

INVESTIGATIONS CONCERNING THE ISOLATION OF  
THE HORMONE PROLACTIN USING IMMUNOCHEMICAL METHODS

by

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## ABSTRACT

The aim of this study was to investigate the use of immunological techniques in the isolation of prolactin.

First, antiserum to ovine prolactin was produced. The rabbits used showed considerable differences in the immune response with regard to the titer of the antiserum and in general the response was weak. Those antisera that were to be used in subsequent studies did not cross-react with other pituitary hormones in agar diffusion tests.

Secondly, two methods were used in attempts to purify prolactin. One was a precipitation technique which involved the dissociation of a precipitated prolactin:anti-prolactin complex. The other was an immunosorption technique. Both techniques were first analysed with a bovine serum albumin (BSA):anti-BSA system. In connection with the first isolation technique, a micro-precipitin curve for prolactin was first established to estimate the ratio of antigen:antibody required to produce maximum precipitation. The precipitation technique was then applied to a prolactin: anti-prolactin system, using glycine-HCl buffer to dissociate the complex. While some prolactin was obtained, the antibody could not be

re-used and it was suspected that the dissociation was incomplete.

Immunosorption gave better results. In this method the antibody fraction was coupled to activated Sepharose. An anti-BSA immunosorbent was found to be capable of binding BSA specifically and could be re-used at least 9 times without loss of activity. When the technique was then applied to a prolactin:anti-prolactin system, much smaller yields of antigen were obtained. When the anti-prolactin immunosorbent was used to isolate prolactin from a sheep pituitary homogenate, some concentration of prolactin was accomplished but also some non-specific protein binding took place.

Finally, the fractions obtained by the immunosorption procedure were quantified using a solid-phase radioimmunoassay as well as the pigeon crop bioassay (local method). Somewhat lower prolactin values were obtained by the bioassay than by the radioimmunoassay. This may indicate a slight reduction in biological activity during immunosorption.

Possible approaches that could be taken to overcome the problems of yield, specificity, and decrease in biological activity are discussed.

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## GENERAL INTRODUCTION

This investigation was prompted by my interest to isolate "salmon prolactin". In a recent review concerning the comparative endocrinology of prolactin, Bern and Nicoll (1968) suggested that prolactin is likely present in the pituitary gland of all vertebrates and that "no other hormone - vertebrate or invertebrate - has so many widely different effects, that is, so many different 'target organs', as have been claimed for prolactin". Although the occurrence of prolactin in the pituitary gland of Pacific salmon has been shown (McKeown and van Overbeeke, 1969; McKeown, 1970), the physiological significance of this proteinaceous hormone remains largely to be elucidated. No successful attempts to isolate this hormone have been published.

Normally, the isolation of a protein from its natural source is achieved by fractionation, and the specific activity of the preparation must be evaluated after each step. In the case of hormones this evaluation would require a bioassay. To date, no bioassay for fish prolactin is available. The observation that "salmon prolactin" cross-

reacts immunologically with antibodies to ovine (sheep) prolactin (McKeown and van Overbeeke, 1969), suggested an alternative possibility to obtain this hormone, namely to use an immunochemical technique.

When using immunochemical methods to remove a protein-antigen or its antibody from a mixture, one benefits from the supposedly highly specific affinity between these two components. The success of such methods obviously depends upon the specificity of the antisera which in turn will depend on the purity of the protein used as antigen.

Very few reports concerning the application of the above technique for the purification of hormones have been published. With regard to prolactin, Emmart and Bates (1968) reportedly succeeded in purifying ovine prolactin as well as prolactin from a teleost, the pollack (Pollachius virens). This last observation is of particular interest since the pollack prolactin was precipitated with anti-ovine prolactin. This would indicate that immunochemical purification methods are useful even in those cases where the immune reaction, which constitutes the basis of this technique, has the nature of a cross-reaction or heterologous reaction (a reaction between an antigen and an antibody to the corresponding

antigen from another species).

For this study, it was decided that prior to attempting the isolation of salmon prolactin, it was necessary to first re-investigate the purification of ovine prolactin. During this phase of the investigation, considerable technical difficulties were encountered. Also, the question arose whether the conjugation and subsequent separation of the hormone-antigen from its antibody would affect its biological activity. The solution of these various problems involved in purifying ovine prolactin constitutes the contents of this thesis.

The report consists of three parts. The first deals with the problems involved in producing antisera to ovine prolactin and the subsequent analysis of these sera. The second and most extensive part concerns investigations into the use of two immunochemical techniques to isolate ovine prolactin. The third part describes the quantification of the isolated prolactin fractions using both a bioassay and a radioimmunoassay.

1. PRODUCTION AND ANALYSIS OF ANTISERA TO BOVINE SERUM ALBUMIN AND OVINE PROLACTIN.

Introduction

The production and analysis of antisera commonly follows standard procedures and is the subject of several textbooks (Campbell et al., 1964; Kabat and Mayer, 1967; Nowotny, 1969). Of major importance is the phenomenon that individuals of the same species respond differently to injection of a particular antigen with respect to titer and specificity of the antiserum (Nowotny, 1969). For this reason it is advisable to immunize a number of animals with the same antigen whenever possible. Of all of these animals, the titer and specificity of the antisera is then analysed for some time, and when one or more adequate producers of antibody are found, the immunization of the others is discontinued.

An antiserum to be used for isolation of a particular antigen must react specifically with this antigen. In a study such as the present one, where the hormone must be isolated from a pituitary homogenate, it is of paramount importance that the antiserum does not react with any other pituitary



hormone. It follows that the hormone-antigen used to produce the antiserum must be of sufficient purity.

One of the isolation procedures used in my study involves precipitation of the antigen with the antibody. Precipitation is the final visible reaction between a soluble antigen and its antibody. This reaction characteristically takes place only when the antigen and antibody are present in a particular ratio (Carpenter, 1965). When either compound is present in excess, the complex will still form but its precipitation is inhibited (Kabat and Mayer, 1967). Because of this phenomenon it was necessary to determine the optimum ratio for prolactin:anti-prolactin.

#### Materials and Methods

Hormone preparations. Two prolactin preparations were used as antigens: Sigma Ovine Prolactin (20 IU/mg), and Ovine Prolactin donated by the National Institute of Health, Bethesda, Md. (NIH-P-S9; 30 IU/mg).

The following hormones were used in tests for specificity of the ovine prolactin antisera: Ovine Growth Hormone (NIH-GH-S9, 1.09 IU/mg); Ovine Luteinizing Hormone (NIH-LH-

Sl3, 0.93 IU/mg); Ovine Follicle Stimulating Hormone (NIH-FSH-S5, 1.42 IU/mg); Ovine Thyroid Stimulating Hormone (NIH-TSH-S5, 1.44 IU/mg); and Porcine Adrenocorticotrophic Hormone (Sigma, 140 IU/mg).

Sheep pituitary homogenate. To prepare a crude prolactin preparation, one hundred sheep pituitary glands were homogenized with 100 ml of 0.01 M phosphate-buffered saline (pH 7.4) in a Virtis tissue homogenizer. The suspension was centrifuged for 30 min. at 20,000 g and the supernatant saved. Agar diffusion tests (Ouchterlony, 1953) showed the presence of prolactin in the supernatant.

Production of antisera. Of seven female New Zealand White rabbits, approximately 6 months old, two were injected with Sigma Ovine Prolactin, one with NIH Ovine Prolactin, and two were kept as uninjected controls to provide a source of normal rabbit serum. The two remaining rabbits were immunized with bovine serum albumin (Sigma BSA, fraction V). The BSA:anti-BSA system was used in the initial investigations of various technical procedures in order to economize on the prolactin antiserum. All antigens were injected in a dosage of 1.5 mg in 1 ml of 0.15 M NaCl and prior to injection each

solution was mixed with an equal volume of Freund's complete adjuvant (Hyland Lab.). The injection of adjuvant with the antigen enhances the production of antibody (Freund and Bonanto, 1944). These 2 ml/injections were given intramuscularly once per week. Three days after the fourth injection, the prolactin-injected rabbits were bled from the marginal ear vein and the titer of the antiserum was estimated as described below. This was continued weekly for 8 weeks after which larger blood volumes of approximately 30 ml were taken from the rabbit producing the highest titer antiserum. This last rabbit was then given monthly booster injections of NIH ovine prolactin. The BSA-injected rabbits were first bled 3 days after the seventh injection to estimate the titer. The rabbit with the highest titer was given monthly booster injections and bled weekly. In all cases the blood collected was allowed to clot, and was then refrigerated for 2 hours, centrifuged at 10,000 g for 5 min., and the serum was removed with a Pasteur pipette. Merthiolate was added as a preservative in a final concentration of 1:10,000 and the serum was stored frozen.

Prolactin antiserum that was kindly made available by Dr. E. Emmart from the National Institute of Health, Bethesda, Md., was also used (antiserum E).

Estimation of titer and determination of specificity.

All titers were estimated by passive haemagglutination (Campbell et al., 1964). Washed sheep erythrocytes were treated with tannic acid and then coated with the antigen. Serial, twofold dilutions of the antiserum sample were prepared and a constant volume of the antigen-coated erythrocytes was added to each of them. The settling patterns were observed for agglutination so that the titer could be obtained.

The prolactin antisera were tested for specificity by Ouchterlony gel diffusion with prolactin (LTH), growth hormone (GH), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and adrenocorticotrophic hormone (ACTH).

Microprecipitin tests. To determine the optimum ratio of antigen to antibody to produce maximum precipitation one can simply add a constant amount of antiserum to a serial, two-fold dilution of the antigen and observe which ratio produces

the most precipitate. Naturally the antigen-antibody complex must be separated from the soluble proteins and this is usually accomplished by centrifugation (Campbell et al., 1964). Such an approach employs larger quantities of both antigen and antiserum than could be afforded in this study. For this reason the microtechnique described by Miquel et al. (1960) was used with some modifications. In this procedure the insoluble complex is separated from the soluble serum proteins using an electrophoretic technique. The procedure was first analysed with a BSA:anti-BSA system and then subsequently applied to prolactin:anti-prolactin. The following description applies to both systems.

In 10, 6 x 50 mm culture tubes, 50  $\mu$ l of a serial two-fold dilution of antigen was prepared, to which was added 50  $\mu$ l of antiserum. The tubes were incubated overnight at 37°C. They were then centrifuged and each supernatant analysed in agar diffusion tests for the presence of excess antigen or antibody. The precipitates were resuspended in 2 drops of 0.01 M phosphate-buffered saline (pH 7.4). The contents of each tube was applied on electrophoresis paper strips (4.5 x 20 cm) with a capillary tube. The paper

used (Schleicher and Schuell, 2043A) was non-absorptive, to allow maximum movement of soluble proteins. Prior to sample applications, the paper strips were dampened with (0.01 M phosphate-buffered saline (pH 7.4)<sup>1</sup>. Electrophoresis was carried out in a cold room (9°C) using prechilled 0.01 M phosphate-buffered saline (pH 7.4). The point of application on the paper strip was moved 4 cm from the center of the electrophoresis chamber toward the cathode and 250 volts were applied for 30 min. The strips were then dried for 30 min., stained for 10 min. with brom phenol blue and de-stained in 3 changes of 1% acetic acid. The insoluble antigen-antibody complex remained at the origin during electrophoresis, well separated from soluble serum proteins (Fig. 1). This stained complex was eluted with an alcohol-NaOH solution (10 ml, 1 M NaOH, 70 ml ethyl alcohol-95%, and 20 ml H<sub>2</sub>O) and the optical density (O.D.) of the eluate determined at 595 nm to estimate the amount of complex present.

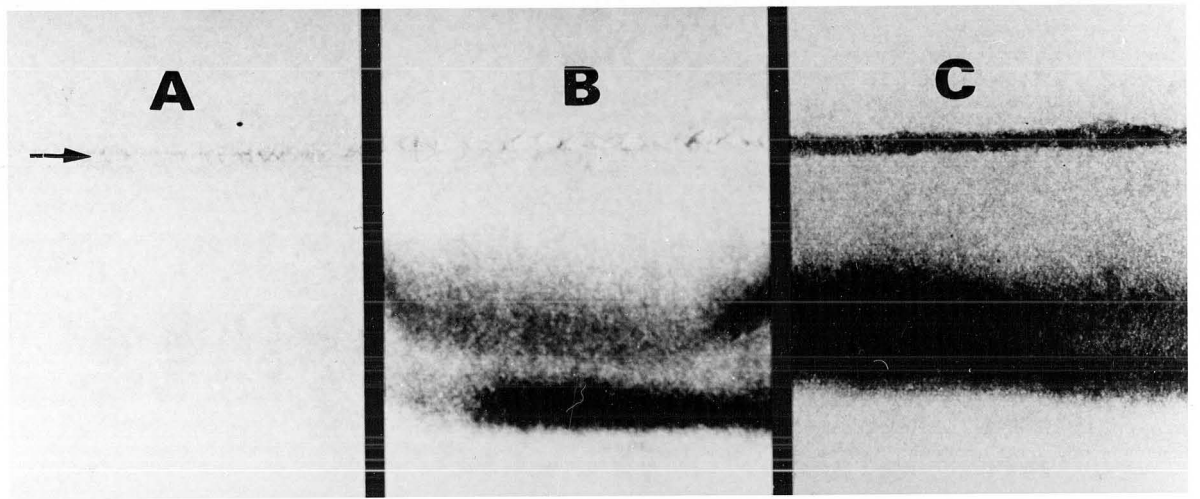
## Results and Discussion

Titer and specificity of antiserum. Considerable

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<sup>1</sup> 0.01 M phosphate-buffer (pH 7.4) dissolved in 0.15 M NaCl

Fig. 1. Electrophoretic separation of a BSA:anti-BSA complex from excess soluble proteins. A: 50  $\mu$ l BSA solution (1 mg/ml) was applied at the origin (arrow). No staining could be detected at this concentration. B: 50  $\mu$ l BSA antiserum. The soluble serum proteins moved toward the anode. C: a mixture containing the insoluble antigen-antibody complex. The insoluble complex remained at the origin, well separated from the soluble proteins that moved towards the anode.





differences were found between the three rabbits injected with prolactin, with respect to the maximum titer of the antisera (Fig. 2). The best response was found in the rabbit injected with NIH prolactin but in all cases the response was rather weak. The titers were much lower than that of antiserum E which was found to be 128,000, while the immunizing procedure used for the production of this last antiserum (Emmart et al., 1963) was not different from that employed in my study. It is unfortunate that due to the scarcity of prolactin, I was unable to immunize a larger number of rabbits. Consequently, my data do not provide information on the causes of variability in the immune response.

It was decided to utilize the antiserum from the NIH prolactin-injected rabbit as well as antiserum E for subsequent investigations and therefore these antisera were tested for specificity. In Ouchterlony tests both antisera were found to give precipitin bands with ovine prolactin, but failed to react with any other pituitary hormone used (Fig. 3).

The titer of the BSA antiserum was found to be between 64,000 and 128,000.

Fig. 2. Variability in immune response by rabbits to ovine prolactin. Titers were estimated by passive haemagglutination test.

