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Immunochemical Studies with Ovine Prolactin. (28788)

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Pierce and Carsten, using a starch gel electrodialysis technique, demonstrated that there are 3 components in highly purified ovine prolactin (N.I.H.-PS-3)(1). Cole and Li(2), using zone electrophoresis on starch gel also obtained 3 components, each of which possessed biological activity according to the standard pigeon crop sac assay method of Riddle, Bates and Dykshorn(3,4). By a similar technique Ferguson and Wallace observed 4 components with crop sac activity (5). Reisfeld *et al*(6) isolated 3 components from ovine prolactin by column chromatography on diethylaminoethyl cellulose (DEAE-C) and noted that component I, which had the greatest biological activity of the 3, also possessed a different C-terminal amino acid group from components II and III. Squire, Starman and Li(7) have studied the sedimentation velocity of ovine prolactin over a wide pH range and observed that despite the high degree of purity, the hormone is polydisperse, having a "multiplicity of biologically active components."

The inhomogeneity of purified ovine prolactin, as demonstrated by these physical-chemical methods has also been supported by recent immuno-chemical studies by Emmart, Bates and Spicer(8), who observed that ovine prolactin (N.I.H.-PS-3), when diluted serially and precipitated by a constant volume of antisera, exhibited 3 zones of precipitation, one zone of higher intensity and 2 of lesser magnitude. Concurrently multiple precipitin bands were observed in Ouchterlony plates. Since these results were in contrast to the single precipitin band in agar gel reported by Hayashida(9), these immuno-chemical studies have been extended to include several preparations of active ovine prolactin, and fractions of prolactin isolated by countercurrent distribution, by chromatography on DEAE cellulose column and by filtration through Sephadex. The following results indicate that fractions prepared by these methods differ in degree of biological activity, in rate of diffusion in agar gel, as well as in immunochemical response to antibody. Furthermore, since antibody to prolactin is capable of precipitating both active prolactin as well as prolactin fractions which have lost their biological activity, the precipitin reaction appears not to be related directly to the biological activity of prolactin.

Materials and methods. Ovine prolactin (N.I.H.-PS-3) with a potency of 15 I.U. per mg was obtained from the Endocrinology Study Section of Nat. Inst. of Health. This lot of purified prolactin was consistently used for immunization in rabbits as previously described(8). Two other ovine prolactin preparations, designated as S-89 and 71713, pre-

pared by Bates, had potencies of 20 and 22 I.U. per mg respectively.

I. Fractionation procedures. (a) Countercurrent distribution of 400 mg of S-89 was carried out using 0.35% dichloroacetic acid *vs* secondary butanol as described by Cole and Li(2). Four fractions were recovered of which 3 had prolactin activity (Fig. 1).

(b) Chromatography on DEAE-C. Two hundred milligrams of prolactin (S-89) were dissolved in 2.2 ml of .005 M sodium glycine buffer at pH 9.5 and placed on a DEAE-C column previously equilibrated with the same buffer. The various components of prolactin were eluted by means of a linear gradient to 0.5 M NaCl in 0.02 M sodium phosphate buffer at pH 6.5, pooled and recovered. Four fractions A, B, C and D were obtained (Fig. 2).

(c) Filtration through Sephadex G-100. A highly purified preparation of ovine prolactin (71713) was filtered on Sephadex G-100 and was resolved into several components (Fig. 3).

II. Bio-assay for the prolactin activtiy was carried out by the systemic crop sac weight method of Riddle, Bates and Dykshorn(3). The results, shown in Table I, represent assays made shortly after preparation of the prolactin and isolation of the prolactin fractions.

