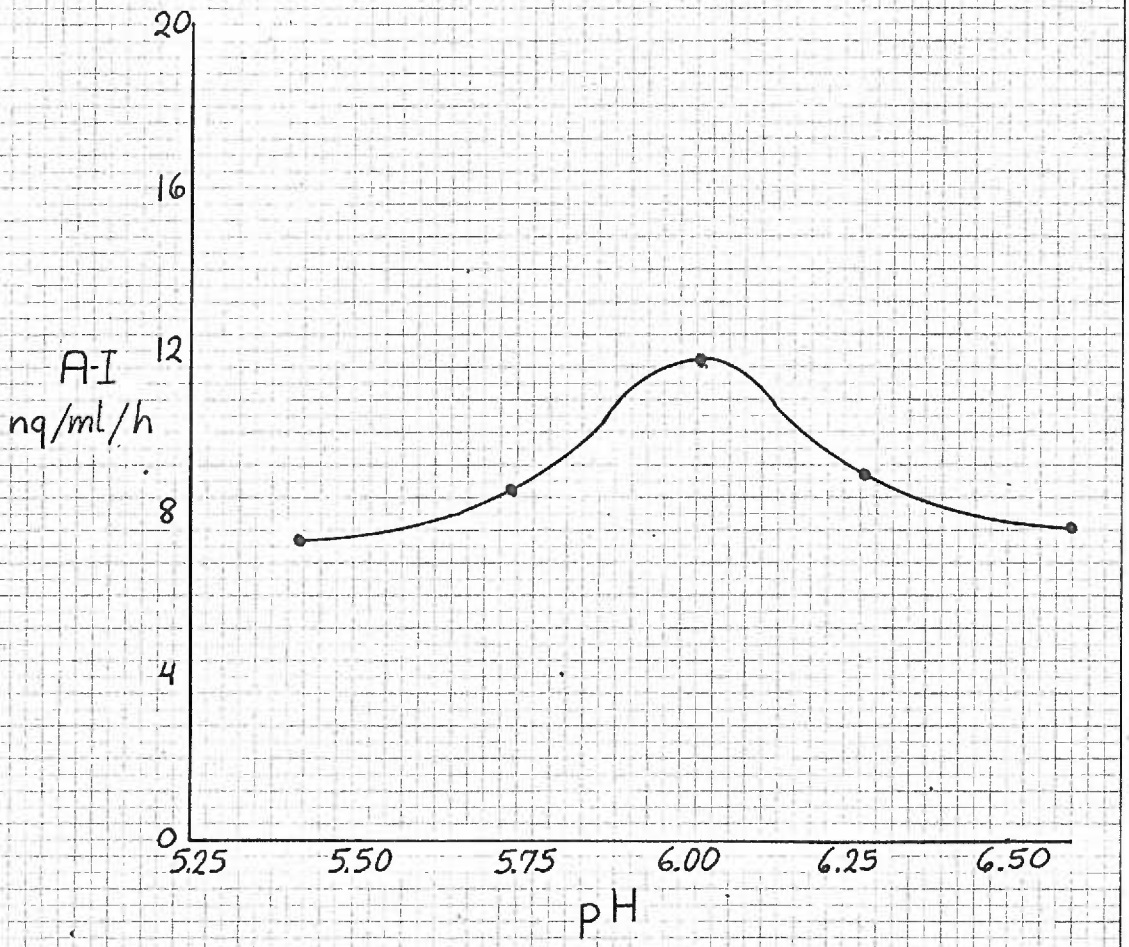

Fig.39. Effect of pH on A-I generation in "control plasma"

Table 11

Effects of EDTA concentration on A-I generation rates

Inhibitor	mg/ml	µmol/ml	A-I(ng/ml/h)
Na_2EDTA	1	2.69	5.7
Na_2EDTA	2	5.38	5.7
Na_2EDTA	3	8.07	5.3

Table 12

Effects of Na and K EDTA on A-I generation rates

Inhibitor	mg/ml	µmol/ml	A-I(ng/ml/h)
Na_2EDTA	1	2.69	2.9
K_2EDTA	1	2.58	2.7

Table 13

Effects of BAL and 8-OH quinoline concentrations on A-I generation rates

Inhibitor	mg/ml	µmol/ml	A-I(ng/ml/h)
8-OH-quinoline	0.19	1.3	5.7
BAL	0.50	4.0	
8-OH-quinoline	1.00	6.9	7.2
BAL	0.25	2.0	