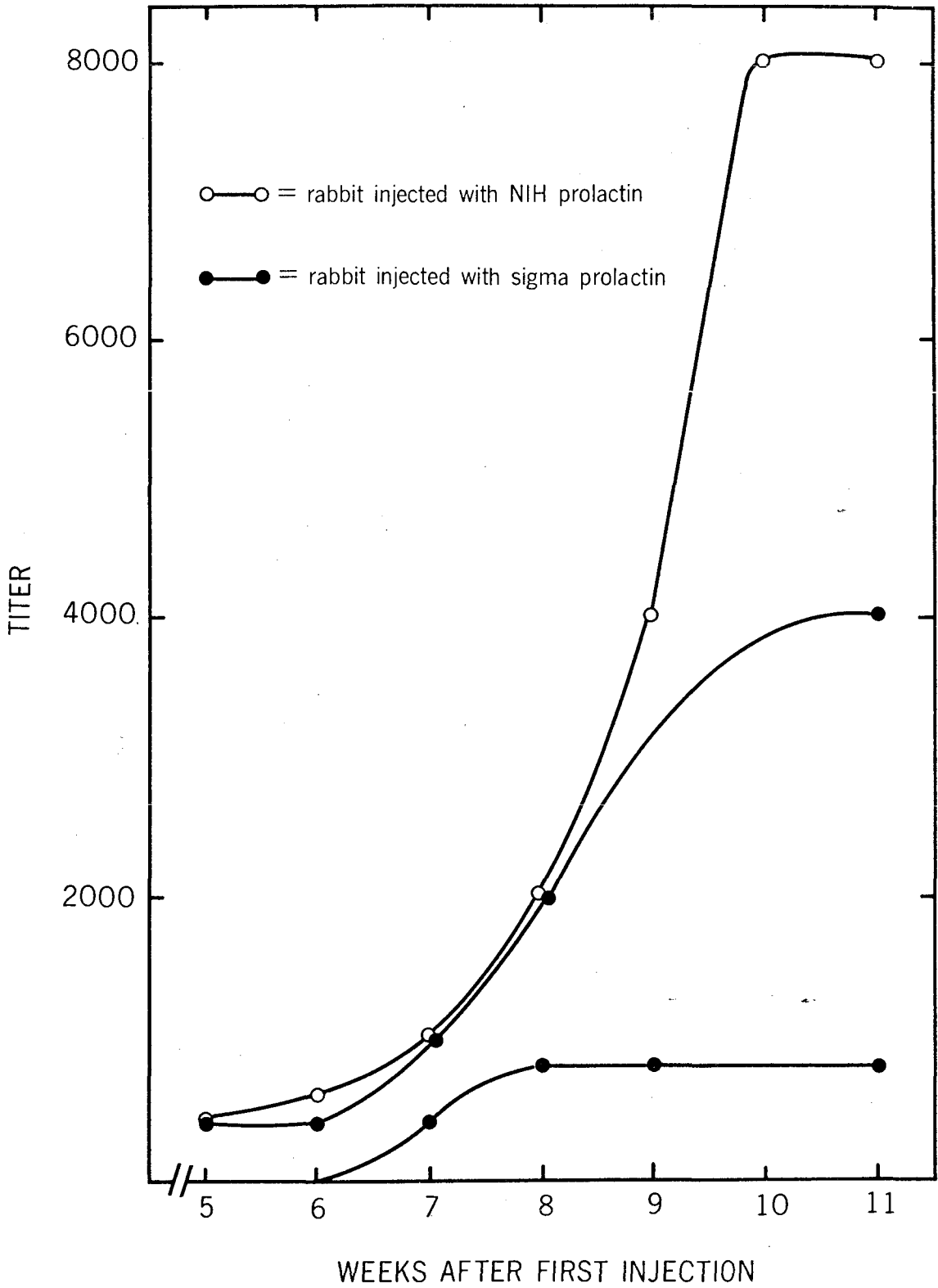
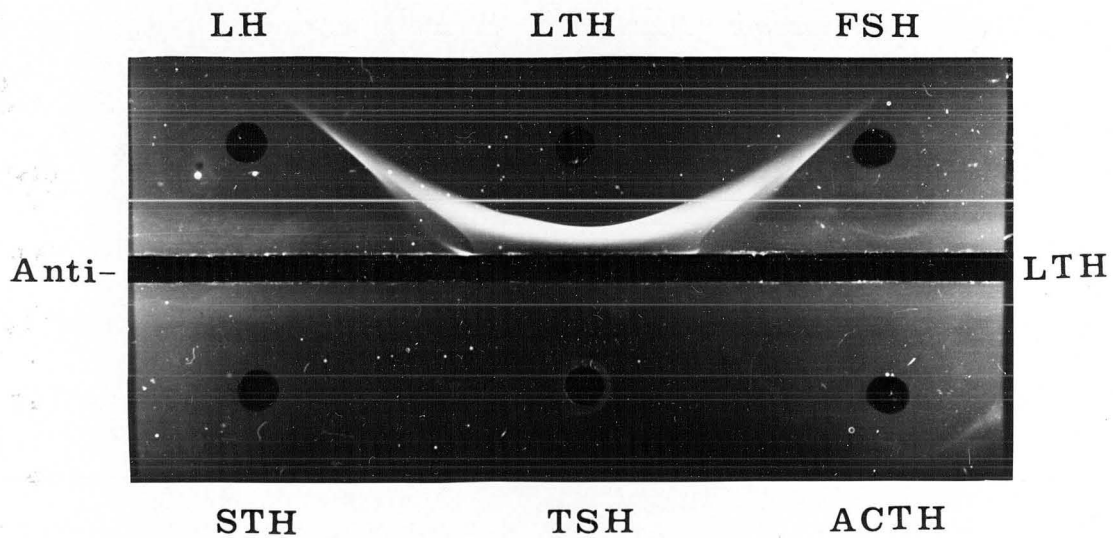


Fig. 3. Gel diffusion test. Antiserum to ovine prolactin (Anti LTH) tested for specificity with the following pituitary hormones: ovine prolactin (LTH), luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (STH), thyroid stimulating hormone (TSH), and porcine adrenocorticotrophic hormone (ACTH). Each well contained antigen in a concentration of 1.0 mg/ml. The antiserum reacted only with prolactin.



Optimum antigen-antibody ratio for precipitation. In a preliminary experiment 50  $\mu$ l of anti-BSA was added to 50  $\mu$ l of a serial twofold dilution of BSA. Maximum precipitation was obtained with a dilution of BSA containing 0.313 mg/ml (Fig. 4). This is consistent with the fact that in the supernatant of this tube neither antigen nor antibody could be detected ("equivalence zone"). In cases where precipitation was low, an excess of either antigen or antibody could be shown (Fig. 4). In determining the precipitin curve for prolactin, antiserum E was used. Maximum precipitation was obtained when 50  $\mu$ l of this serum was added to a solution of prolactin containing 0.0625 mg/ml (Fig. 5). Again, maximum precipitation was found to coincide with the "equivalence zone"; where smaller amounts of precipitate were formed, either antigen or antibody was present in excess in the supernatant (Fig. 5).

These results are consistent with the now well-documented opinion that an excess of either antigen or antibody inhibits precipitation of the antigen-antibody complex (Kabat and Mayer, 1967). It is generally agreed that there exists a bivalence or multivalence of the antigen, the anti-

Fig. 4. Precipitin curve for BSA and its antiserum. In each tube 50  $\mu$ l of antiserum was added to 50  $\mu$ l of a serial, twofold dilution of antigen.

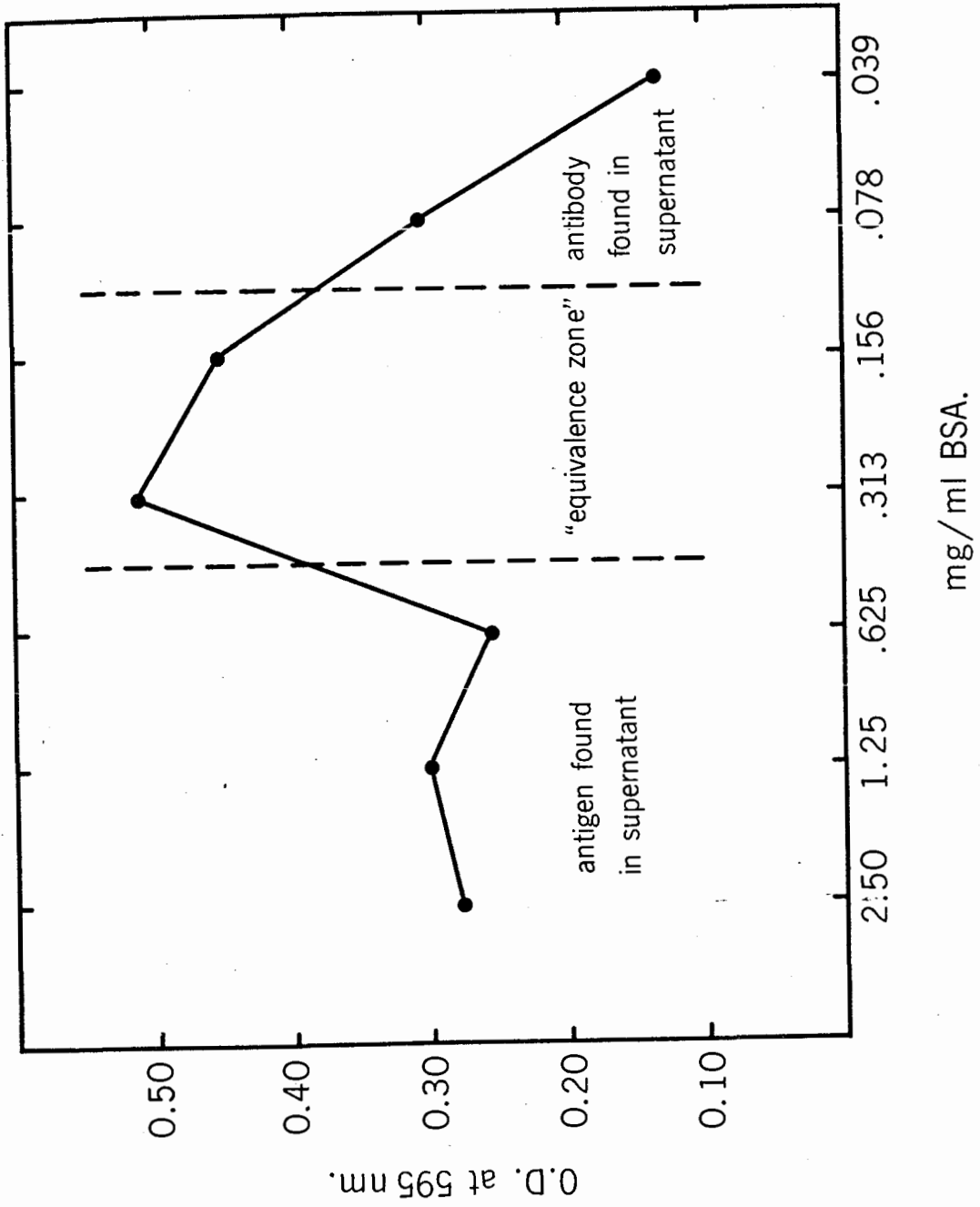
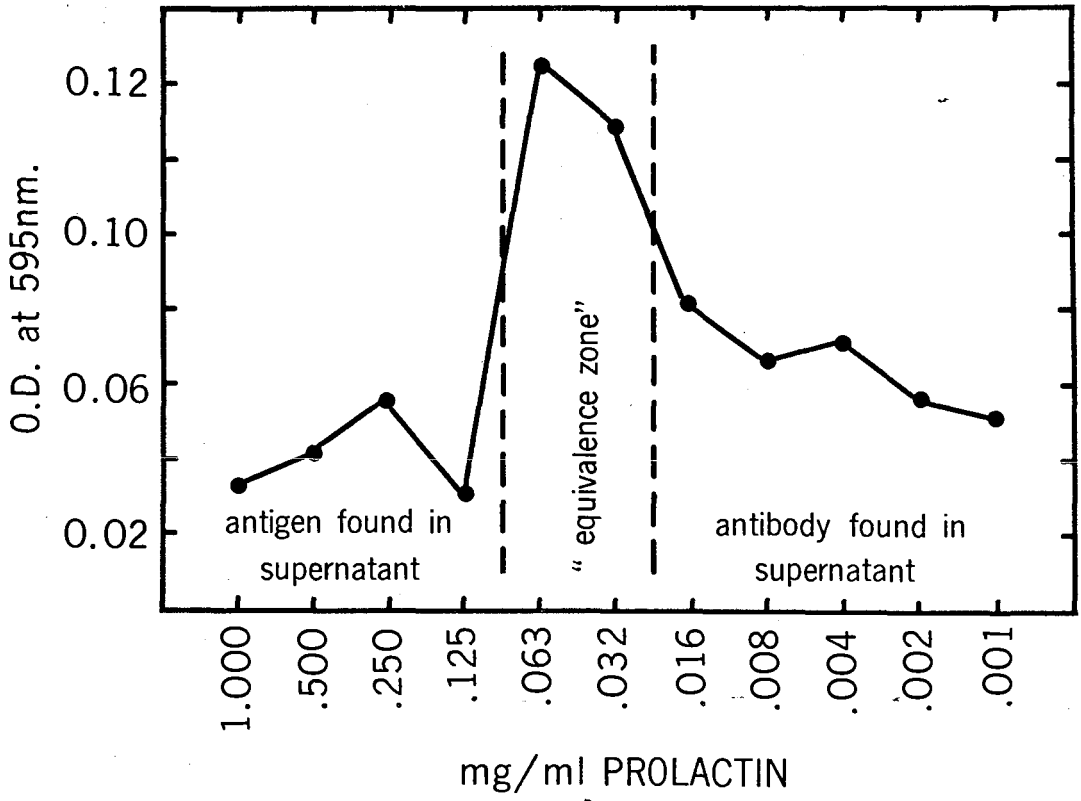




Fig. 5. Precipitin curve for ovine prolactin and its antiserum. In each tube 50  $\mu$ l of antiserum E was added to 50  $\mu$ l of a serial, twofold dilution of antigen.



body or both, which makes possible the union of these components in variable proportions. When antigen and antibody are available in a proper ratio, a three-dimensional lattice is formed which is insoluble. With other proportions of these components, complexes of a different geometrical configuration are formed which are soluble to varying degrees (see Carpenter, 1965).

The above data concerning maximum precipitation of prolactin were used to estimate the prolactin concentration in the sheep pituitary homogenate. The dilution of this homogenate at the equivalence zone was assumed to have the same prolactin concentration (0.0625 mg/ml) as that at the equivalence zone obtained with the purer prolactin-anti-prolactin system (see Fig. 5) since the same antibody solution was used. The results indicate that the prolactin content of the pituitary homogenate is approximately 0.125 mg/ml. This result will be further used in the isolation of prolactin as reported in Chapter 2.

## 2. THE IMMUNOCHEMICAL PURIFICATION OF BOVINE SERUM ALBUMIN AND OVINE PROLACTIN.

### Introduction

In general there exist two approaches to isolate an antigen immunochemically. The first, the so-called "precipitation technique", involves precipitation of the antigen by antiserum, uncoupling of the antigen-antibody complex, and the separation of the two components by electrophoresis, column chromatography or another appropriate method. The second technique is a modification of the first one and is known as "immunosorption". Here, the antibody is first made insoluble by either coupling it to an insoluble, inert matrix, or by polymerizing it. Both procedures have been successfully applied in the isolation of antigens and antibodies, but in recent studies, immunosorption is more commonly employed. This general preference for immunosorption is related to three disadvantages inherent to the precipitation technique. First, precipitation is a secondary reaction and does not in all cases follow the formation of the antigen-antibody complex. This then limits the use of the precipitation technique. Furthermore, satisfactory precipitation of this complex requires the antigen and

antiserum to be present in a proper ratio (see previous chapter). Finally, the separation of the soluble components after dissociation of the complex is often difficult, especially if they have similar characteristics. Immunosorption, on the other hand, is based solely on the primary reaction of antigen and antibody, and is therefore ratio-independent. Also, with this method the separation of the reactants after dissociation is facilitated by the fact that one of them is insoluble.

A useful immunosorbent must have the following properties (Silman and Katchalski, 1966): (1) it should specifically bind a given protein from a mixture; (2) it should possess a high capacity for binding that protein; (3) it should release the bound material quantitatively under conditions sufficiently mild to allow full retention of its specific biological activity; (4) it should be reusable. In most cases, immunosorbents are prepared by covalently linking either antibody or antigen to an insoluble carrier. Proteins can also be made insoluble by adsorption to inert carriers, inclusion inside the lattice of a gel, or by covalent cross-linkage (polymerization).

This subject is extensively reviewed by Silman and Katchalski (1966).

A problem common to both immunosorption techniques and procedures involving precipitation is that of dissociating the antigen-antibody complex. In most cases this is accomplished at a pH of 2 to 3. Frequently employed solvents are acetic acid (Robbins et al., 1967; Akanuma et al., 1970; Anderson et al., 1970) and glycine-HCl buffer (Avrameas and Ternynck, 1967a; Childlow et al., 1968; Emmart and Bates, 1968). In some cases effective dissociation requires more acidic solutions such as 0.5 M HCl (Webb and Lapresle, 1961) and even 1 M and 3 M HCl (Cuatrecasas, 1969).