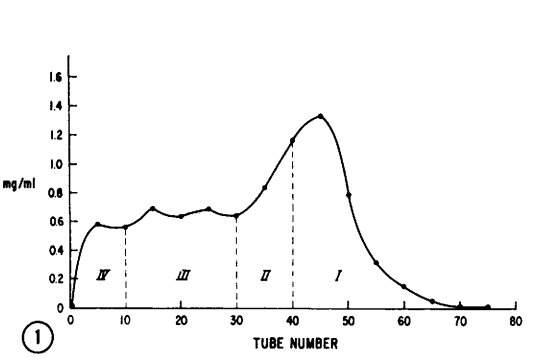
III. Antisera. (a) Development of antibody. Prolactin (N.I.H.-PS-3) was administered intramuscularly to rabbits in bimonthly doses of 10 mg. The initial dose was inoculated with complete Freund's adjuvant and thereafter with incomplete adjuvant. Serum samples were obtained before sensitization and at successive intervals during the course of inoculation. As previously reported, an increase in per cent gamma globulin accompanied the gradual rise in antibody titer(8). When antiserum of high titer was obtained (approximately after administration of 60-80 mg), serum samples were pooled so that all precipitin reactions to the various prolactin fractions could be made with antiserum of uniform potency. One milliliter of pooled antisera was capable of completely inhibiting the crop sac stimulating activity of 150 μg (3 units) of prolactin.

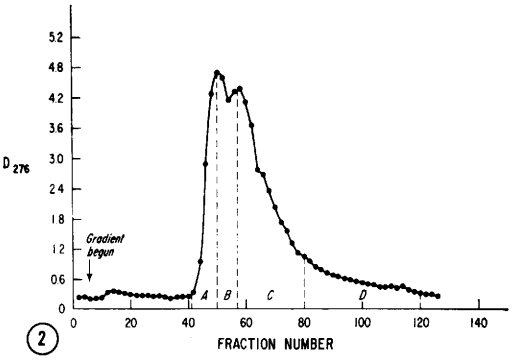
(b) Precipitin reaction (ring test). The ability of antiserum to precipitate the antigen was based upon a ring test *in vitro* using a constant volume of rabbit antiserum against prolactin antigen or antigen fraction in 2-fold dilution. Nitrogen determinations of the washed precipitates from each successive sample were carried out by the Miquel chromatographic method using a Coleman spectrophotometer(10).

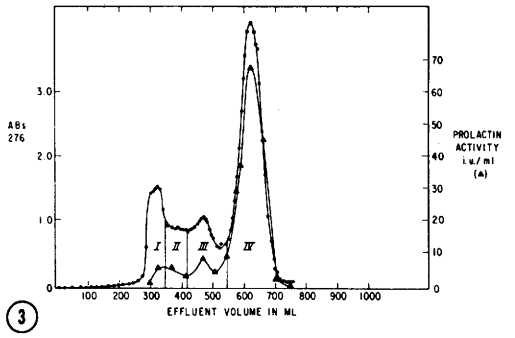
Precipitin reactions with prolactin fractions, prepared by the 3 procedures, were similarly carried out in a total test volume of 0.2 ml. The tubes contained the antigen in 2-fold dilution, 5.0 to 0.006 μg in buffered 0.15 M saline pH 7.4, and 0.1 ml of pooled antiserum. Nitrogen values of the washed precipitates demonstrated zones of optimal precipitation. The mean of these values from replicate experiments was expressed as optical density, and plotted against the concentration of the antigen per ml (Fig. 4, 5, 6).