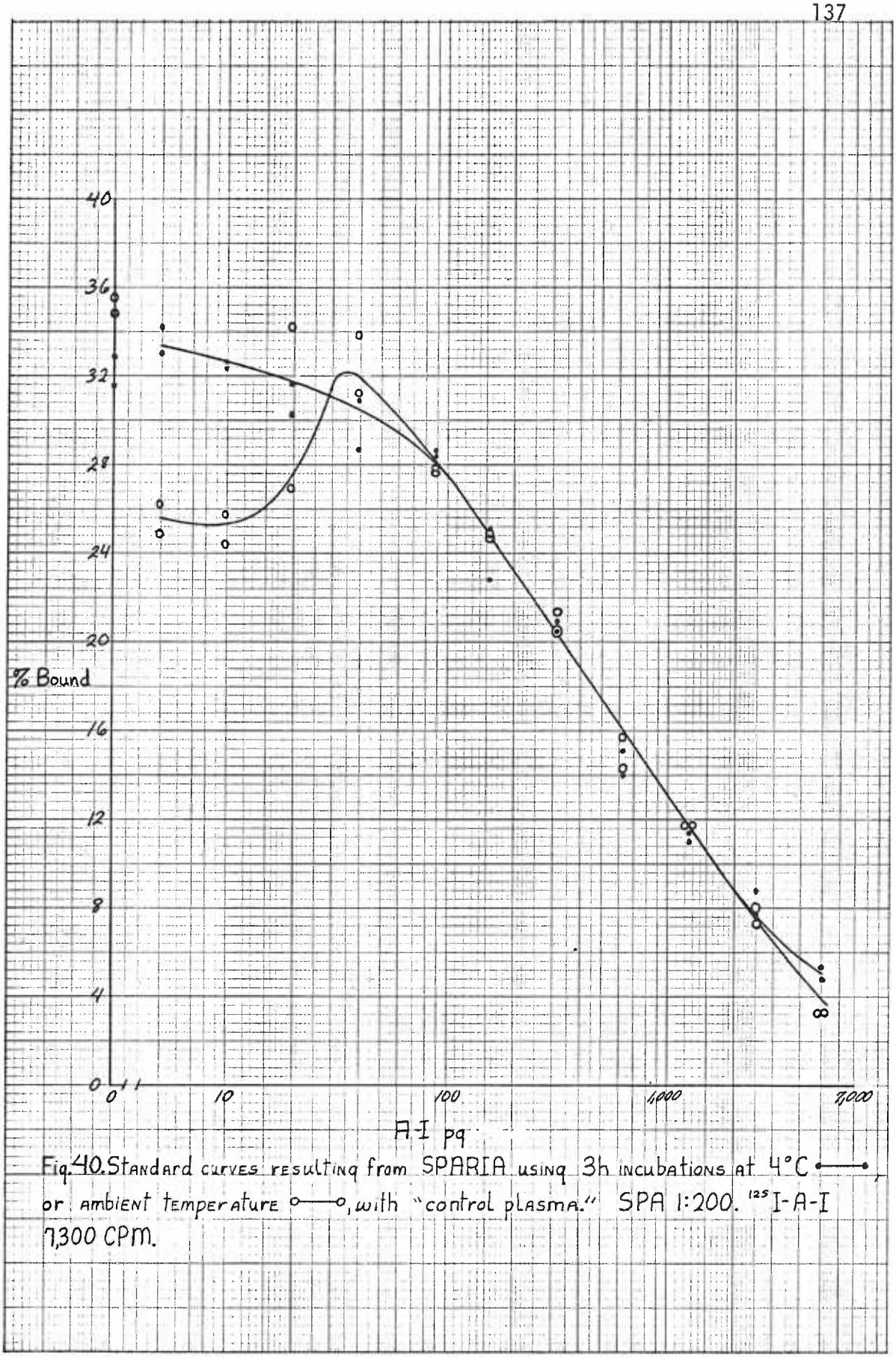

Fig. 40. Standard curves resulting from SPARIA using 3h incubations at 4°C ●——●, or ambient temperature o——o, with "control plasma." SPA 1:200. ^{125}I-A-I 7,300 CPM.

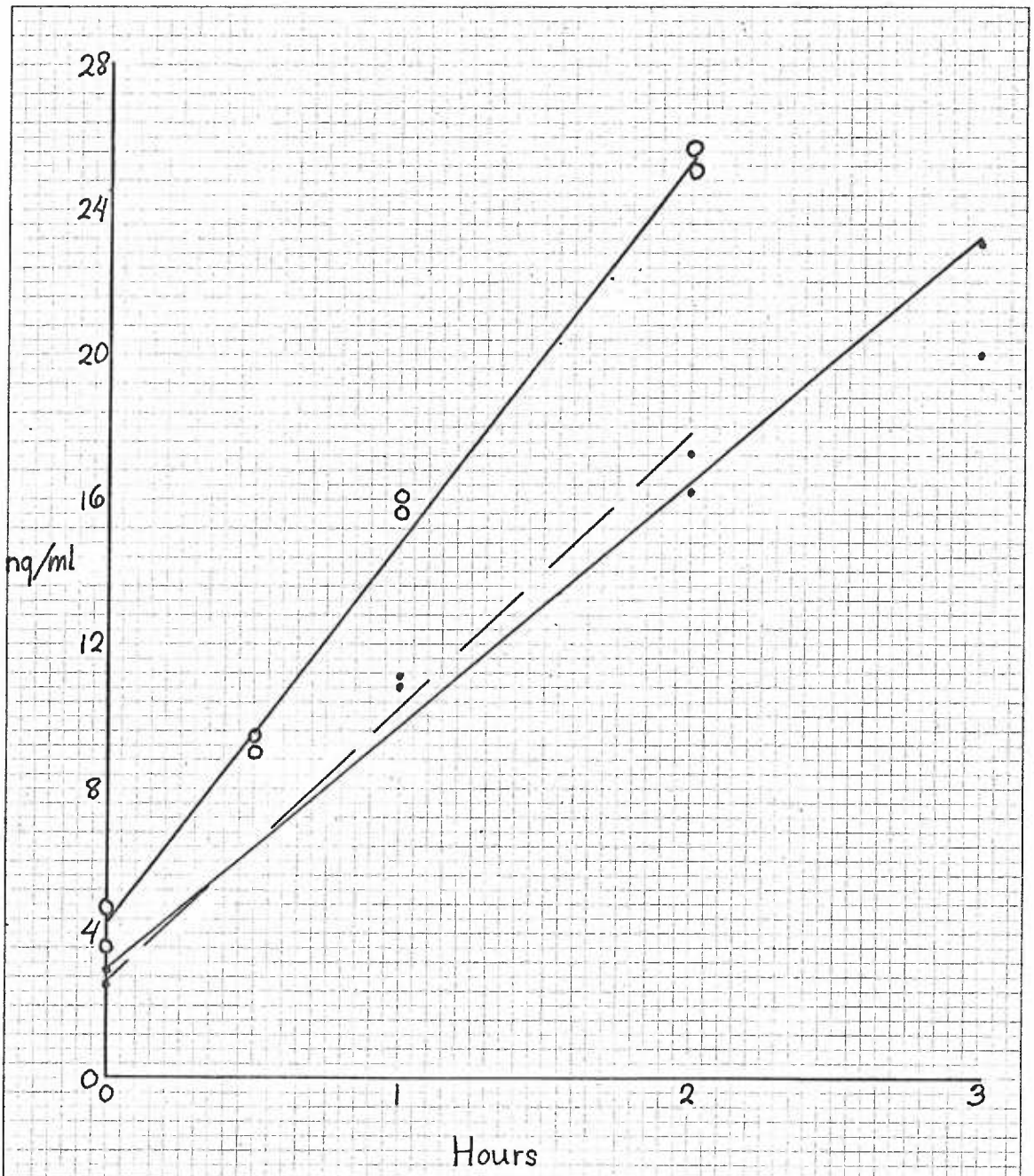

Fig. 41. Effects of shorter sampling intervals on graphic calculation of renin activity. •—• 60, 120, 180 min, •- - -• 60, 120 min plot, o—o 30, 60, 120 min

Table 14

Within day precision of RIA

	A-I pg/50µl	N	$\bar{x}$ pg/50µl	s pg/50µl	SEM pg/50µl	Range pg/50µl
without plasma	216	6	222.3	13.5	5.5	206-238
with plasma	54	6	61.4	15.7	6.4	33-74.5
	216	6	208.0	17.9	7.3	177-232
	216	24	219.0	15.7	3.2	180-240
	863	6	895.8	69.7	28.4	795-975

Table 15

Precision of renin activity determination

	N	$\bar{x}$ ng/ml/h	s ng/ml/h	SEM ng/ml/h	Range ng/ml/h
within day	10	10.2	0.62	0.20	9.1-11.1
between day	12	9.0	1.6	0.46	7.2-11.8

Table 16

Percent recovery of A-I from plasma

A-I added (pg/50µl)	N	$\bar{x}$	s	SEM	Range
328	6	101.1	6.9	2.8	91.5-111.3

Table 17

Sensitivities at various levels of A-I with 95% confidence limits

A-I (pg)	N	2s (pg)	2s (ng/ml/h)
"zero" (estimated)	--	<20	<0.4
54	6	31.4	0.63
216	6	35.8	0.71
216	24	31.4	0.63
863	6	139	2.8