Dissociation of antigen-antibody complexes at near neutral pH has recently been accomplished with thiocyanate, perchlorate, or iodide by Dandliker et al. (1967). It is felt by these authors that these "chaotropic ions" disrupt hydrophobic, ionic, and hydrogen bonds, probably involved in the linkage of antigens to antibodies. Avrameas and Ternynck (1967a; 1967b) showed that 3.5 M and 5 M iodide solutions are as effective as acid (pH 2.2) in removing antibody from its immunosorbent and that the iodide-eluted

antibody appeared to be more stable. In a similar study, Bata et al. (1964) successfully employed urea solutions to elute antibody.

In all cases, the dissociation of antigen-antibody complexes requires conditions which may cause denaturation or partial hydrolysis of the proteins. For this reason it is necessary to determine the effect of the chosen procedure on the biological activity of the reactants. When dealing with hormones, therefore, the final product must be bioassayed.

To date, very little has been published concerning the use of immunochemical procedures to purify hormones. Removal of contaminating proteins by immunological precipitation has been used to purify growth hormone (Li et al., 1962; Grumbach and Kaplan, 1962). Of particular interest is the study by Emmart and Bates (1968) who reported the immunochemical purification of ovine and piscine prolactin by precipitation of these proteins with anti-ovine prolactin serum. The antigen-antibody complex thus formed was dissociated with glycine-HCl buffer (pH 2.3) and the two components were separated by passage through a Sephadex G-100 column. The only known hormone that has been isolated immunochemically from its

natural source (blood plasma) is insulin (Akanuma et al., 1970). In this case, insulin antibody was coupled covalently to Sepharose after activating the Sepharose with cyanogen bromide (CNBr). The polysaccharide portion of the Sepharose is presumably acted upon by the cyanogen halide, forming imino carbonic esters and these in turn react with the primary amino groups of proteins (Porath et al., 1967).

Because of the similarity of my study, aimed at immunochemically isolating prolactin, to that of Emmart and Bates (1968), it was decided to first investigate the precipitation method used by these investigators. In view of the results, an immunosorption technique was employed in later stages of the work.

### Materials and Methods

Precipitation technique. One mg of ovine prolactin (NIH-P-S9, 30 IU/mg) was dissolved in 1 ml of 0.01 M phosphate-buffered saline (pH 7.4) and added to 15 ml anti-ovine prolactin serum. These amounts were derived using the microprecipitation test (see Chapter 1). After 12 hours (overnight) the precipitate was centrifuged, washed twice with 0.01 M phosphate-buffered saline (pH 7.4) and finally dissolved in 4 ml of 0.2 M



glycine-HCl buffer (pH 2.3). This solution was then passed through a Sephadex column (1.3 x 90 cm) that had previously been filled to 60 cm with Sephadex G-100 and equilibrated with the glycine-HCl buffer. The chromatographic separation was carried out at 9°C and the eluate was monitored with a U.V. analyser (Uvicord). Initially 200 fractions (2.8 ml each) were collected. Since however, absorption was recorded only in the first 40 aliquots, only those were subsequently analysed using a Perkin-Elmer spectrophotometer (model no. 139). The absorbing fractions were then neutralized with 0.5 M NaOH and tested for the presence of antigen or antibody in agar diffusion tests.

Immunosorption technique. The technique used was a modification of the method of Akanuma et al. (1970). The globulin fraction of the antiserum was precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , washed, dissolved in and dialyzed against saline for 24 hours (Campbell et al., 1964). The protein concentration of this preparation was then determined using the Biuret test (Campbell et al., 1964). Subsequently, the  $\gamma$ -globulin was coupled to Sepharose (Sepharose 2B, Pharmacia). The Sepharose was washed with distilled water and then activated with CNBr (1 ml of a 25 mg/ml CNBr solution per 2 g wet

weight Sepharose) for 6 min. The reaction mixture was kept at pH 11-12 using 2 M NaOH. The activated Sepharose thus formed was washed by filtration with ice-chilled distilled water and 0.1 M NaHCO<sub>3</sub>. It was then mixed with the globulin (1.4 g wet weight Sepharose/10 mg protein), and a 0.14 M NaCl-0.1 M NaHCO<sub>3</sub> solution was added (1 ml/10 mg protein). The gel was stirred gently in an ice bath for 20 hours and finally washed by filtration with saline and 0.01 M Tris-HCl buffer (pH 8.4) to remove unbound proteins.

The immunosorbent was poured into a column (0.9 x 15 or 0.9 x 60 cm) and equilibrated with 0.01 M Tris-HCl buffer (pH 8.4). On this column, the antigen was applied, and the column was washed with a volume of buffer equal to approximately twice the void volume of the column. The column was then eluted with an uncoupling agent (acetic acid, citric acid, HCl, or urea; see below). The eluate was monitored with a U.V. analyser and collected in 2.8 ml aliquots. All steps of this immunosorption procedure were carried out at 9°C. The protein concentration of each fraction was then determined using a Perkin-Elmer U.V. spectrophotometer. When acid uncoupling agents were used, the absorbing fractions were immediately

neutralized with NaOH. When urea was used to uncouple bound antigen, the urea-containing tubes were dialyzed for 24 hours in three 4-liter changes of saline at 4°C. When pure protein preparations were added to immunosorbent columns, the U.V. absorption of the eluates was taken as a measure of protein concentration. When the pituitary homogenates were applied, the Biuret test was used to estimate protein concentrations.

Some fractions were analysed by disk-gel electrophoresis. The procedure used was similar to that described by Steward et al., 1965. The gels were 7½% acrylamide and were run at pH 8.7. The major deviation from this technique was that the running gels were polymerized using ammonium persulfate and the samples were applied in a glycerol solution (Shapiro et al., 1967). The gels were stained overnight with Coomassie brilliant blue and de-stained with a methanol-acetic acid solution (Weber and Osborn, 1969).

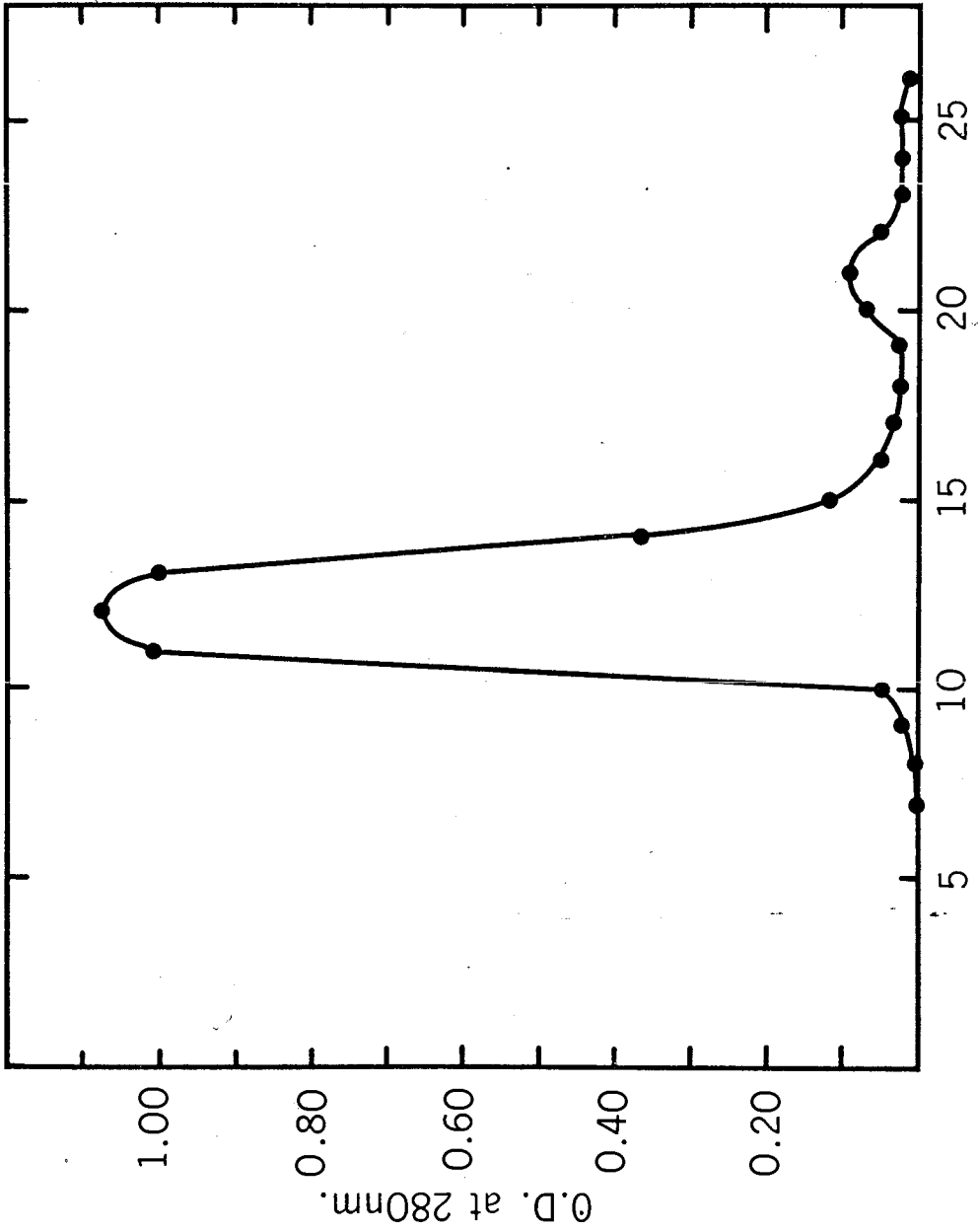
## Results and Discussion

Experiment 1. Separation of ovine prolactin using the precipitation technique. The results of the chromatographic

separation are presented in Fig. 6. Two peaks were obtained. The results of testing the neutralized aliquots from each peak for the presence of antigen and antibody are shown in Table I. The second peak contained immunologically active prolactin and the material with the highest optical density at 280 nm (tube 21) also produced the strongest precipitin band in the Ouchterlony test. The first peak, however, gave no precipitin reaction with either antigen or antibody. About 1 hour after neutralizing the tubes of this peak, a white flocculant precipitate resembling an antigen-antibody complex began to form. This could indicate that peak 1 contains antigen-antibody complex that has been dissolved but only partly dissociated by the glycine-HCl buffer, and that, when the pH is raised to 7.5, it becomes insoluble. To test this idea, the precipitate formed was washed, re-dissolved in glycine-HCl buffer, and again passed through the Sephadex G-100 column. As before, two peaks were obtained but the second peak was barely visible. It did, however, contain prolactin as was shown in agar diffusion tests. The material of the first peak again precipitated after neutralizing it.

The appearance of two peaks, the second one containing

Fig. 6. Sephadex G-100 gel filtration of the dissociation products of an ovine prolactin:anti-ovine prolactin precipitate, using glycine-HCl buffer (pH 2.3). Optical density was measured at 280 nm. Each tube contained 2.8 ml. The void volume of the column is 25 ml.



TUBE NO.

TABLE 1 - Gel diffusion tests for the presence of antigen and antibody in Sephadex G-100 gel filtrates.

Tube No. 1,	Reaction with antigen	Reaction with antibody
11	-	-
12	-	-
13	-	-
14	-	-
15	-	-
20	-	+
21	-	++
22	-	+
23	-	±

I refer to figure 6

symbols - (-) = no precipitation band; (±) = faint precipitation band; (+) = clear precipitation band; (++) = strong precipitation band.

prolactin, is similar to the results obtained by Emmart and Bates (1968). They, however, showed the first peak to consist of anti-prolactin and the question arises why in my study, the material in the first peak probably contained antigen-antibody complex rather than free antibody. It should be realised, however, that in Emmart and Bates' study excess antigen was used in the precipitation reaction whereas in my study, antigen and antibody were added in a ratio to produce optimum precipitation. Bata et al. (1964) suggested that when either antigen or antibody is present in excess, the precipitate differs from that formed at the equivalence zone and that in the first case dissociation of the complex takes place more readily.

The discrepancy between my results and those obtained by Emmart and Bates was not investigated further. Perhaps by employing different uncoupling agents this technique might have been more successful. It was decided however, to try an immunosorption procedure in view of the obvious advantages mentioned above.

Experiment 2. Separation of BSA with immunosorption using different eluting agents. In the first test 100 mg of anti-BSA  $\gamma$ -globulin was reacted with 14 g wet weight of



activated Sepharose. The gel was suspended in a 0.9 x 15 cm column and equilibrated with 0.01 M Tris-HCl buffer (pH 8.4). To this anti-BSA column was added 4 mg BSA in 1 ml of 0.01 M Tris-HCl buffer (pH 8.4) and the column was washed with approximately 30 ml of Tris-HCl buffer to remove the excess BSA. The column was then eluted with 1 M acetic acid with the objective of uncoupling the bound BSA. The results are shown in Fig. 7. It was estimated that approximately 25% of the BSA came through the column as excess (peak 1, tubes 1-9). However, of the 3 mg possibly bound to the column, only 0.9 mg was eluted with acetic acid (peak 2, tubes 11-18). It seems therefore that the uncoupling of BSA was incomplete and it was decided to try more acidic eluting agents in a subsequent test.

A new immunosorbent was prepared (112.5 mg anti-BSA-globulin with 15 g activated Sepharose). Again 4 mg BSA was added. The column was eluted successively in 3 stages: first with 0.5 M citric acid, then with 1 M citric acid, and finally with 1 M HCl. The bound BSA was obtained in 3 fractions (Fig. 8). Apparently, both citric acid solutions removed it incompletely, while the remainder was eluted by 1 M HCl. The total amount recovered was 3.8 mg or approximately 95%.

Fig. 7. Immunosorption of BSA by anti-BSA-Sepharose and subsequent elution with 1 M acetic acid. Four mg BSA in 1 ml of Tris-HCl buffer was applied. The first peak represents excess BSA not bound by the immunosorbent (estimated as 1 mg protein). The second peak contains the BSA eluted with acetic acid (estimated as 0.9 mg protein). The dotted line represents the pH of the eluate. Each tube contained 2.8 ml.