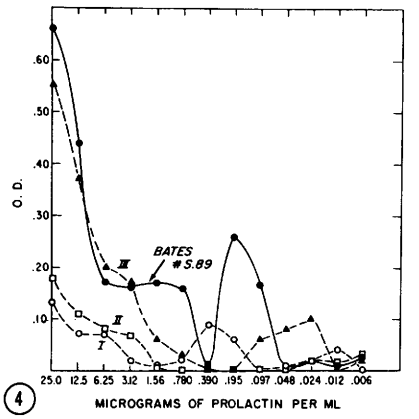
(c) Precipitin reaction in agar. Precipitin reactions between prolactin, prolactin fractions, and rabbit anti-prolactin serum were carried out in Noble agar using the Ouchterlony plate method(11). The prolactin and prolactin fractions were dissolved in buffered 0.15 M saline and added to the cups surrounding the central cup containing the antibody, so that each received 12.5 μg of the antigen. The distance in millimeters from the antigen cup to the precipitin bands was measured by a glass millimeter grid described elsewhere(12). When placed below the Petri dish and illuminated from below, the distance between each precipitin band and the antigen cup appeared constant under uniform conditions. By this means, as well as by the continuity of the precipitin bands of adjacent cups, it was possible to determine the presence or absence of the slow "5 mm" and the fast "6 mm" diffusing components of the antigen.

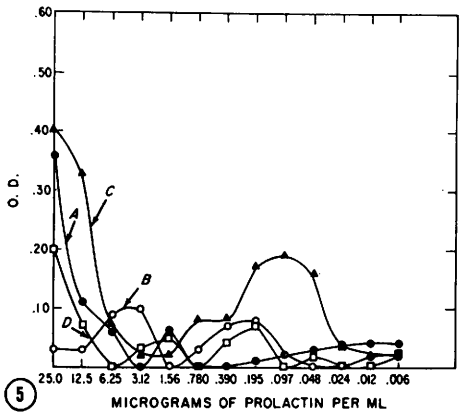
Results. Antibody binding to ovine prolactin. In the test tube all precipitin reactions with purified prolactin (N.I.H.-PS-3) and pooled homologous antisera gave similar results. The optical densities of the precipi-











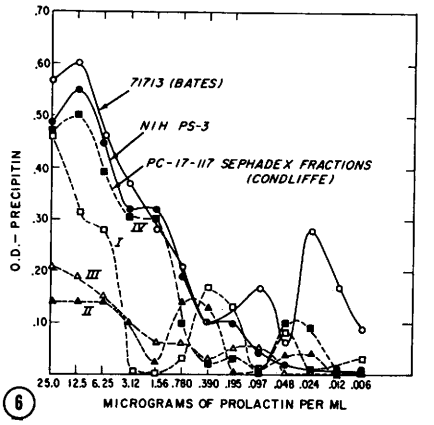


FIG. 1. Fractionation of ovine prolactin (S-89) by countercurrent distribution. After 160 transfers fractions I, II, III and IV were recovered. Activity of these fractions shown in Table I. Abscissae: mg per ml recovered; ordinates: tubes pooled for each fraction.

FIG. 2. Fractionation of ovine prolactin (S-89) by chromatography on DEAE-C column at pH 9.5. Abscissae: optical density read at 276 mμ; ordinates: fraction number in A, B, C and D. For activity see Table I.

FIG. 3. Fractionation of ovine prolactin (71713) by filtration through Sephadex G-100. Abscissae: optical density; ordinates: effluent volume per ml. ●—mg protein/ml; ▲—prolactin activity, I.U./ml.

FIG. 4. Precipitation by antiserum of prolactin (S-89, Bates) and fractions obtained by countercurrent distribution (Condliffe) (See Fig. 1). Abscissae: optical density of precipitins; ordinates: μg of prolactin per ml. Note multiple zones of precipitin in fractions I, II and III as well as in original unfractionated prolactin (S-89).

FIG. 5. Precipitin reaction of fractions A, B, C and D (Condliffe) prepared from prolactin (S-89, Bates) by chromatography on DEAE-C column. Abscissae: optical density of precipitated protein; ordinates: μg of prolactin per ml. The curves show multiple zones of absorp-

tion of precipitins obtained with fractions A, B, C and D.

FIG. 6. Precipitin reactions of sheep prolactin (NIH-PS-3); 71713 (Bates) and Sephadex fractions PC 17-117 (Condliffe) I, II, III and IV. Fraction IV contained most of the active material. Abscissae: optical density of precipitated protein; ordinates: μg of prolactin per ml. Both starting material and fractions show multiple zones of maximal precipitation.