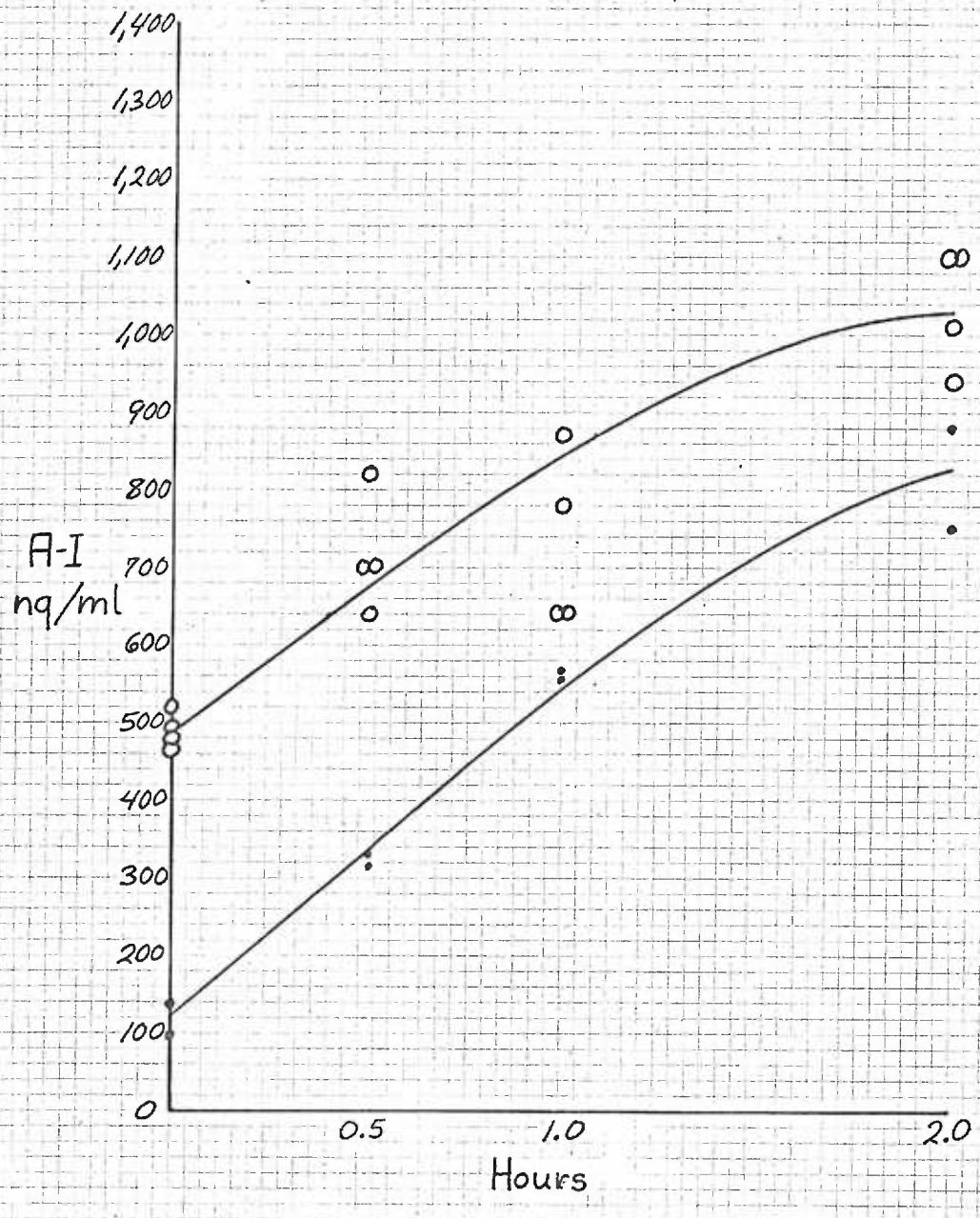

Fig. 42. Recovery of A-I vs. time in plasma containing A-I (328pq/50ul) o—o and plasma containing "assay buffer" •—•.

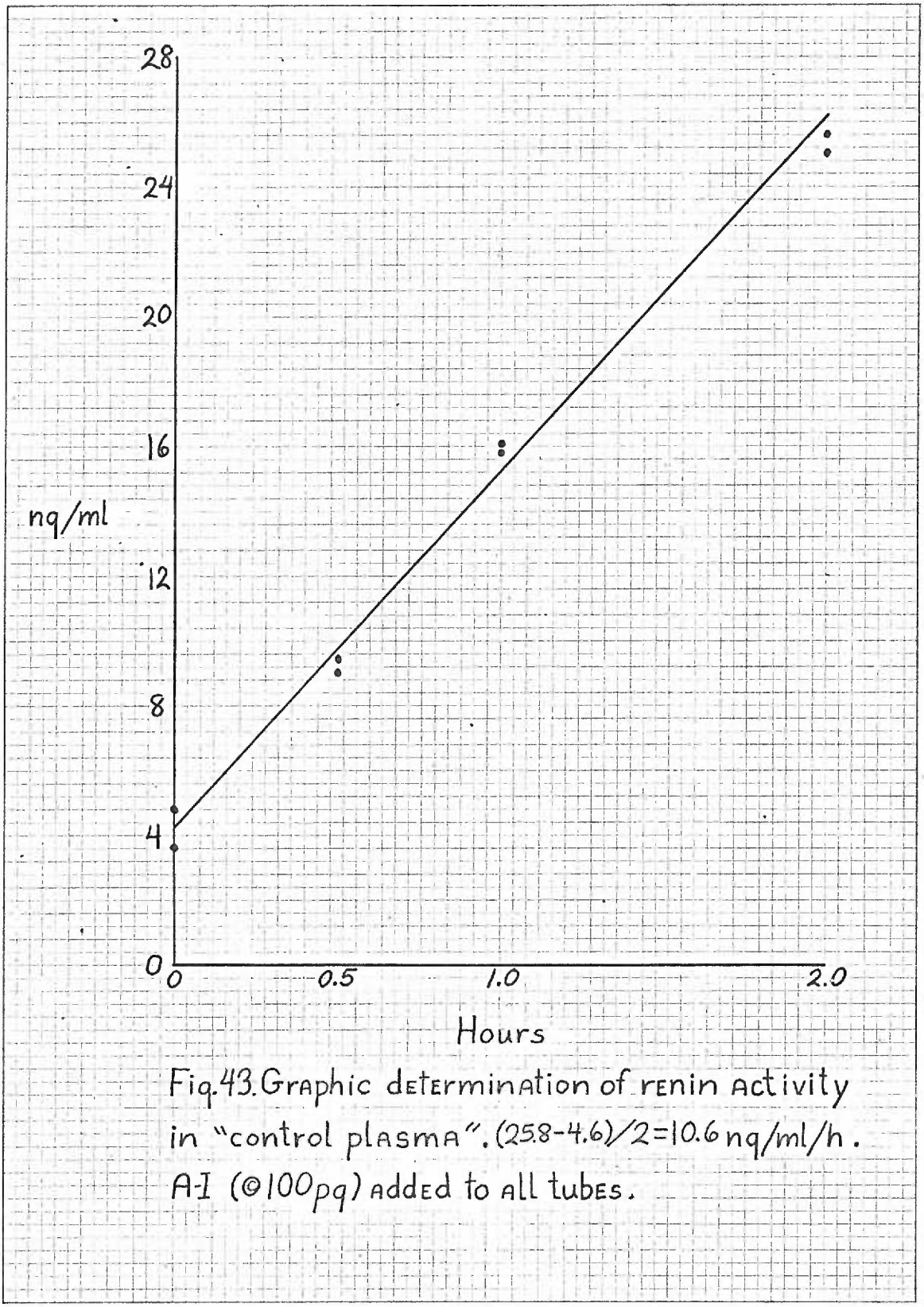

Fig. 43. Graphic determination of renin activity in "control plasma". (25.8-4.6)/2=10.6 nq/ml/h. A-I (@100pq) added to all tubes.