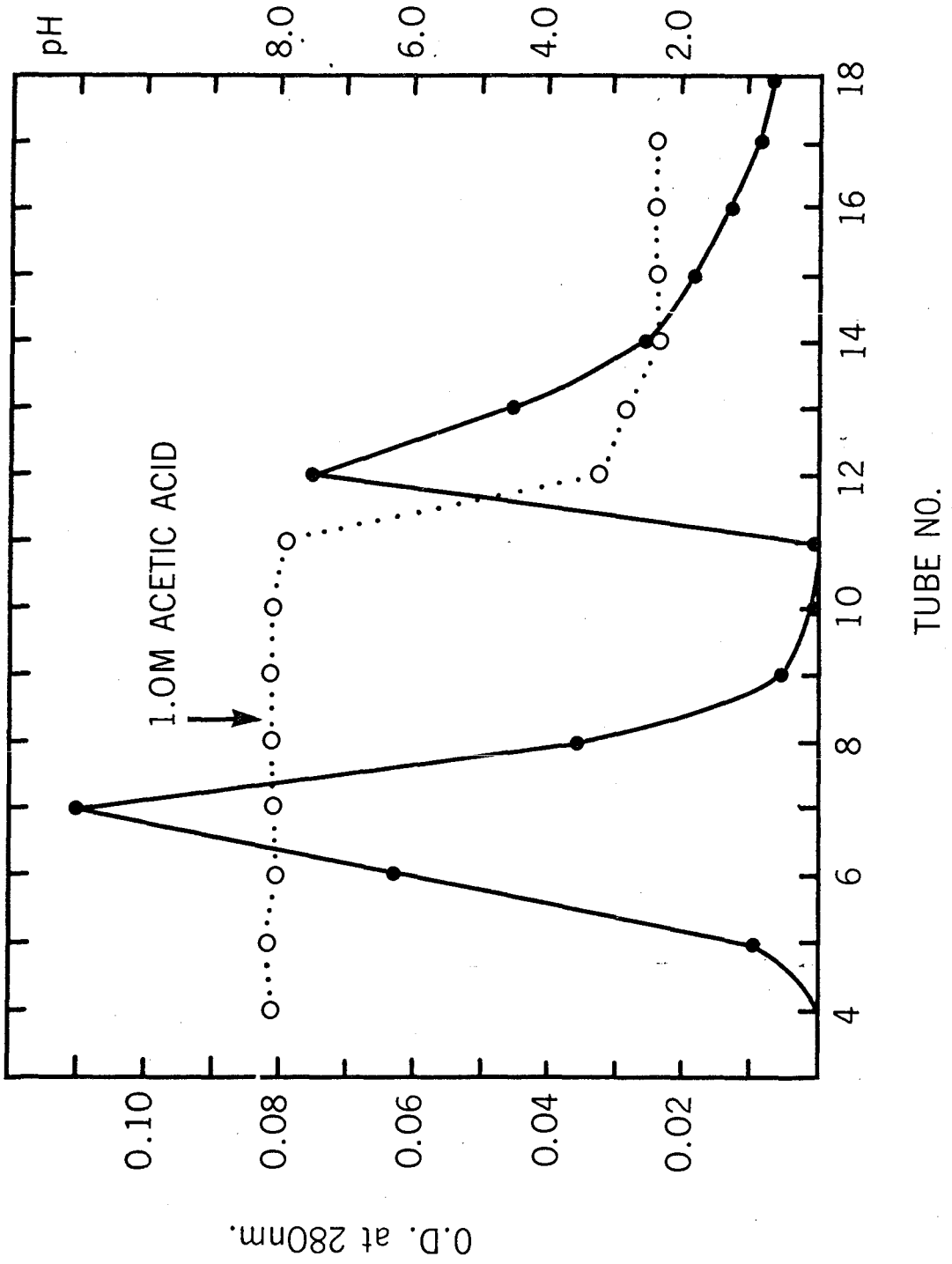
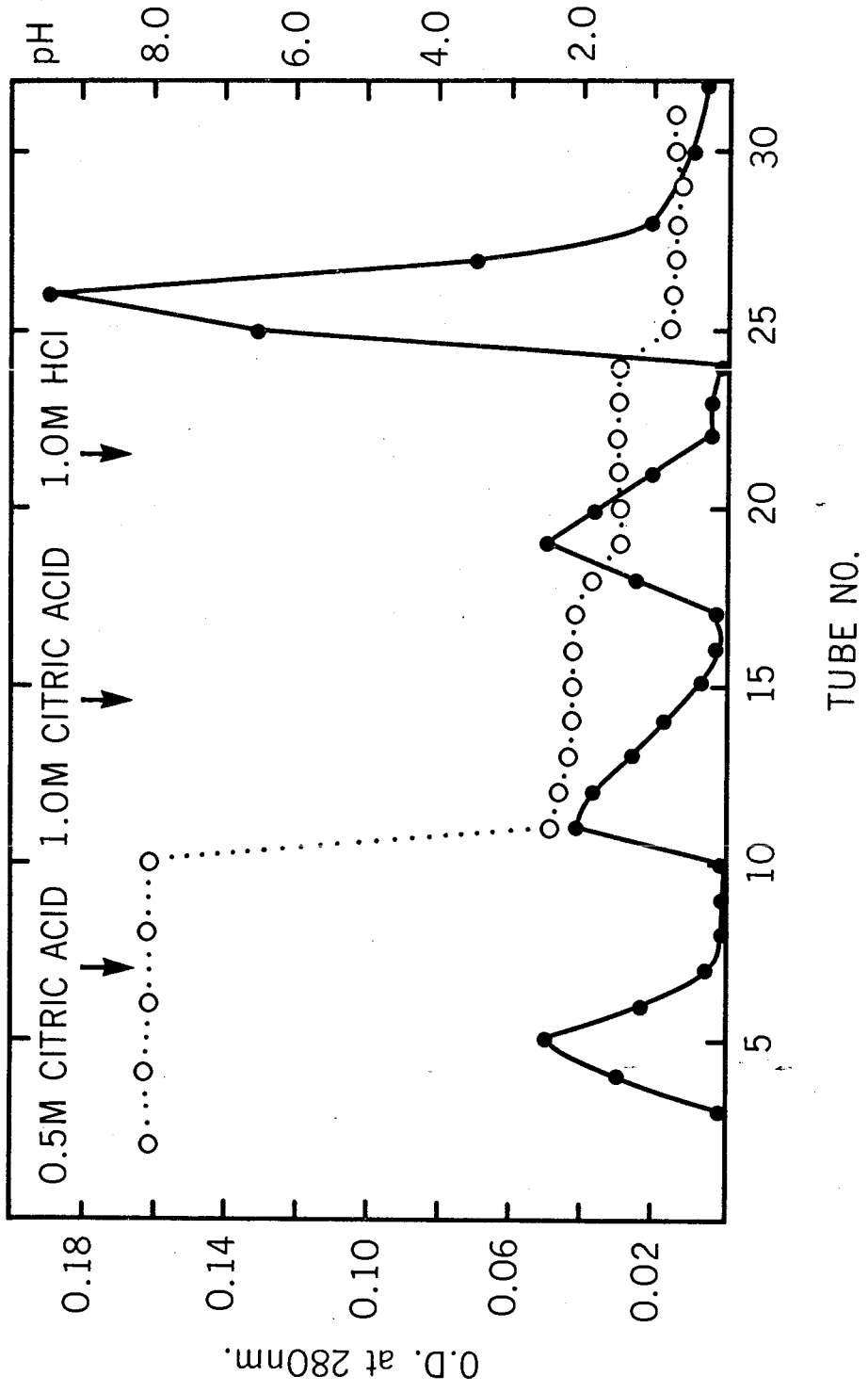


Fig. 8. Immunosorption of BSA by anti-BSA-Sepharose and subsequent stepwise elution with 0.5 M citric acid, 1.0 M citric acid, and 1.0 M HCl. Four mg of BSA in 1 ml of 0.01 M Tris-HCl buffer was applied to the column. The first peak represents excess BSA not bound by the immunosorbent and estimated as 0.5 mg protein. 0.5 M and 1.0 M citric acid eluted 0.6 and 0.7 mg protein respectively; the remainder, approximately 2 mg, was eluted by 1 M HCl. The dotted line represents the pH of the eluate. Each tube contained 2.8 ml.



Finally, this last test was repeated using a new immunosorbent (100 mg anti-BSA  $\gamma$ -globulin with 14 g activated Sepharose) but with 1 M HCl only as an eluting agent. The results were compared to those of a control test, using an immunosorbent prepared by reacting 100 mg of control rabbit  $\gamma$ -globulin with 14 g activated Sepharose. In the control experiment all of the BSA applied came through the column with the Tris-HCl buffer wash and none was bound (Fig. 9). Of the 4 mg of BSA that was applied to the anti-BSA column, 2.5 mg was bound and subsequently eluted with 1 M HCl (Fig. 9). The tubes from this HCl peak were neutralized and tested in agar diffusion tests. All gave a positive reaction with homologous antiserum. An aliquot of the tube with the highest optical density at 280 nm (tube 11) was tested in disk-gel electrophoresis. The electrophoretic pattern was identical to that of untreated BSA (Fig. 10).

Although the results of these agar diffusion and disk-gel electrophoresis tests suggest that the separated BSA was not affected, I felt that 1 M HCl could possibly hydrolyze proteins and might therefore not be a suitable uncoupling agent. Thus, I investigated other uncoupling agents which

Fig. 9. Immunosorption of BSA by anti-BSA-Sepharose and subsequent elution with 1 M HCl. Four mg BSA in 1 ml Tris-HCl buffer was applied to both the experimental and control column. Solid line represents excess BSA (first peak, approximately 1.3 mg protein) and HCl- eluted BSA (second peak, approximately 2.5 mg protein). The broken line represents a control test whereby BSA was applied to a column containing normal rabbit  $\gamma$ -globulin-Sepharose. The dotted line represents pH of the eluate. Each tube contained 2.8 ml.

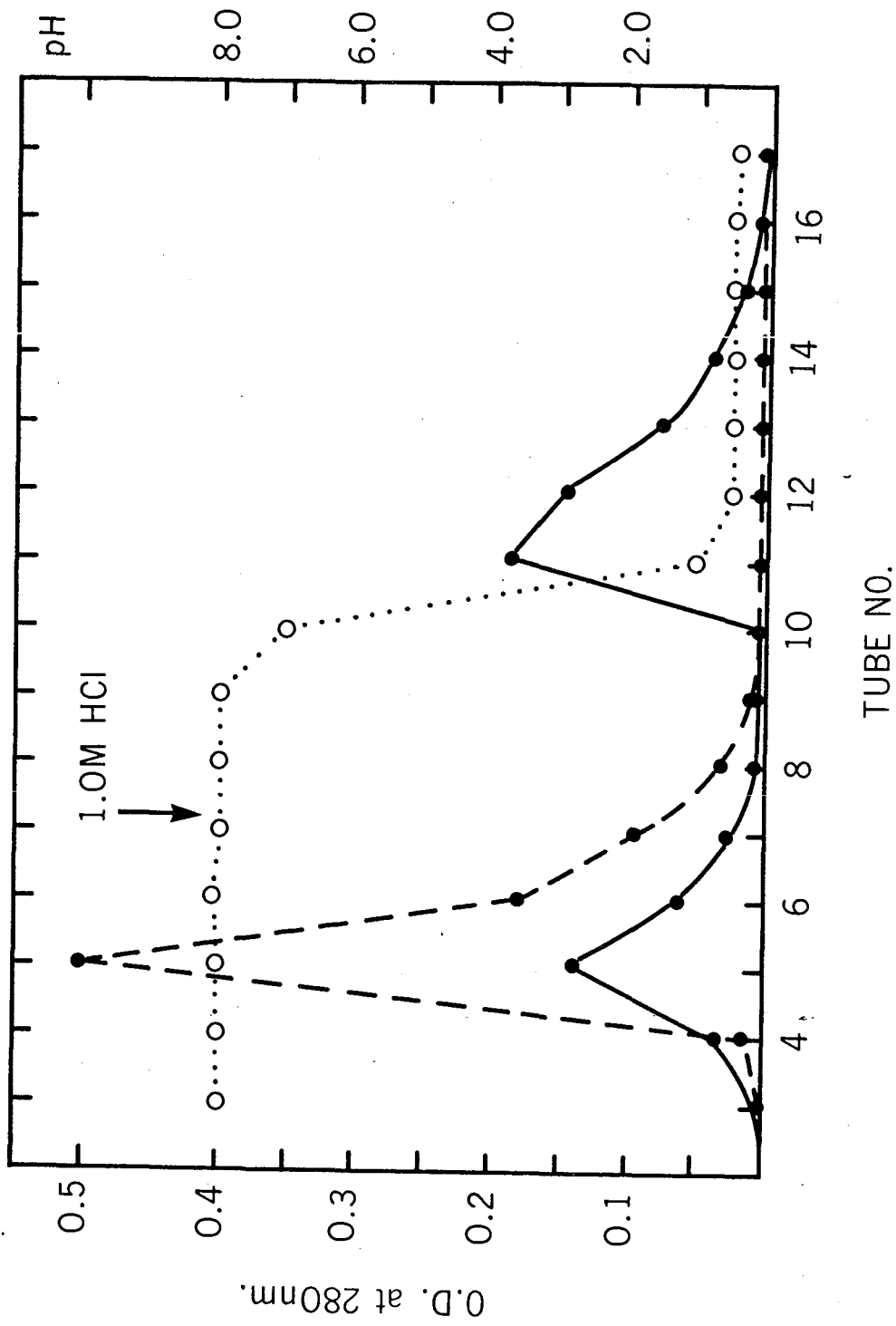
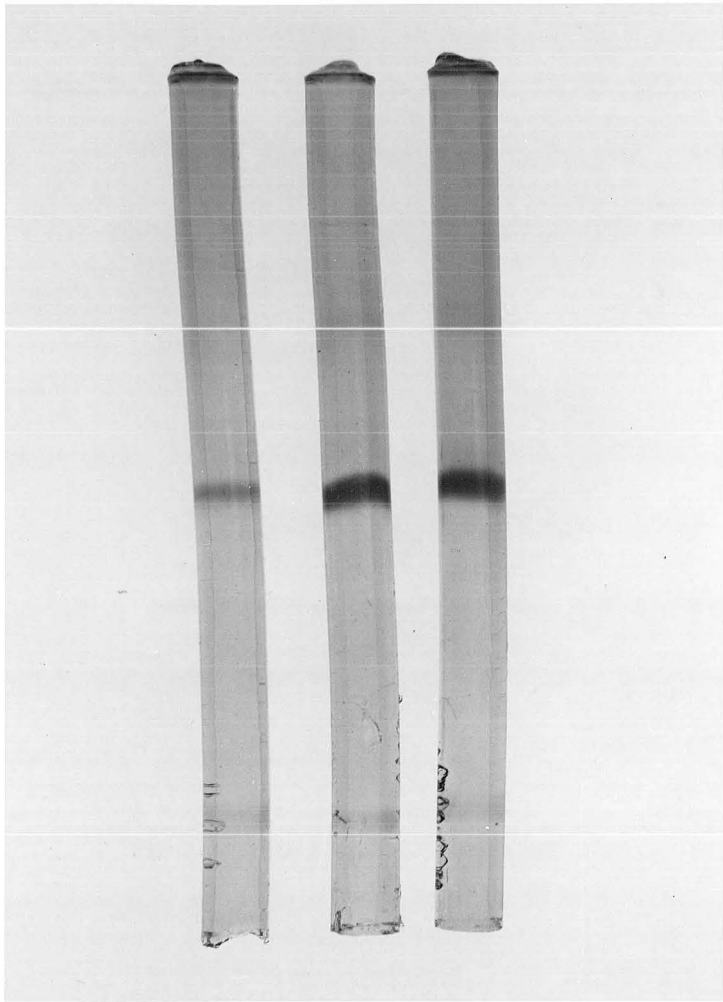




Fig. 10. Disk-gel electrophoresis of HCl- eluted (left),  
urea- eluted (center), and untreated (right) BSA.  
The gels were stained with Coomassie brilliant  
blue.

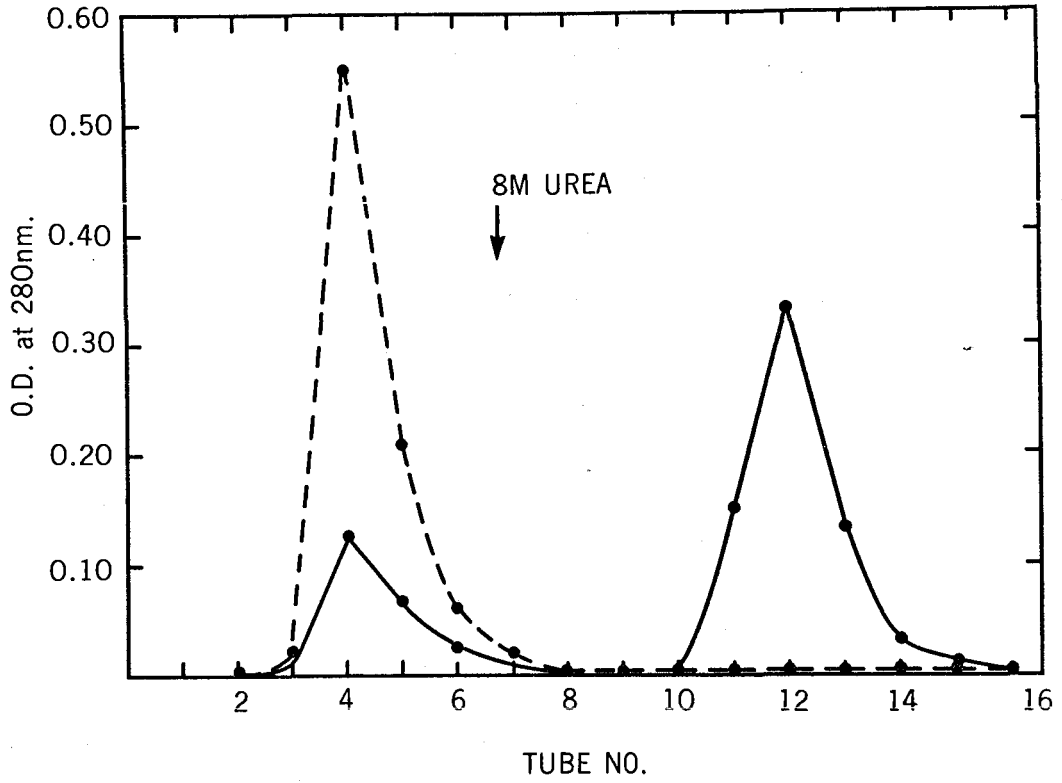


are presumably less drastic. "Chaotropic ions" have been successfully applied to elute antibody from its immunosorbent (Dandliker et al., 1967; Avrameas and Ternyck, 1967a, b). However, I found such ions, notably thiosulfate and iodide in concentrations of 2.0 M to 5.0 M, to dissolve the Sepharose. Another molecule related to these "chaotropic ions" in that it, too, disrupts the intramolecular and intermolecular non-covalent bonding of proteins, is urea (Schachman, 1963). Urea, usually in a concentration of 8 M, is commonly used to unfold proteins (see Mahler and Cordes, 1968). This process is moreover reversible as, upon removal of the urea, the native state of the protein is restored (Epstein et al., 1963; Mahler and Cordes, 1968).