TABLE I. Immunochemical Reactions of Ovine Prolactin and Prolactin Fractions to Pooled Anti-Prolactin Rabbit Sera.

(A)		Precipitin—ring test			Precipitin bands visible in Noble agar (Ouchterlony)	
Prolactin, purified (PPL)	Activity, I.U./mg	No. precipitin zones	Max optical density	Conc antigen, μg/ml	No. precipitin bands	Distance from band to antigen cup (mm)
						mm
N.I.H.-PS-3	15.0	3	.55 —	12.50	3	6 } close together
			.32 —	1.56		5 } close together
			.10 —	.195		4 — faint, rarely seen
Bates (B) #S-89	20.0	3	.66 —	25.00	2	6
			.17 —	1.56		5
			.26 —	.19		
Bates (B) #71713	22.0	3	.60 —	12.50	3 or 4	6 — may split in two
			.17 —	.09		5 — diffuse
			.28 —	.02		4 — faint, rarely seen
(B)	Fractionation by counter-current distribution from #S-89					
Condliffe (P.C.)—I	23.0*	2	.13 —	25.00	1	6
			.09 —	.39		
(P.C.)—II	20.0*	2	.20 —	25.00	2	6
			.07 —	3.12		5
(P.C.)—III	13.5*	3	.55 —	25.00	2	6 — trace
			.27 —	3.12		5
			.10 —	.02		
(C)	Fractionation by chromatography on DEAE-C from #S-89					
Condliffe (P.C.)—A	20.0*	2	.36 —	25.00	2	6
			.06 —	1.56		5 — strongest
(P.C.)—B	13.0*	2	.10 —	3.12	1	5
			.08 —	.19		
(P.C.)—C	12.0*	3	.40 —	25.00	1	5
			.08 —	.78		
			.19 —	.09		
(P.C.)—D	0 *	3	.20 —	25.00	0	0
			.05 —	1.56		
			.07 —	.19		
(D)	Fractionation by gel filtration through Sephadex G-100 from #71713					
Condliffe (P.C.)—17-117-I	3.1	4	.45 —	25.00	2	6
			.28 —	6.25		5 — trace
			.17 —	.39		
			.08 —	.04		
(P.C.)—17-117-II	5.9	3	.14 —	25.00	2	6
			.14 —	.78		5 — trace
			.04 —	.02		
(P.C.)—17-117-III	10.2	3	.21 —	25.00	2	6 } close together
			.06 —	.78		5 } close together
			.05 —	.19		
(P.C.)—17-117-IV	23.0	3	.50 —	12.50	2–4	4 — faint
			.30 —	1.56		5 & 6 (either of which may split)
			.10 —	.04		

* Value obtained at time of isolation. Subsequent reassay showed loss of activity (see text).