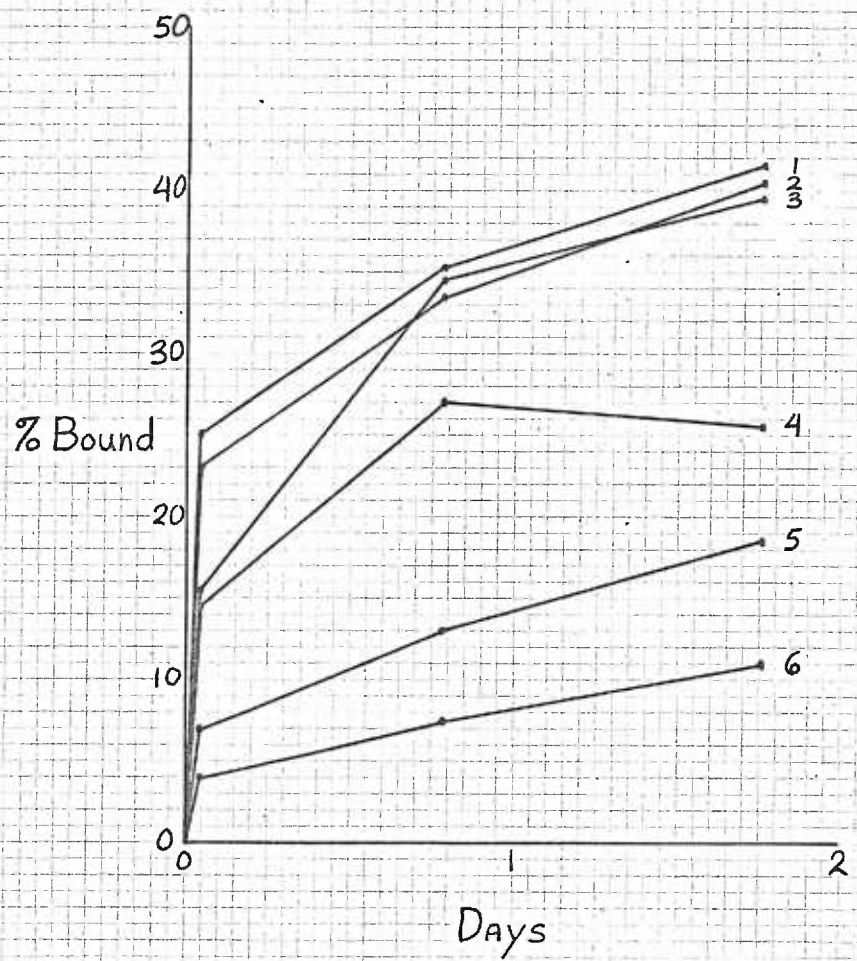

Fig. 44. Rate of coupling of ^{125}I-A-I (2.5 ng, 12,000 CPM) to activated Sephadex G-25-40 (100 mg) in various buffers.

0.1 M NaH_2PO_4 pH		0.1 M $NaHCO_3$–Na_2CO_3 pH	
1.	8.0	4.	8.1
2.	7.0	5.	9.6
3.	6.0	6.	10.9

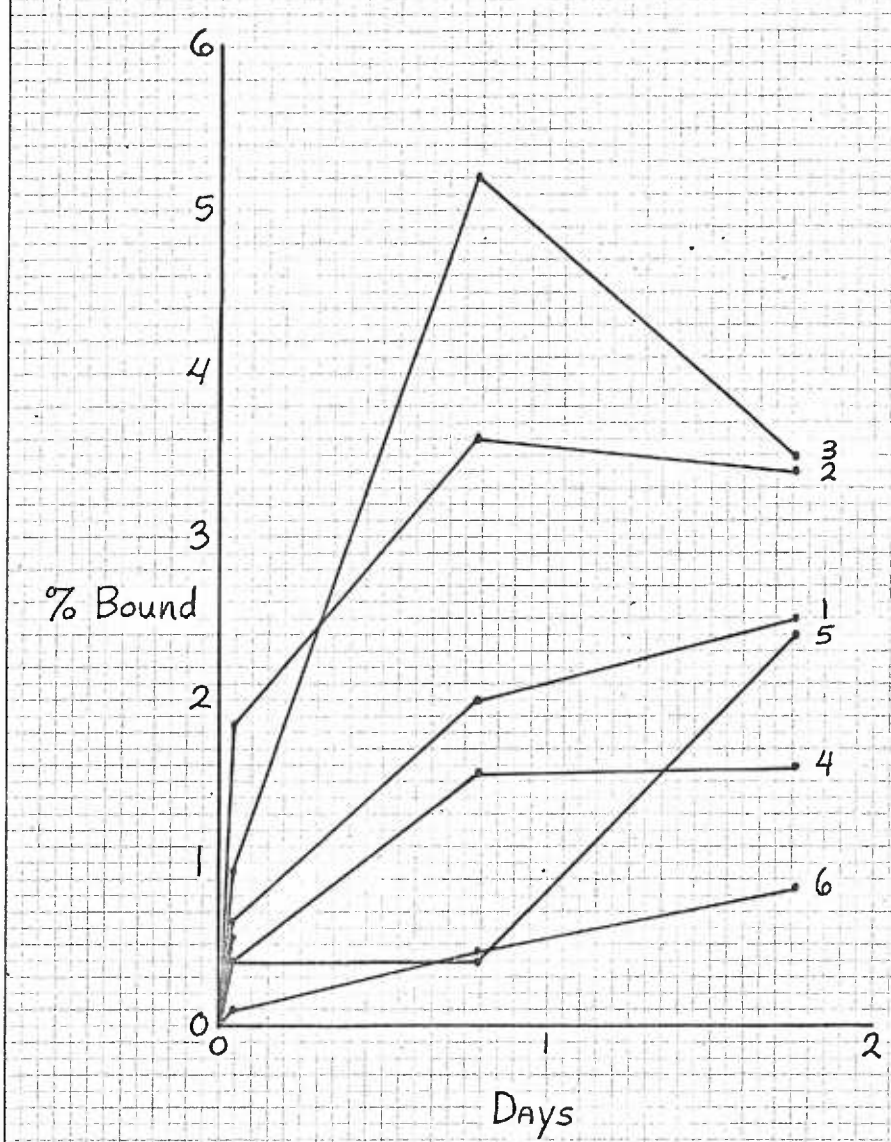

Fig. 45. Rate of coupling of ^{125}I-A-I (2.5 nq, 12,000 CPM) to 12X75 mm plastic tubes in various buffers.