To determine the efficiency of 8 M urea as an uncoupling agent, an anti-BSA column was prepared as before (100 mg anti-BSA  $\gamma$ -globulin with 14 g activated Sepharose) and 8 M urea was used to elute the bound antigen. The results were compared to a control test using an immunosorbent prepared by reacting 100 mg of control rabbit  $\gamma$ -globulin with 14 g activated Sepharose. It appeared that 8 M urea is just as effective as 1 M HCl in uncoupling the BSA from its immunosorbent (Fig. 11). In fact, the urea-eluted BSA

Fig. 11. Immunosorption of BSA by anti-BSA-Sepharose and subsequent elution with 8 M urea. Four mg BSA was applied to the control and the anti-BSA column. Solid line represents the excess BSA (first peak, approximately 1.2 mg protein) and urea- eluted BSA (second peak, approximately 2.8 mg protein). The broken line represents the control test whereby BSA was applied to a column containing normal rabbit  $\gamma$ -globulin-Sepharose. Each tube contained 2.8 ml.

-38b-



was obtained in a smaller volume than that of the HCl-eluted protein. After dialysis to remove the urea, the solutions comprising the urea peak were tested for antigen activity in agar diffusion and all tubes gave strong precipitin bands with BSA antiserum. An aliquot of the tube with the highest optical density at 280 nm (tube 12) was tested in disk-gel electrophoresis. The electrophoretic pattern was identical to that of untreated BSA (Fig. 10) which suggests that no denaturation had occurred during the procedure. The column could be re-used up to 10 times with no loss in binding capacity. This indicates that 8 M urea had no effect on the anti-BSA immunosorbent either.

Bata et al. (1964) also applied urea solutions to dissociate antigen-antibody complexes; they eluted anti-BSA from a BSA-cellulose immunosorbent. In this case, however, the highest concentration used was 6 M and this solution was found to be less effective than glycine-HCl buffer (pH 3.0) in removing immunologically bound antibody.

To test the specificity of the anti-BSA immunosorbent, 2 mg of ovine prolactin (Sigma) in 1 ml 0.01 M Tris-HCl buffer (pH 8.4) was applied to the column. A negligible

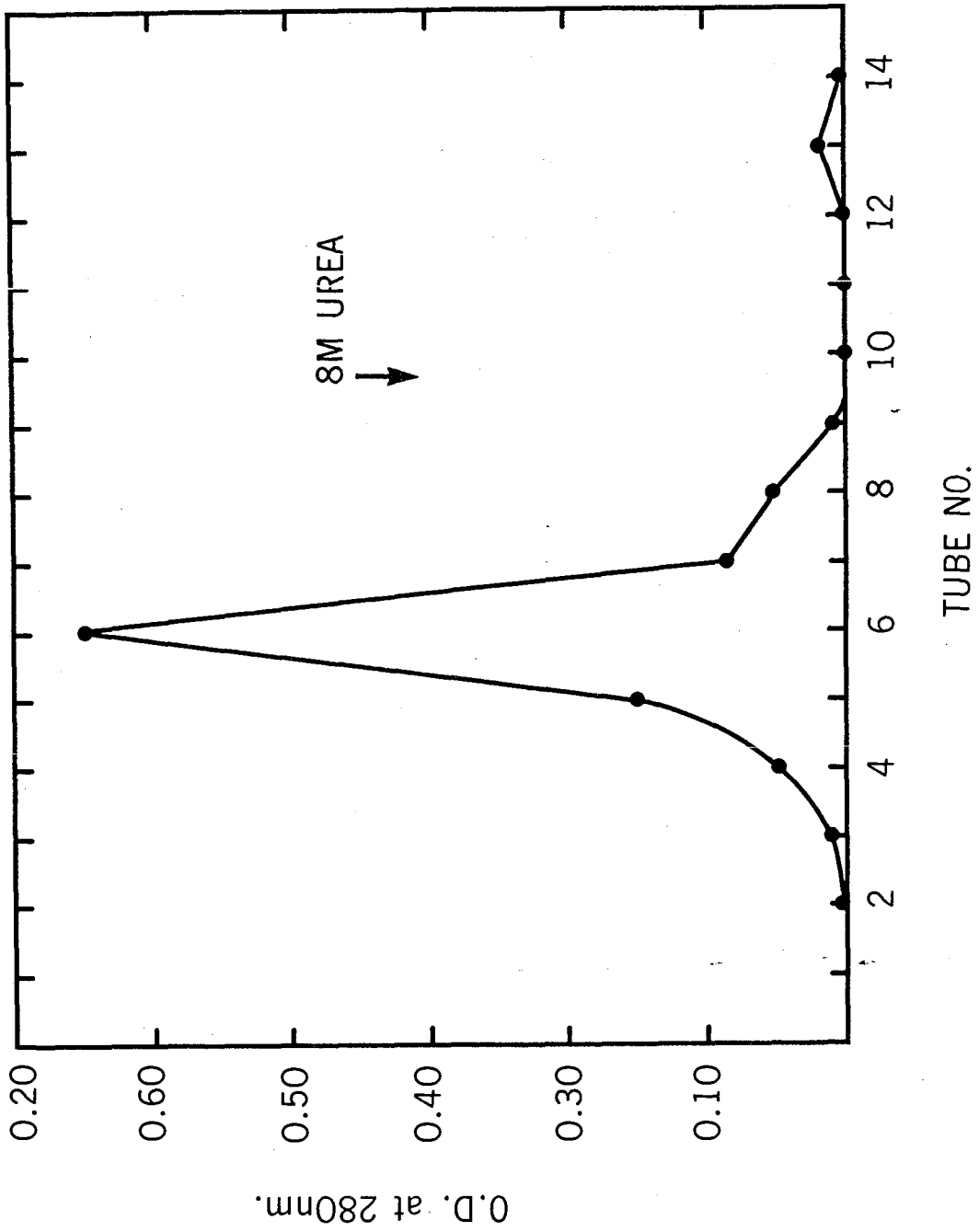
amount of the prolactin appeared to be bound (Fig. 12) and it seems therefore, that the binding of BSA in the previous tests was not the result of unspecific protein adsorption.

These last tests indicated that an immunosorbent prepared by covalently linking antibody to Sepharose has the properties required to isolate an antigen. The anti-BSA-Sepharose had a high, specific capacity to bind BSA. The bound antigen could be eluted under mild conditions and, on the basis of agar diffusion and electrophoresis experiments, the BSA did not appear to be affected by this process. Finally, the immunosorbent was re-usable. It was decided therefore, to apply this procedure to the purification of prolactin.

Experiment 3. Separation of prolactin with immunosorption. The antiserum used in this test was that produced with NIH-ovine prolactin as an antigen, and had a titer of 8000 (see Chapter 1). The anti-prolactin immunosorbent was prepared by reacting 300 mg of anti-ovine prolactin  $\gamma$ -globulin with 37 g activated Sepharose. The resulting gel was poured into a 0.9 x 90 cm column, and in the first test (Test 'a), 2 mg Sigma ovine prolactin in 1 ml of 0.01 M Tris-

Fig. 12. Specificity test of anti-BSA-Sepharose. Three mg ovine prolactin (Sigma) was applied to the column; 8 M urea was used as an uncoupling agent (compare with Fig. 10). Each tube contained 2.8 ml.





HCl buffer (pH 8.4) was applied. The bound antigen was uncoupled with 8 M urea. A control immunosorbent was prepared by reacting 290 mg normal rabbit  $\gamma$ -globulin with 37 g activated Sepharose. In the control test, a negligible amount of prolactin was bound whereas about 0.6 mg prolactin was eluted from the anti-prolactin column (Fig. 13). When twice as much prolactin was added to the same column (Test b), the amount of prolactin bound did not substantially increase (Fig. 14). This then indicates that the capacity of the anti-prolactin immunosorbent is approximately 0.6 mg. The column retained this capacity during 6 successive tests. The specificity of the anti-prolactin immunosorbent was tested by applying BSA to the column. No binding could be demonstrated.

Although the above data indicate the usefulness of an immunosorption technique for the isolation of prolactin, the yield was low when compared to the binding of BSA by its homologous immunosorbent. This relatively low capacity of the anti-prolactin immunosorbent is very likely to be ascribed to the low titer of the prolactin antiserum.

Finally, the same anti-prolactin column was used in an attempt to isolate prolactin from a crude sheep pituitary

Fig. 13. Immunosorption of ovine prolactin by anti-ovine prolactin-Sepharose and subsequent elution with 8 M urea. Two mg prolactin (Sigma) was applied to both the control and the anti-prolactin column. Solid line represents excess prolactin (first peak, approximately 1.1 mg protein) and urea-eluted prolactin (second peak, approximately 0.6 mg protein). The broken line represents a control test whereby BSA was applied to a column containing normal rabbit  $\gamma$ -globulin-Sepharose. Each tube contained 2.8 ml.

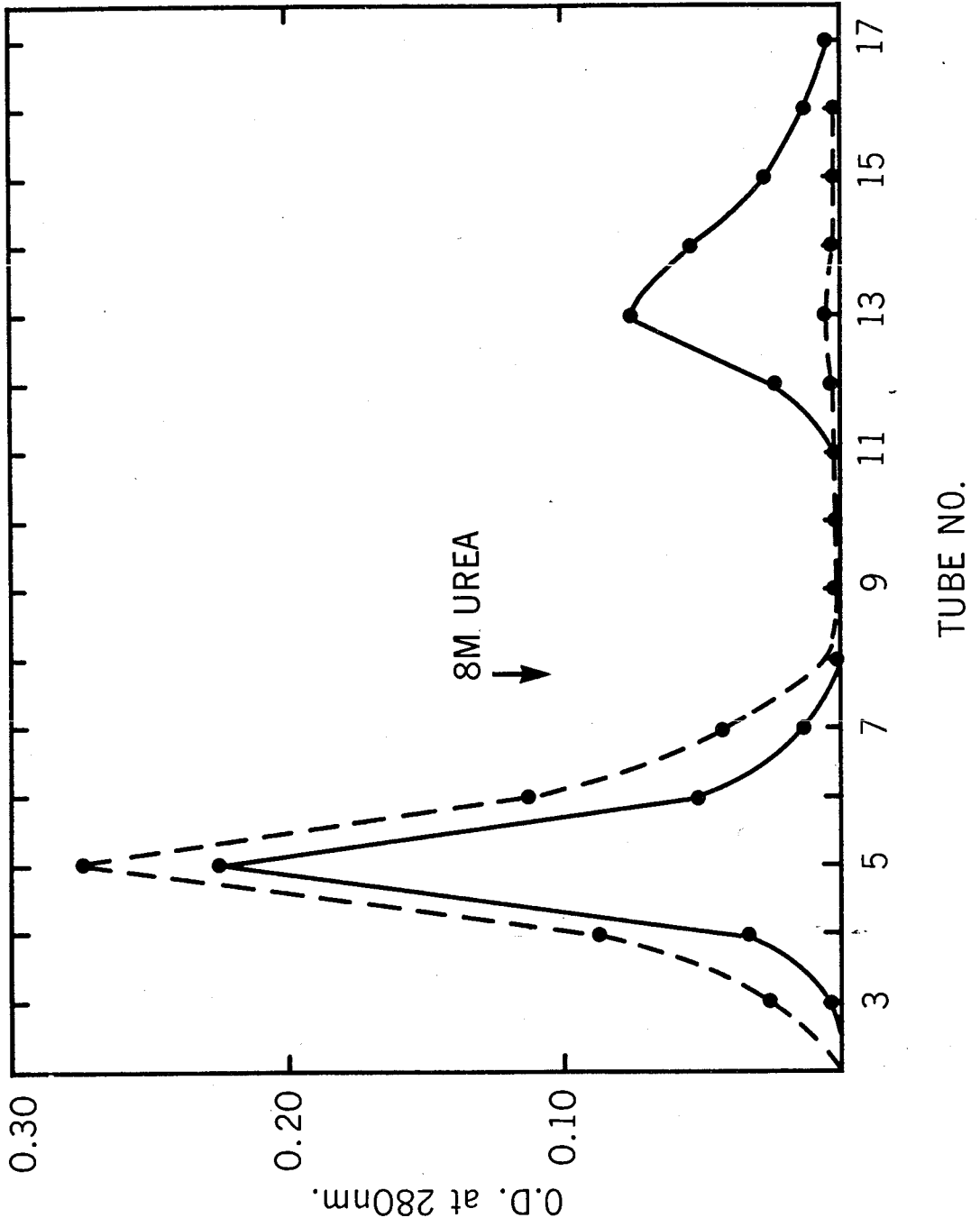
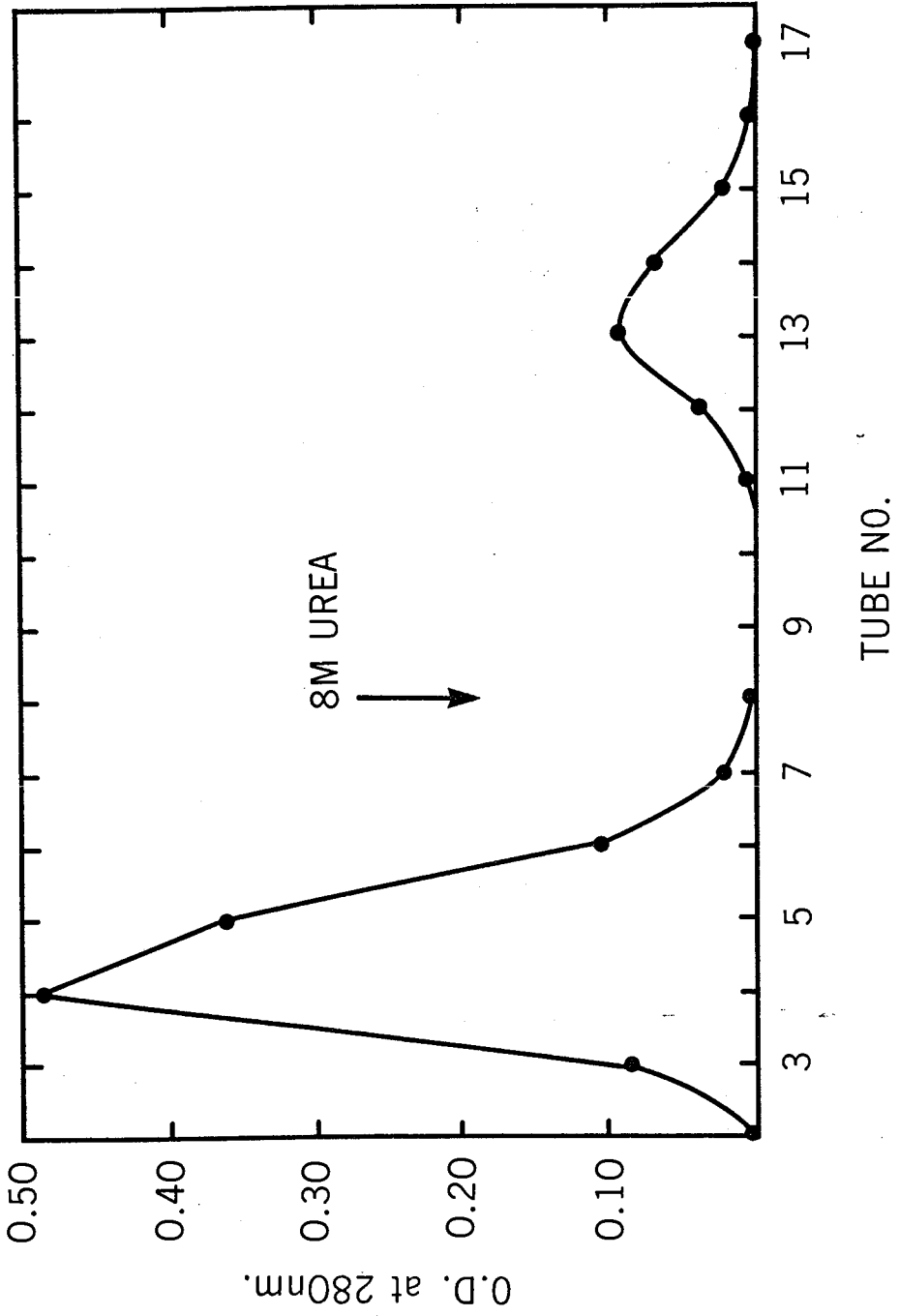