0.1M NaH_2PO_4 pH

1. 8.0
2. 7.0
3. 6.0

0.1M $NaHCO_3$-Na_2CO_3 pH

4. 8.1
5. 9.6
6. 10.9

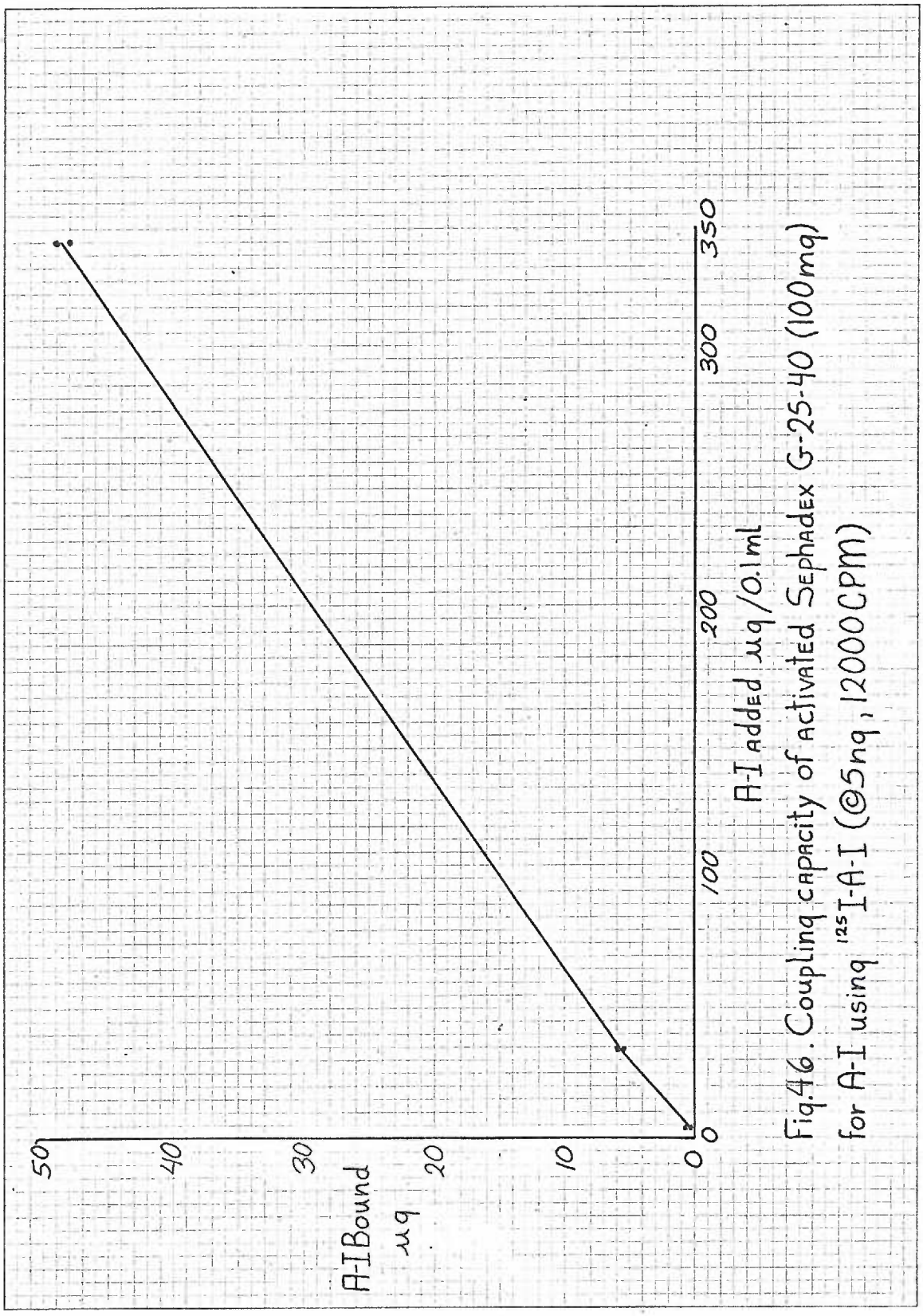

Fig.46. Coupling capacity of activated Sephadex G-25-40 (100mg) for A-I using ^{125}I-A-I (@5ng, 12000CPM)

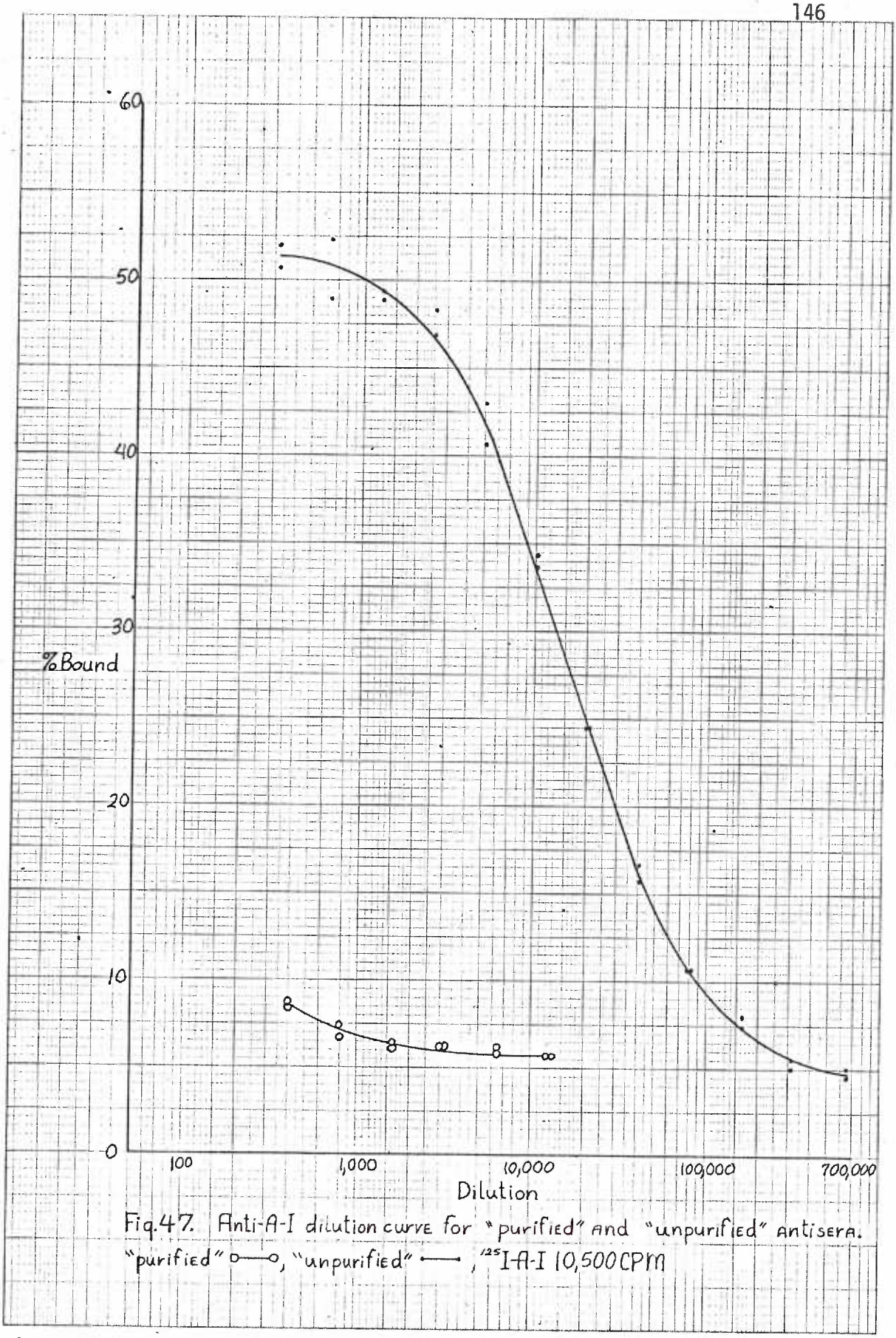

Fig. 47. Anti-A-I dilution curve for "purified" and "unpurified" antisera. "purified" o—o, "unpurified" ——, ^{125}I-A-I 10,500 CPM

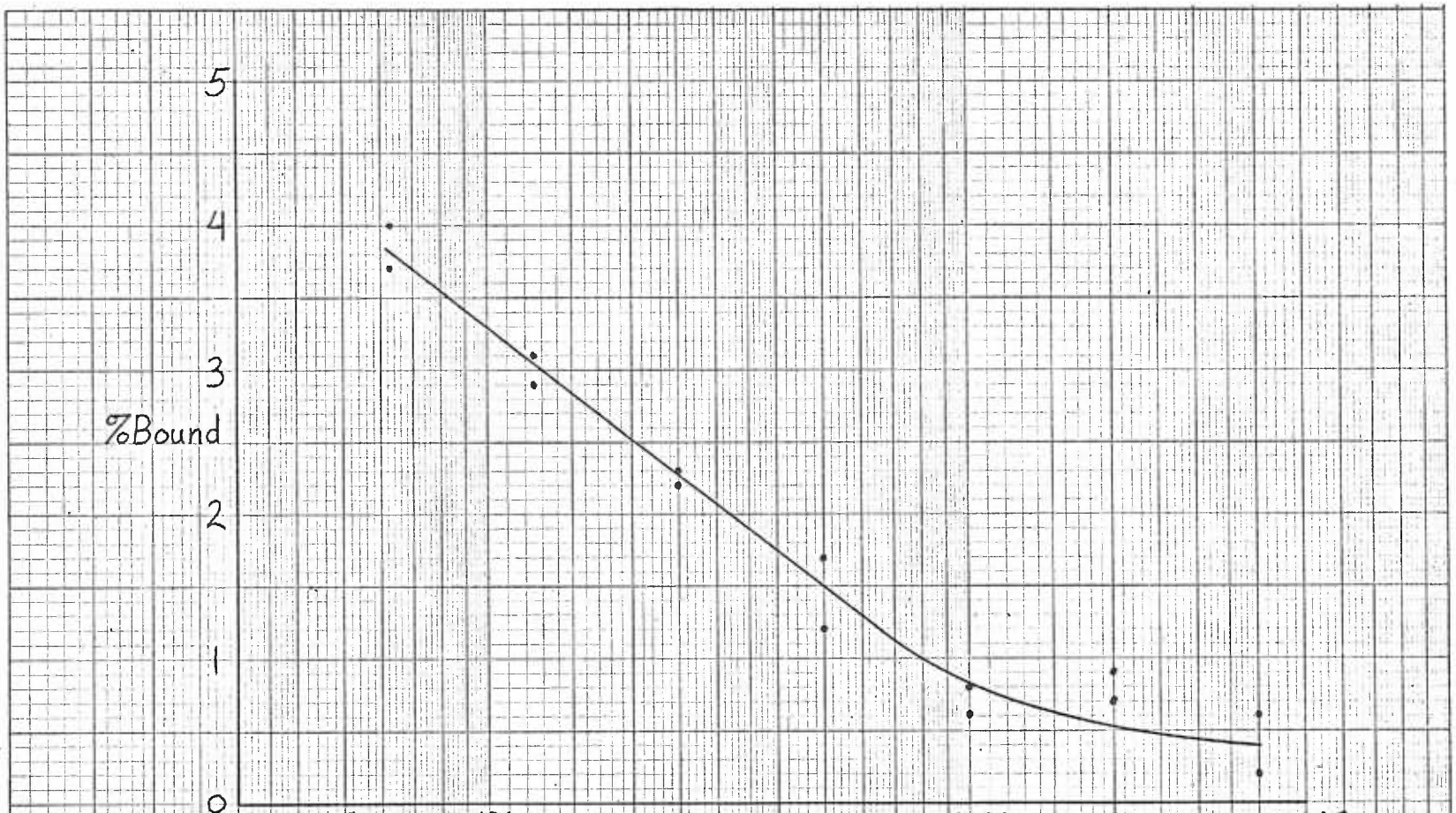

Fig. 48. "Purified" anti-A-I dilution curve after concentration by cone ultramicrofiltration. ^{125}I-A-I 12,000 CPM