Fig. 14. Immunosorption of ovine prolactin by anti-ovine prolactin-Sepharose and subsequent elution with 8 M urea. Four mg prolactin (Sigma) was applied. The excess prolactin was estimated as 3.2 mg while that eluted by urea was estimated as 0.70 mg (compare with Fig. 13). Each tube contained 2.8 ml.



homogenate. In view of the estimated capacity of the column (0.6 mg) and since the microprecipitin curve (Chapter 1) showed that 10 ml of sheep pituitary homogenate contains approximately 1 mg prolactin, in this test (Test c) 10 ml of the homogenate was applied to the anti-prolactin column. This presumably represents excess antigen. Ten ml homogenate was also applied to the control column (Test d). The results of these 2 tests are illustrated in Figs. 15 and 16. A considerable amount of protein was bound and subsequently eluted from both the control and the anti-prolactin column. It seemed likely that these peaks are comprised of more than one protein. A slight red coloration of these eluates indicates the presence of hemoglobin as a contaminant. To further investigate the possibility that a mixture of proteins was bound by these columns, the fraction obtained from the anti-prolactin column, was analysed by column chromatography. Eleven ml of this fraction was concentrated by dialysis in a saturated solution of dextran to a volume of 2.5 ml. This solution was then applied to a Sephadex column (1.5 x 90 cm) that had previously been filled to 80 cm with Sephadex G-100 and equilibrated with 0.05 M phosphate buffer

Fig. 15. Immunosorption of prolactin from a sheep pituitary homogenate by anti-ovine prolactin-Sepharose. Ten ml of homogenate was applied. The first peak represents extraneous protein not bound by the column. The second peak represents the protein eluted with urea and estimated to contain approximately 6.2 mg protein. Each tube contained 2.8 ml.



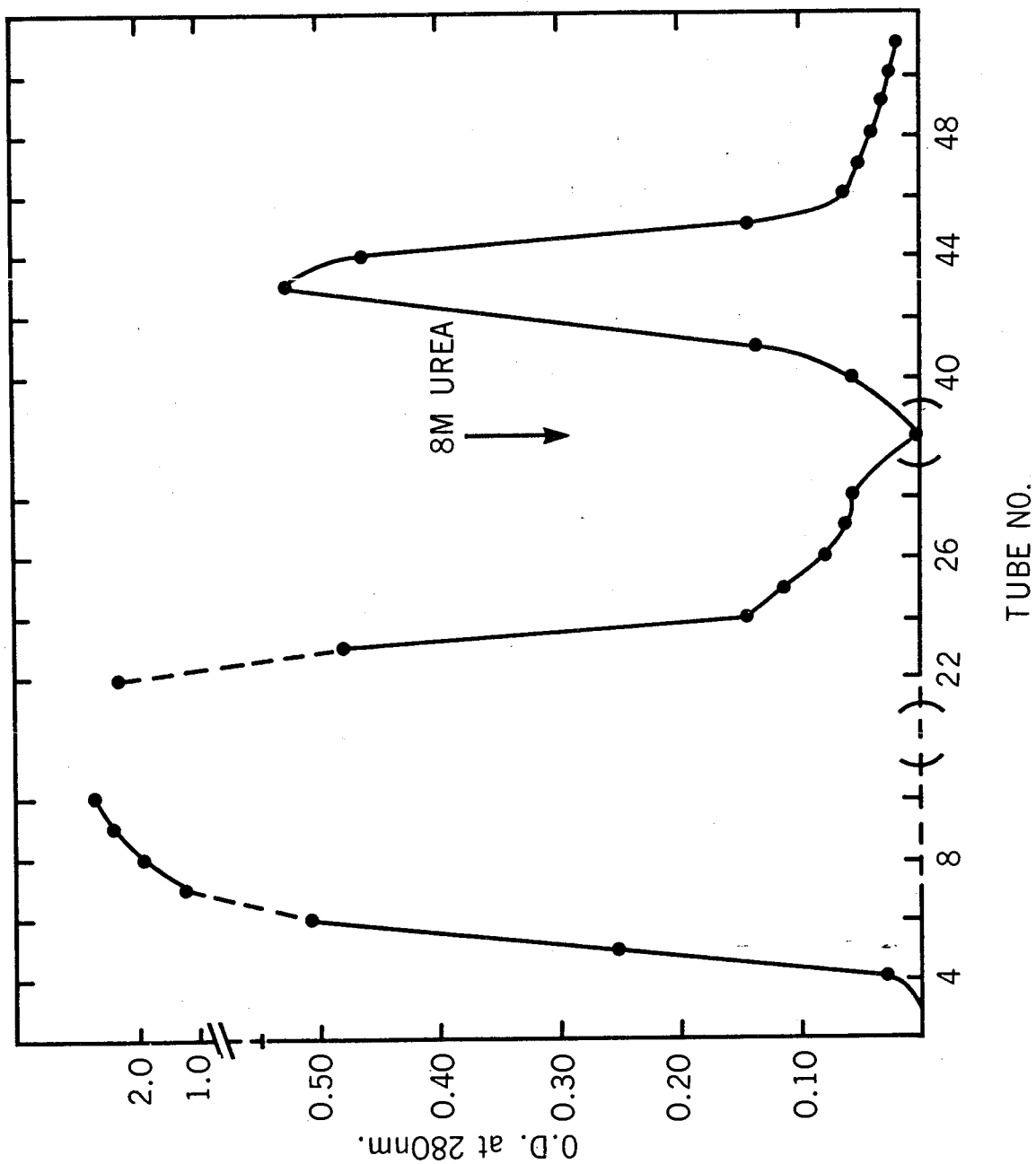
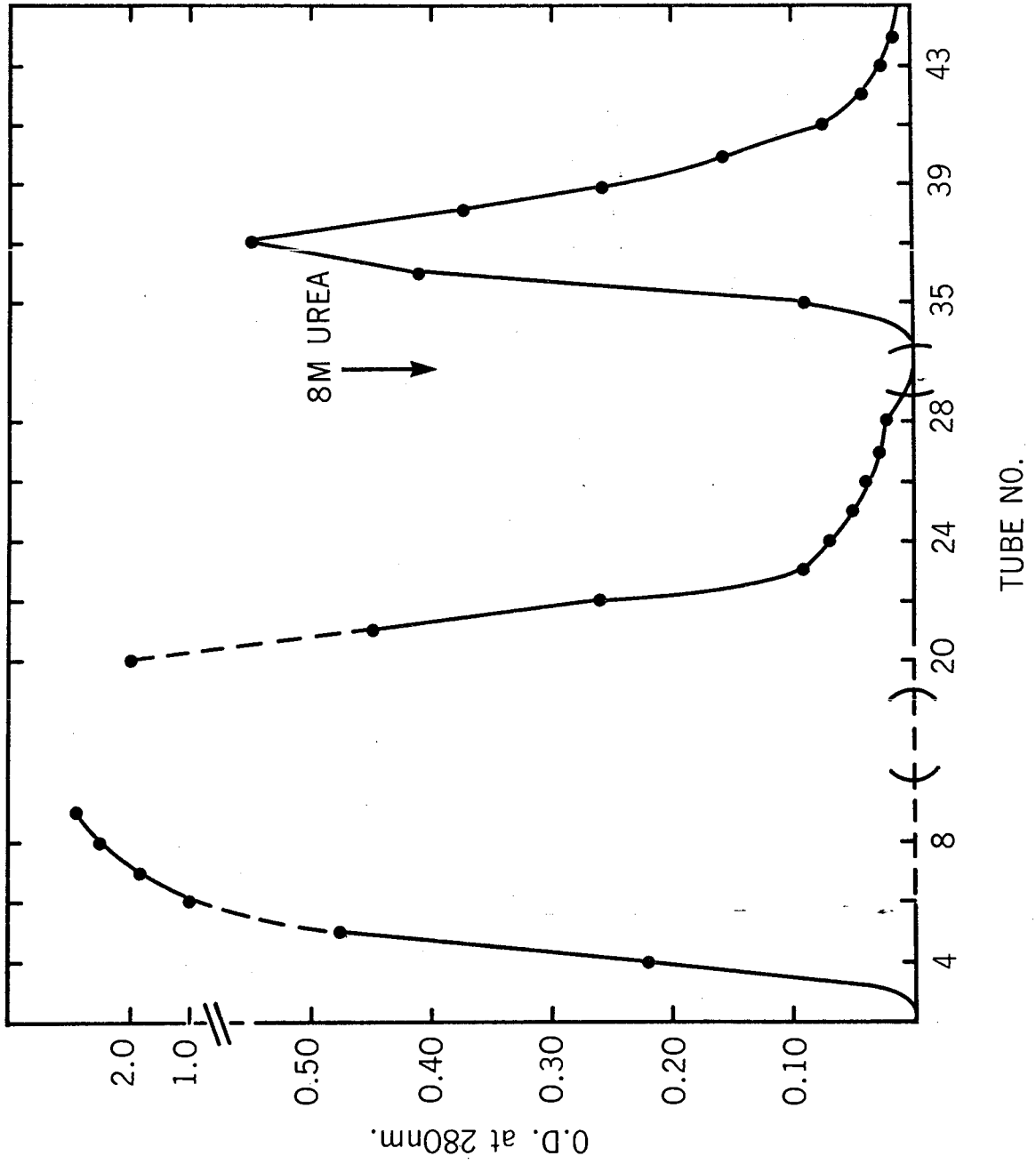
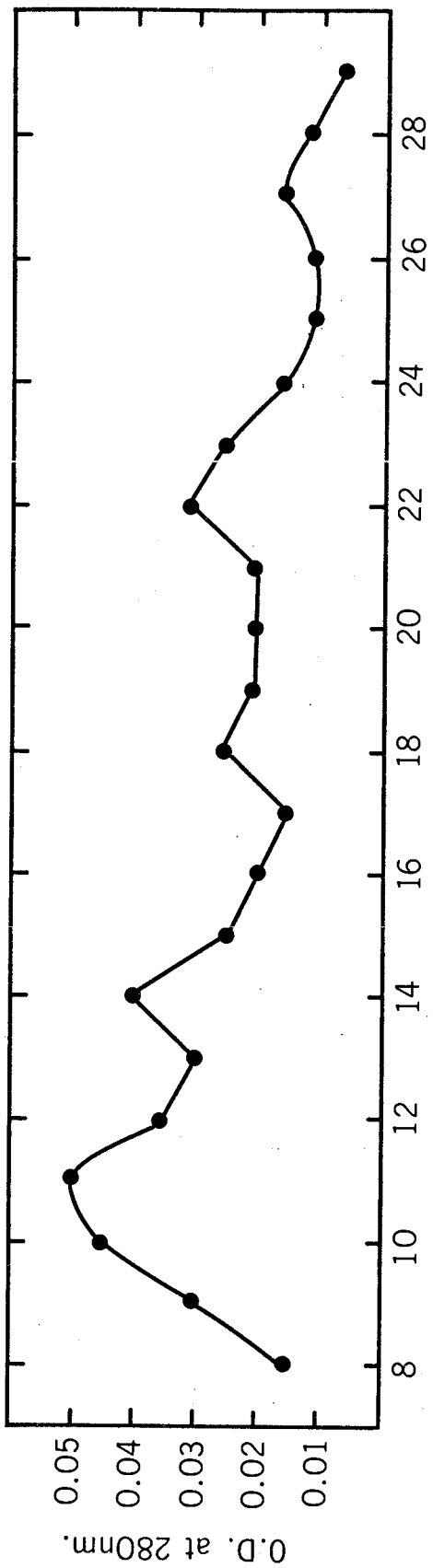


Fig. 16. Control test for the immunosorption of prolactin from a sheep pituitary homogenate. The immunosorbent was prepared with control rabbit  $\gamma$ -globulin. Ten ml homogenate was applied. Each tube contained 2.8 ml (compare with Fig. 15).



(pH 7.5). The eluate was collected in 2.8 ml fractions and the optical density at 280 nm of each tube was measured. At least 5 components were separated by this procedure, which further indicates that the anti-prolactin column had bound a mixture of proteins (Fig. 17). This observation is discussed further in Chapter 3.

Fig. 17. Separation by Sephadex G-100 filtration of proteins present in an urea-eluted fraction from the anti-prolactin column (see Fig. 15); 11 ml of this total fraction was concentrated by dextran to 2.5 ml before applying it to the Sephadex column (2.5 x 90 cm). Each tube contained 2.8 ml.



TUBE NO.

O.D. at 280nm.

### 3. BIOASSAY AND RADIOIMMUNOASSAY OF PROLACTIN OBTAINED BY IMMUNOSORPTION.

#### Introduction

In the previous chapter, the amount of protein in the various fractions was estimated by U.V. absorption or by the Biuret test. These methods, however, lack both the specificity and the sensitivity required for measuring the amounts of prolactin. Therefore, it was decided to use a radioimmunoassay to quantify this hormone. This method seemed most suitable since it may be considered very unlikely that the isolation procedure employed would have interfered with the capacity of the prolactin to be bound by its antibody. The results of the radioimmunoassay were also compared with those of a bioassay to determine whether immunosorption and subsequent elution has any effect on the specific hormonal activity of prolactin.

Radioimmunoassays have been successfully applied in the quantitative analysis of a number of hormones. Their main advantages, as compared with bioassays, are greater sensitivity and relative simplicity. The basic principle of the radioimmunoassay involves competition between radioactive isotope-

labeled and unlabeled antigen for the antibody. The amount of labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled antigen. Specific radioimmunoassay techniques have been developed to measure prolactin in the following mammalian species: rat (Kwa and Verhofstad, 1967a; Niswender et al., 1969); mouse (Kwa and Verhofstad, 1967b); sheep (Arai and Lee, 1967; Brayant and Greenwood, 1968; McNeilly, 1971); cow (Johke, 1969; Raud and Odell, 1971) and man (placental lactogen) (Leake and Burt, 1969).

In general, the various radioimmunoassays available differ primarily in the means by which labeled antibody-bound antigen is separated from labeled unbound antigen. The method used in my study is the "solid phase technique" of Catt and Tregear (1967). This procedure involves coating plastic tubes with antibody. The ability of these antibody-coated tubes to specifically bind antigen forms the basis of the assay.

Several bioassays are available for mammalian prolactin. They stem from this hormone's trophic actions on the mammary gland, the crop sac of pigeons, and the corpus luteum of



rats and mice (for a review, see Nicoll, 1967; Bern and Nicoll, 1968). Pigeon crop assays are most widely employed, whereby prolactin is administered either systemically by subcutaneous or intramuscular injection, or locally by intradermal injection over one or both sides of the crop (Nicoll, 1967). The crop sac response in the systemic method is quantified by weighing the lateral lobes of the organ (Riddle et al., 1933). In the local method of Lyons (1937) the response is determined by measuring the affected area of the crop-sac. Although this local technique is much more sensitive than the systemic method, it has the disadvantage of being subjective. Nicoll (1967) introduced a modification of the local method which made this technique more objective and improved considerably on its sensitivity. In view of the presumably small quantities of prolactin in my samples, it was decided to use this last bioassay.