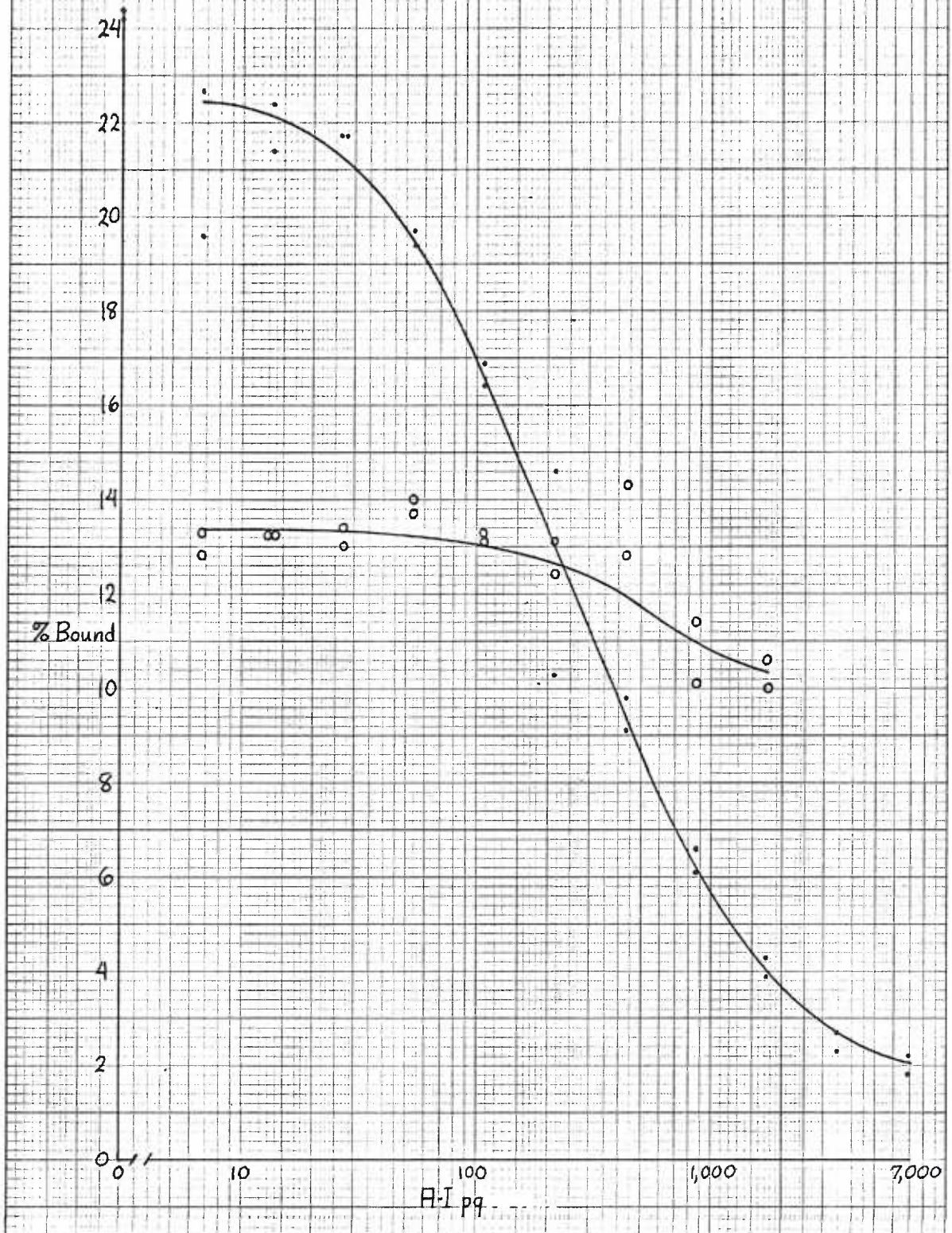

Fig. 49. Standard curves resulting from SPARIA (SPA 1:200) •——•, or free antibody RIA using concentrated "purified" anti-A-I o——o. ^{125}I-A-I 13,000 CPM.

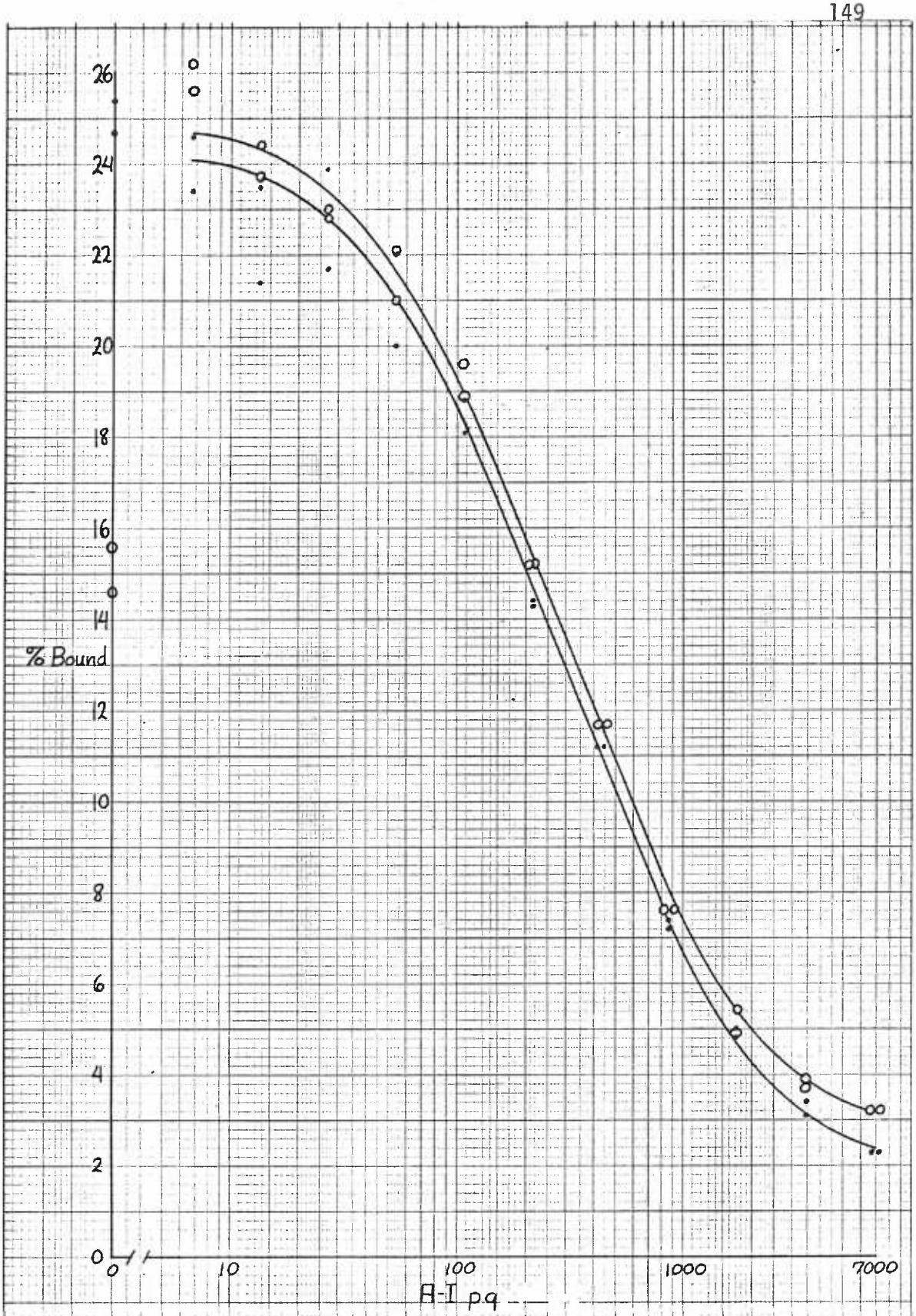

Fig. 50. Standard curves resulting from SPARIA (SPA 1:200) ⟶, or free antibody RIA using Na_2SO_4 purified anti-A-I (1:5,000) o——o. ^{125}I-A-I 10,000 CPM.