### Materials and Methods

Radioiodination of prolactin. The procedure followed was similar to that used by Greenwood et al. (1963) for the iodination of growth hormone. To 1 mCi of a Na<sup>125</sup>I solution

(Amersham), was added 12.5  $\mu$ l of 0.5 M phosphate buffer (pH 7.5) containing 2.5  $\mu$ g ovine prolactin (NIH-P-S9, 30 IU/mg) and 25  $\mu$ g chloramine-T dissolved in 25  $\mu$ l 0.05 M phosphate buffer (pH 7.5). The chloramine-T was used to oxidize the iodide to iodine which could then react with the tyrosine residues of the prolactin. After 20 seconds, 50  $\mu$ l of sodium metabisulfite in 50  $\mu$ l of 0.05 M phosphate buffer (pH 7.5) was added to convert unreacted iodine back to iodide. This reaction mixture was then diluted with 300  $\mu$ l of a carrying solution of potassium iodide (1 mg/ml) in 0.05 M phosphate buffer (pH 7.5).

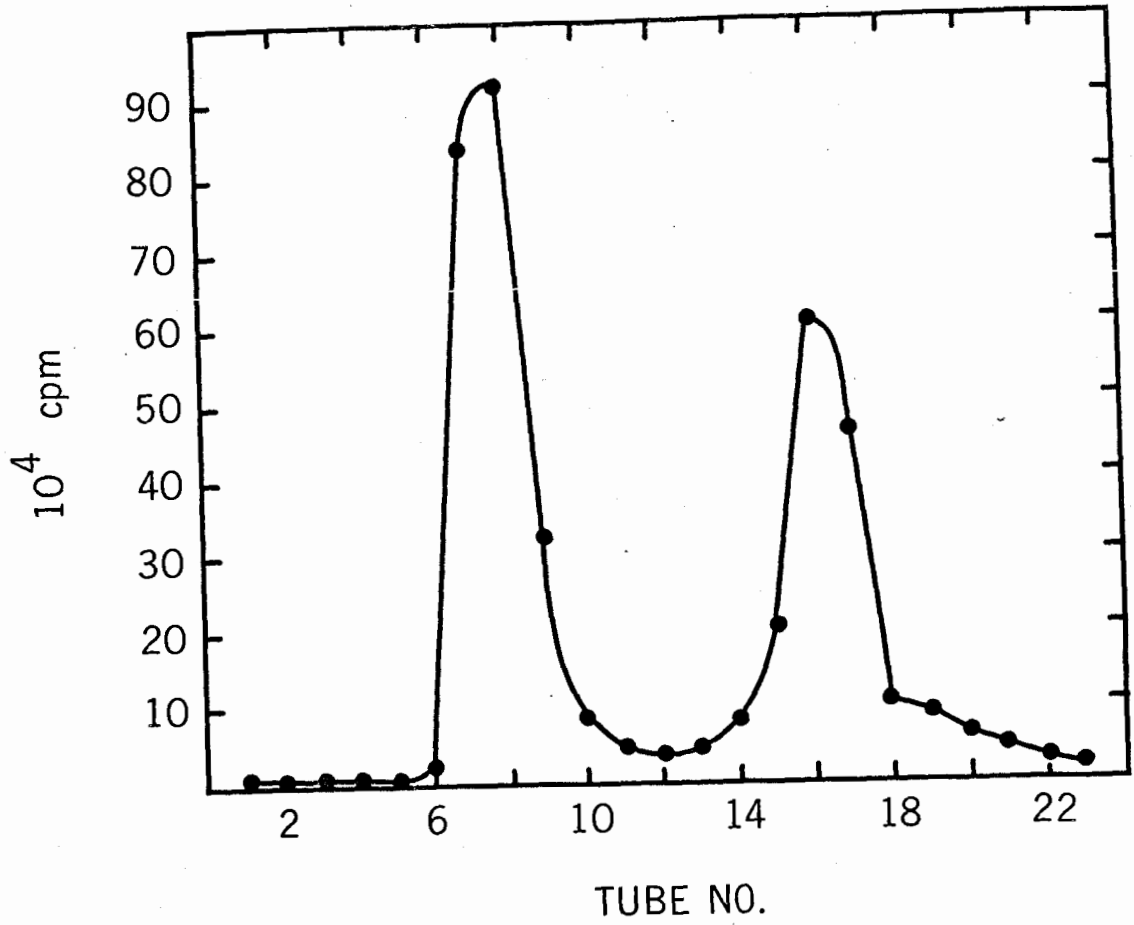
The reaction mixture was then transferred to a column (0.9 x 30 cm) of Sephadex G-50 to separate labeled prolactin from the unreacted iodide. The column was equilibrated with 0.07 M barbitone buffer (pH 8.6) and prior to use 20 mg of human serum albumin in 1 ml barbitone buffer was passed through it. This protein becomes partially adsorbed to the column and thereby prevents adsorption of the labeled hormone. The column was eluted with the barbitone buffer and each 1.0 ml-fraction was collected in a solution of 1.0 ml of human serum albumin (50 mg/ml) in barbitone buffer. Ten  $\mu$ l of each

fraction was transferred to a 12 x 75 mm test tube and its radioactivity counted using a 3 in. x 3 in. NaI (Tl) well crystal connected to a single channel analyzer (Hewlett Packard Co.). Those fractions which contained most of the labeled hormone (tubes 7 and 8, see Fig. 18) were pooled and diluted with a solution of human serum albumin (0.5 mg/ml) in barbitone buffer to a dilution of 300,000 counts/ml.

Radioimmunoassay. The procedure followed for the solid-phase method was similar to that of Catt and Tregear (1967). For this type of assay 12 x 75 mm disposable polystyrene test tubes (Lab-Tek) were coated with 1.0 ml of an antiserum preparation diluted 1:50 with 0.025 M carbonate-bicarbonate buffer (pH 9.0). Adsorption of the antibody to the test tube continued for 2 hr at room temperature. To remove the unadsorbed antibodies, the coating solution was poured out and the tubes were washed three times with saline. Finally, the tubes were washed once with 5% (v/v) normal rabbit serum to block the remainder of the test tube surface. The tubes thus coated were used immediately or stored frozen for future assays.

To establish a standard curve, ten 0.6 ml samples con-

Fig. 18. Separation of  $^{125}\text{I}$ -labeled ovine prolactin from  $^{125}\text{I}$  iodide on a Sephadex G-50 column. Ten ml from each tube was counted. Background count was approximately 1200 cpm.

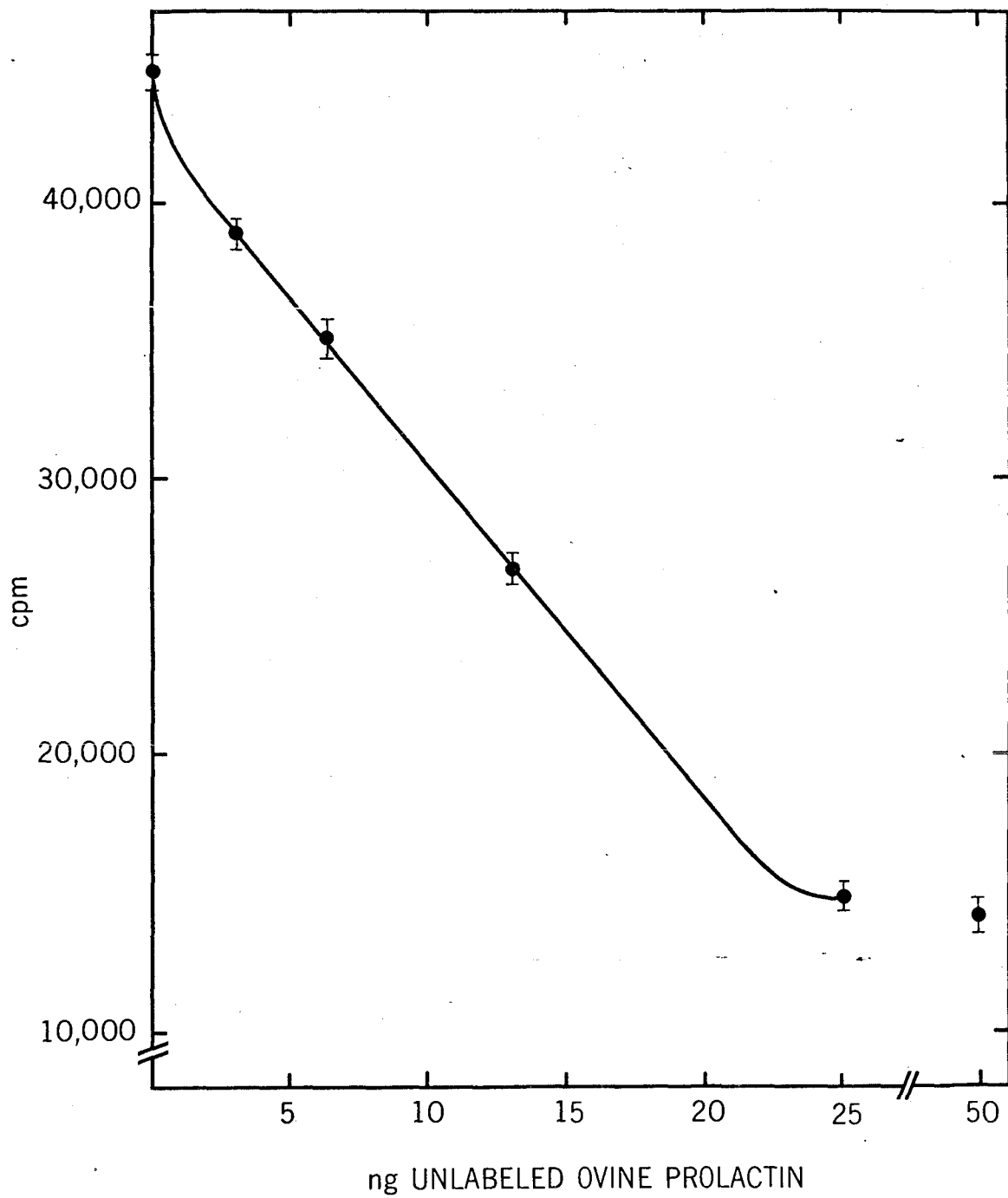


taining 0 to 200 ng ovine prolactin (NIH-p-S9, 30 IU/mg) were added in triplicate to the antibody-coated tubes. Labeled prolactin was then added (150,000 cpm in 0.5 ml) and the tubes were incubated for 16 hr at room temp. After pouring out the reaction mixture and washing twice with tap water, the tubes were placed in a protective capsule and counted in the gamma well detector for 1 min. The standard curve is shown in Fig. 19. The sensitivity range for the procedure was approximately 2-25 ng ovine prolactin which is comparable to that found by Arai and Lee (1967) and McNeilly (1971). The fractions to be analysed were assayed at three different dilutions to ensure that the prolactin concentration would be within the sensitivity range.

Pigeon-crop assay. The procedure was largely the same as that described by Nicoll (1967). Six to eight-week-old White-King pigeons were obtained from a local dealer and were plucked in the crop region. For each dosage, 3 birds were used. Each bird was injected intradermally over both sides of the crop sac. The injection sites were marked to ensure that during subsequent injections and dissection, the locations could be identified. The injection volume was 0.1 ml and this was administered in the morning (9-10 AM) and

Fig. 19. Standard curve for ovine prolactin (NIH-P-S9; 30 IU/mg) obtained by a solid-phase radioimmunoassay. The data are presented as the mean of three determinations  $\pm$  the standard deviation.

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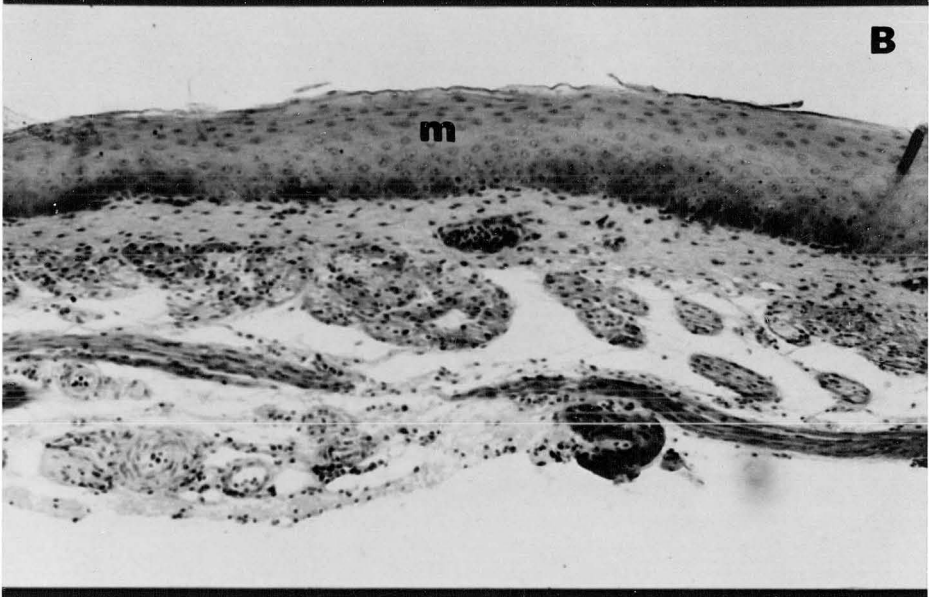
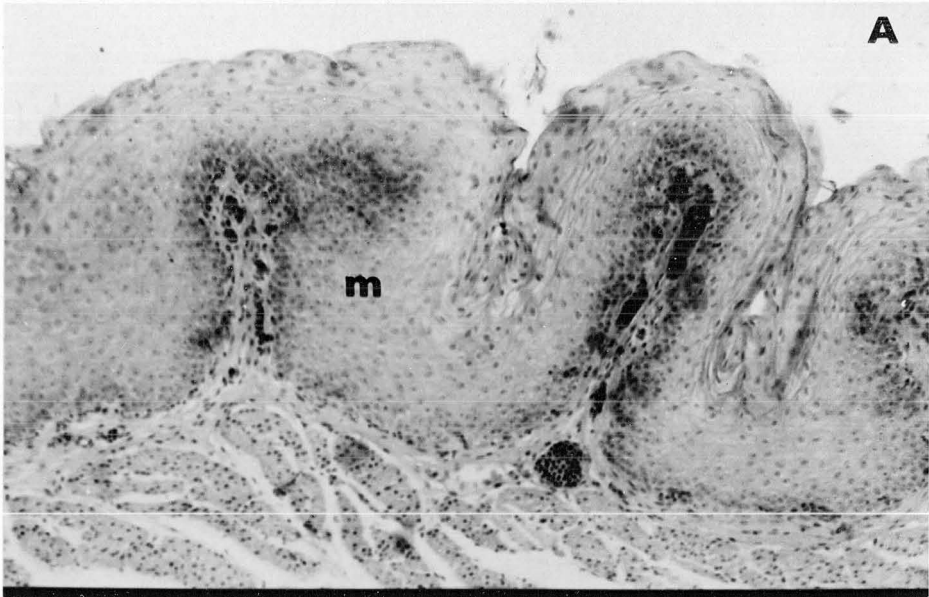


evening (4-5 PM) for 2 consecutive days. On the third day (18-24 hours after the last injection), the birds were killed by chloroform. Control birds were injected with 0.15 M NaCl. Prolactin was always dissolved in 0.15 M NaCl and the same solvent was used to dilute the fractions to be analysed.

After the birds were killed, the entire crop-sac was removed and halved by a median incision. Each hemicrop was mounted on the holding apparatus (Nicoll, 1967), in such a way that the marked injection site was central on the metal screen, the mucosal tissue facing upward. Suction was then applied to the apparatus and the mucosal epithelium, which lies within the perimeter of the metal disk was scraped from the underlying submucosal tissue. The holding apparatus used in this work was identical to that used by Nicoll except a stainless steel filter support (Millipore) was used as the top of the cylinder rather than scintered stainless steel. Fig. 20 shows a cross sectional view of the hemicrop tissue in an area near the injection site of a control bird, a control bird after scraping, and a prolactin-injected bird.