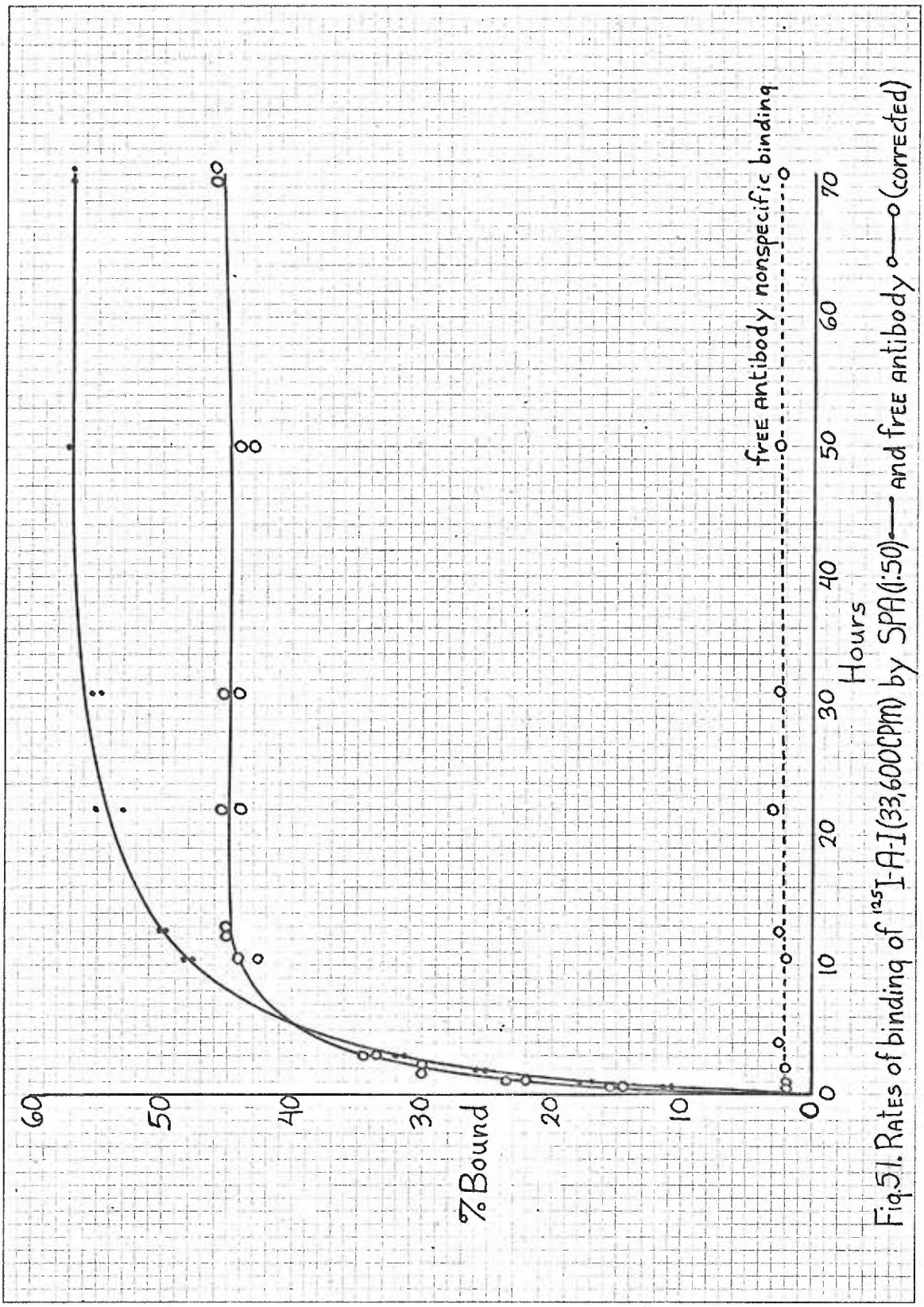

Fig. 51. Rates of binding of ^{125}I-A-I (33,600 CPM) by SPA (1:50) — and free antibody o—o (corrected)

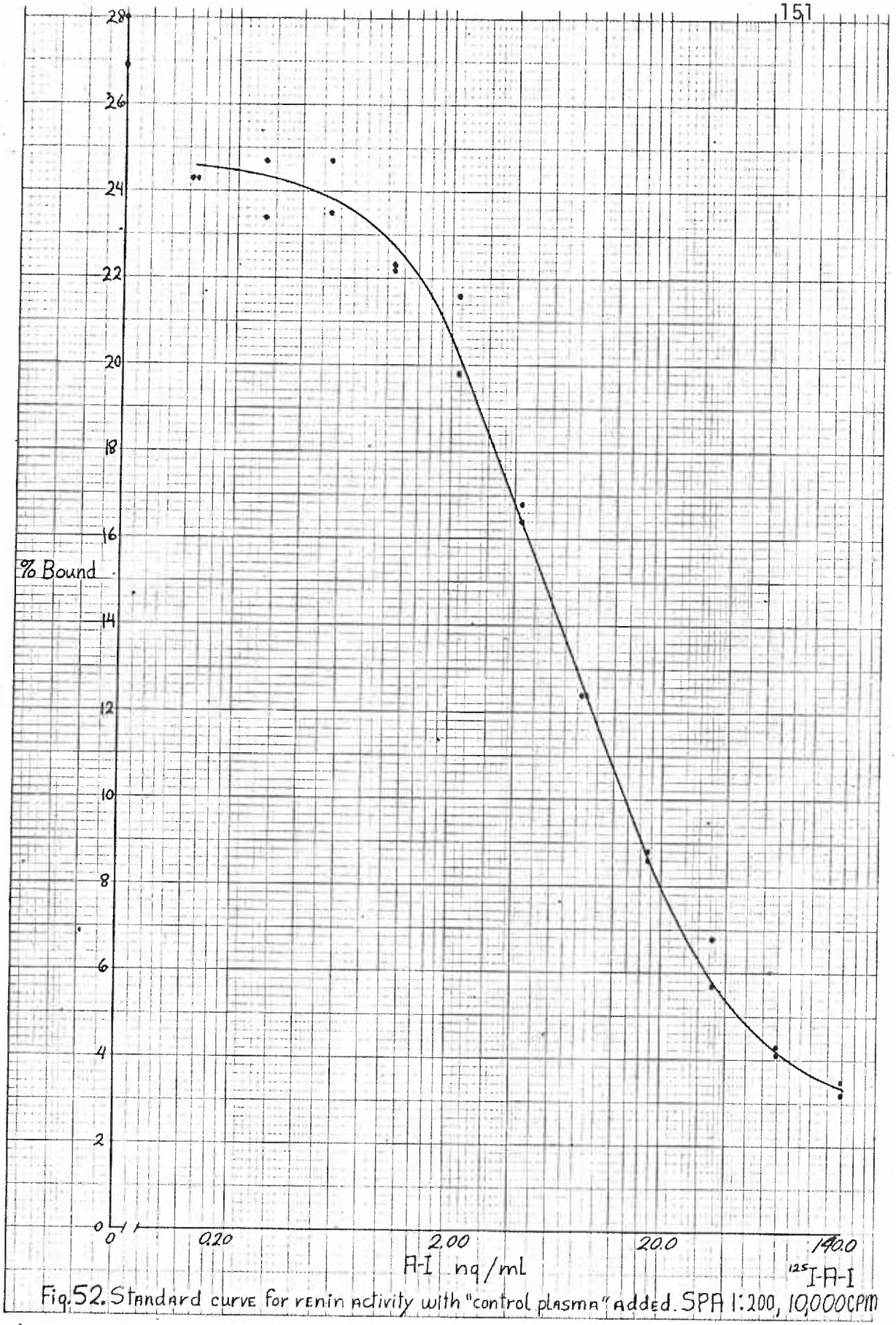

Fig. 52. Standard curve for renin activity with "control plasma" added. SPA 1:200, 10,000CPM

Table 19

Percent recovery of A-I by several methods of RIA of A-I for renin activity

A-I added (ng/ml)	$\bar{x}$	s	SEM	CV (%)	N	Ref.
6.56	101.1	6.9	2.8	--	6	Present study
1,5	91	10	--	--	10	55
2,3,4,5	100.5	11	--	--	4,5	56
2	84	4	--	--	11	12
2	85.6	--	--	2.6	6	34

Table 20

Sensitivities of several methods of RIA of A-I for renin activity

Lower limit (pg)	Other levels (pg)	2s (ng/ml)	2s (ng/ml/h)	Ref.
ca. 20	--	--	0.4	Present study
--	54	0.63	0.63	
--	216	0.71	0.71	
--	216	0.63	0.63	
--	863	2.8	2.8	
ca. 20	--	--	0.2	56
ca. 80	--	--	--	12
ca. 200	--	--	--	33

In the present study, crossreactivity could be evaluated in a number of ways. The effects of the removal of N or C terminal amino acids from A-I could be systematically compared on a standard curve basis. Likewise species specific A-I molecules such as val^5-angiotensin-I could be evaluated. Renin substrate or tetradecapeptide may crossreact. The specific effects of the introduction of 1 or 2 atoms of iodine into the A-I molecule could also be determined.