A variable in this method concerns the ultimate degree

Fig. 20. Comparable transversal sections of the pigeon crop sac in areas near the injection sites. A: bird injected with urea-eluted Sigma prolactin; B: Saline-injected control bird. Note the strong hypertrophy of the mucosa (m) in A. C: submucosal layer of the crop sac in control bird after mucosa has been scraped off. Note that due to considerable stretch of this tissue on the holding apparatus, this submucosal layer appears much thinner than in B. All tissues were fixed in Bouin's fixative; sections were stained with hematoxylin-eosin. x 300.



of stretch of the mounted tissue and, consequently, the actual surface area of the mucosa. For this reason, a modification was introduced. Not only was the mucosal epithelium collected and weighed, but also the submucosal layers of the same area. The variability introduced by slight differences in the size of the area analysed should be eliminated if the ratio between these two weights is used as the index of response. All samples of tissue were collected on pre-weighed aluminum pans, dried overnight at 100°C and weighed to the nearest 0.1 mg. The modification described above was compared with the original method in the composition of the standard curve.

To establish a standard curve, dosages of 12.5, 25, 50, and 100 mU of ovine prolactin (NIH-P-S9; 30 IU/mg) were administered. Table III shows the response for each dose expressed as mucosal dry weights and as the ratio of this weight to the dry weight of the underlying tissue. There is less variation within each group (smaller standard deviations) and the differences between the groups (analyzed by Student's t-test) are more significant when the results are expressed as a ratio. This last parameter was therefore used in drawing the standard curve (Fig. 21). The dose-response relationship

TABLE II - Response of the pigeon crop sac mucosa to 4 dosages of ovine prolactin

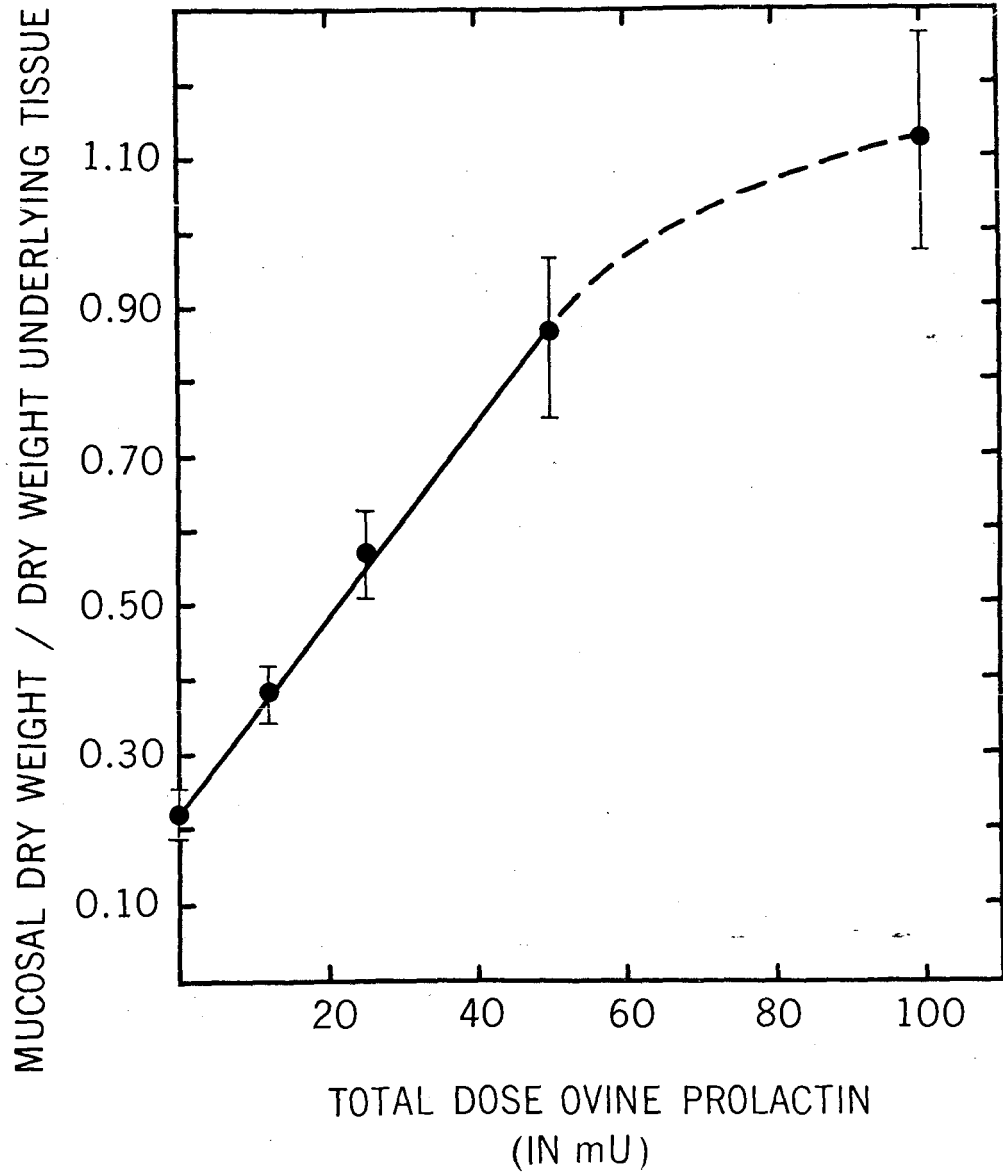
NIH prolactin dose in mU	number of hemicrops analysed	average mucosal dry weight	ratio mucosal dry weight to dry weight of underlying tissue.
control (saline)	4	6.3 ± 1.0 <sup>1</sup> N/S <sup>2</sup>	0.22 ± 0.03 p < 0.005
12.5	4	7.4 ± 1.0 p < 0.05 <sup>3</sup>	0.38 ± 0.04 p < 0.005
25.0	6	12.3 ± 1.5 p < 0.05	0.57 ± 0.06 p < 0.005
50.0	4	15.7 ± 2.5 p < 0.05	0.86 ± 0.11 p < 0.05
100.0	4	19.8 ± 2.1	1.12 ± 0.15

1 data presented as mean ± standard deviation

2 N/S = not significant by Student's t test

3. p values obtained by Student's t test

Fig. 21. Standard curve for ovine prolactin (NIH-P-S9; 30 IU/mg) obtained by the pigeon crop bioassay. Response is presented as the mean  $\pm$  the standard deviation. (See also Table III).



was linear up to a dose of 50 mU. This is in agreement with the findings of Nicoll (1967) who showed a similar sensitivity range, namely 1.5 to 37.5 mU ovine prolactin. Presumably, at higher dosages the time required for the hormone to exert its activity is the limiting factor.

### Results and Discussion

Immunosorption of Sigma prolactin. The results of the bioassay are compiled in Table III. Using the NIH prolactin as a standard (see Fig. 21) it appeared that the Sigma prolactin preparation had an activity of  $12.6 \pm 3.2$  I.U. per mg. Bioassay of the urea-eluted Sigma prolactin showed that this material had an activity of  $12.8 \pm 1.7$  I.U. per mg of protein. The assay of this fraction was conducted at two dilutions to determine whether the hormone shows a dose-response relationship. The importance of such a relationship was stressed by Nicoll (1967), who reported that non-prolactin protein was capable of causing an increase in mucosal dry weight but that this effect was in no way dose-dependent. My values do reflect a dose-response relationship. Radio-immunoassay of the urea-eluted preparation indicated that



TABLE III -- Bioassay of prolactin preparations

preparation assayed	mucosal response <sup>1</sup>	calculated amount of prolactin (mU)	total amount of prolactin eluted (IU)	calculated prolactin activity of preparation assayed (IU/mg protein)
Sigma prolactin (sample injected = 1.25µg)	0.43 ± 0.11	15.8 ± 4.05		12.6 ± 3.2 IU/mg
fraction obtained from immunosorption of sigma prolactin <sup>2</sup>	diluted 1:40	5.6 ± 0.66	8.4 ± .99	
	diluted 1:20	12.6 ± 1.60	9.5 ± 1.20	
			av = 8.95 ± 1.19	12.8 ± 1.7 IU/mg <sup>3</sup>
Sheep pituitary homogenate	diluted 1:120	5.6 ± 0.55		1.7 ± 0.17 IU/ml homogenate = 0.11 ± 0.01 IU/mg <sup>4</sup>
	diluted 1:60	21.0 ± 2.10		3.2 ± 0.32 IU/ml homogenate = 0.20 ± 0.02 IU/mg
				av. = 0.16 ± 0.016 IU/mg
fraction obtained from immunosorption of sheep pituitary homogenate (undiluted) <sup>5</sup>	0.475 ± 0.07	19.0 ± 2.80	1.23 ± 0.18	0.20 ± 0.014 IU/mg <sup>6</sup>

1 response expressed as the ratio of mucosal dry weight to dry weight of submucosal tissue (mean ± standard deviation)

2 urea peak (peak 2) from experiment 3 - Test b (see Fig. 14)

3 calculated on the basis that the total protein of this fraction = 0.70 mg.

4 protein of sheep pituitary homogenate was estimated as 16.2 mg/ml by the Biuret test

5 urea peak (peak 2) from experiment 3 - Test c (see Fig. 15)

6 calculated on the basis that the total protein of this fraction = 6.15 mg

it contained a total of 450  $\mu$ g prolactin (Table IV). Since the standard NIH prolactin had an activity of 30 IU/mg, the urea-eluted fraction should, in theory, possess a total prolactin activity of 13 IU. As this fraction was estimated at 0.7 mg protein, its expected prolactin activity per mg protein would then be 19.3. However, its actual activity as determined by the bioassay is lower ( $12.8 \pm 1.7$  IU/mg). This then, is an indication that there has been some loss of biological activity. Support for this possibility comes from a comparison of the calculated activities of the Sigma preparation before and after immunosorption ( $12.6 \pm 3.2$  and  $12.8 \pm 1.7$  IU/mg respectively). The Sigma prolactin very likely contained impurities (cf. its activity with that of the NIH preparation). Presumably, these contaminating proteins would not be bound to, and subsequently eluted from the immunosorbent, so that the eluted protein fraction should be of a higher purity and consequently have a higher activity per mg protein. The fact that the activity had not markedly increased, might be explained by assuming that the hormonal activity of this prolactin had been somewhat reduced by the immunosorption procedure.

TABLE IV - Radioimmunoassay of prolactin fractions obtained during immunosorption

Experiment <sup>1</sup> - Fraction	dilution <sup>2</sup>	cpm <sup>3</sup>	ng prolactin counted	total prolactin in fraction (µg)
Experiment 3 - Test b 4 mg ovine prolactin - peak 2 applied to anti-prolactin column (see Fig. 14)	1:1000	20,247 ±450	18.0	450
Experiment 3 - Test c 10ml sheep pituitary homogenate applied to anti-prolactin column (see Fig. 15)	1:5000	40,350 ±828	2.0	100
	1:500	35,440 ±515	6.5	150
Experiment 3 - Test d 10ml sheep pituitary homogenate applied to control column (see Fig. 16)	1:500	27,430 ±958	12.8	475
	1:200	43,434 ±612	<2.0	<11.0

- 1 Experiment numbers refer to Chapter 2
- 2 Only the dilution giving a count rate within the sensitivity range of the standard curve is shown
- 3 Read from standard curve (see Fig. 19)

Immunosorption of sheep pituitary homogenate. As was reported in Chapter 2, immunosorption of sheep pituitary homogenate resulted in an unexpectedly large amount of protein in the urea-eluted fraction from both the control and the anti-prolactin column. Radioimmunoassay of these fractions showed that 150  $\mu$ g prolactin was obtained from the anti-prolactin column while the control column bound a negligible amount of hormone (<11  $\mu$ g). It can be concluded from these results that the nature of prolactin binding must be different from that concerning the non-prolactin proteins. The binding of prolactin by the anti-prolactin column was most likely a result of specific immunosorption while the binding of the other materials by both the control and the anti-prolactin column was probably the result of non-specific protein-protein or protein-gel interaction.

The yield of prolactin obtained from the anti-prolactin column was surprisingly low: the same column bound 450  $\mu$ g of prolactin from the Sigma preparation. The homogenate added to the column (10 ml) was estimated to contain 1 mg prolactin on the basis of the microprecipitin test (see Chapter 1). This last quantity is in agreement with the results of the

bioassay of the sheep pituitary homogenate which indicated a prolactin content of about 2.4 IU/ml homogenate (see Table II) which is equivalent to 0.8 mg prolactin/10ml. The low prolactin-yield might be the result of interference, e.g. by steric hindrance of the antigen-antibody reaction, by the non-specific binding of other proteins. The problem of non-specific binding of proteins can, in all likelihood, be minimized by partly purifying the sheep pituitary homogenate prior to immunosorption.

It is interesting to compare the results of the bioassay of the urea-eluted prolactin fraction with those of the radioimmunoassay. The total hormonal activity of this fraction was estimated as  $1.23 \pm 0.18$  IU (Table III). On the other hand, the theoretical activity of 150  $\mu$ g prolactin should be 4.5 IU. This indicates that in this experiment too, a slight loss of specific hormonal activity may have occurred.

A last consideration concerns the purification of prolactin. The homogenate is estimated to possess an activity of  $0.16 \pm 0.016$  IU/mg protein while the eluted fraction had an activity of  $0.20 \pm 0.014$  IU/mg protein. Since moreover,

some loss of hormonal activity had presumably taken place, as mentioned above, it is evident that some degree of purification of the prolactin preparation had been accomplished.

## EPILOGUE

Most reports dealing with immunosorption procedures indicate that the solid phase antigen or antibody is highly stable and re-usuable. The results from my study seem to confirm this observation and it may therefore be concluded that for the isolation of prolactin, the immunosorption technique is to be preferred to the precipitation technique.

It seems however, that before this technique can be successfully applied to the isolation of "salmon prolactin", three problems have to be solved. The first problem is that concerning the yield. Less than 1 mg prolactin was bound by an immunosorbent prepared from 300 mg anti-prolactin  $\gamma$ -globulin whereas 2-3 mg BSA could be obtained from an immunosorbent prepared with only 100 mg anti-BSA  $\gamma$ -globulin. These yields are in all likelihood related to the antibody titer since the BSA antiserum had a much higher titer than the prolactin antiserum. A high-titer-antibody may be of particular importance in the case of a cross-reaction between "salmon prolactin" and anti-ovine prolactin since this heterologous reaction is much weaker than the homologous reaction (McKeown, 1970).

Secondly, there is the problem of specificity. My attempts to extract prolactin from the pituitary homogenate suffered from a high degree of binding of extraneous proteins. This binding was likely of a non-immunological nature. While an initial purification step should minimize the unspecific binding it is unlikely that it would abolish it. Anderson et al. (1970) also showed non-specific binding of extraneous proteins to a bromacetyl-cellulose immunosorbent. These workers showed that a 3 M urea solution would remove the contaminating proteins without eluting the immunologically bound antigen. The latter was then eluted with acetic acid. This approach should be investigated for the Sepharose immunosorbent used in my study. If this specificity problem cannot be minimized to a satisfactory level, a new immunosorption procedure will have to be investigated.

Thirdly, the apparent loss of specific hormonal activity during the immunosorption procedure requires further investigation. It must be determined what part of the procedure affects this hormone. Urea treatment might have some denaturing effect, but preliminary experiments have shown that it affects neither the hormonal nor the immunological



activity of ovine prolactin. This particular aspect is in agreement with the general observation that effects of urea on protein molecules are reversible (Epstein et al., 1963; Mahler and Cortez, 1968). Another aspect that should be investigated is the effect of freezing and thawing on the prolactin molecule. It was inevitable that during this procedure isolated prolactin solutions were frozen and thawed, sometimes repeatedly. It is generally agreed that protein preparations remain more stable if they are lyophilized before storing (Mahler and Cordes, 1968). Perhaps then, if the isolated prolactin fractions are immediately concentrated and lyophilized, the loss of prolactin activity would be minimal.

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