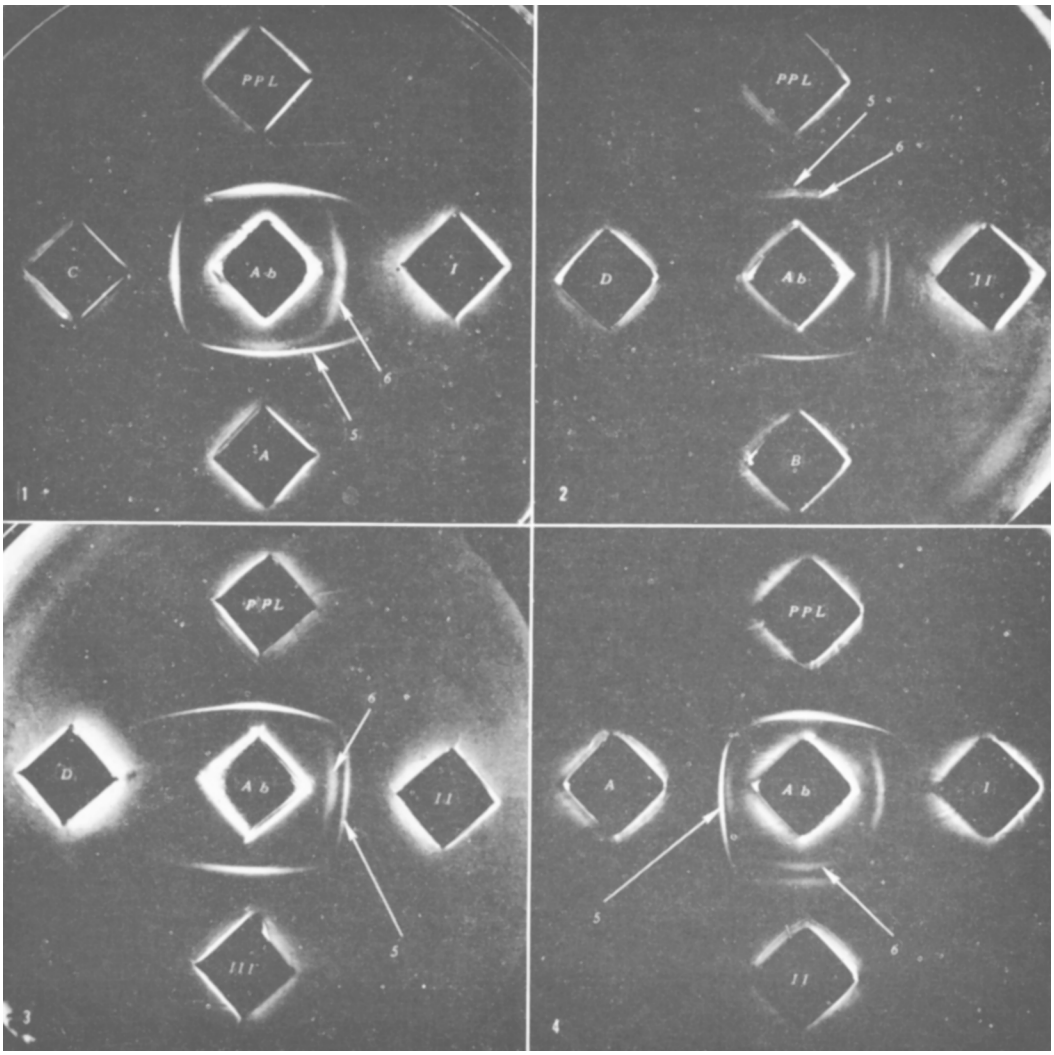


FIG. 7. Comparison of precipitin reactions in agar gel using pooled antisera to purified prolactin (PPL - NIH-PS-3) and fractions of prolactin (S-89, Bates) shows presence or absence of components which diffuse at different rates as indicated by precipitin bands at 5 and 6 mm from the cup containing the antigen. Fractions I, II and III prepared by countercurrent distribution and A, B, C and D by chromatography on DEAE-C column. The continuity of the lines with those of adjacent cups indicates similarity of components in Petri dishes 1, 2, 3 and 4. Photographed at 7 days.

tated protein indicated the presence of 3 zones of precipitation, descending in intensity with dilution of the antigen (Fig. 6—N.I.H.-PS-3). Three zones of absorption were also obtained when the same lot of antiserum was used to precipitate the other 2 lots of ovine prolactin, S-89 and 71713 (Bates). However, these latter preparations in high dilution both exhibited a rise in optical density of precipitins (Fig. 4 and 5), suggesting the presence of antigenic compounds with greater precipitin activity than are demonstrable with the N.I.H.-PS-3 preparation.

In agar gel prolactin (N.I.H.-PS-3) exhibited one wide precipitin band or frequently two close together, which were 5 and 6 mm from the cup containing the antigen (Fig. 7—1 and 2, top cups). The precipitin band made its appearance as a faint, short,

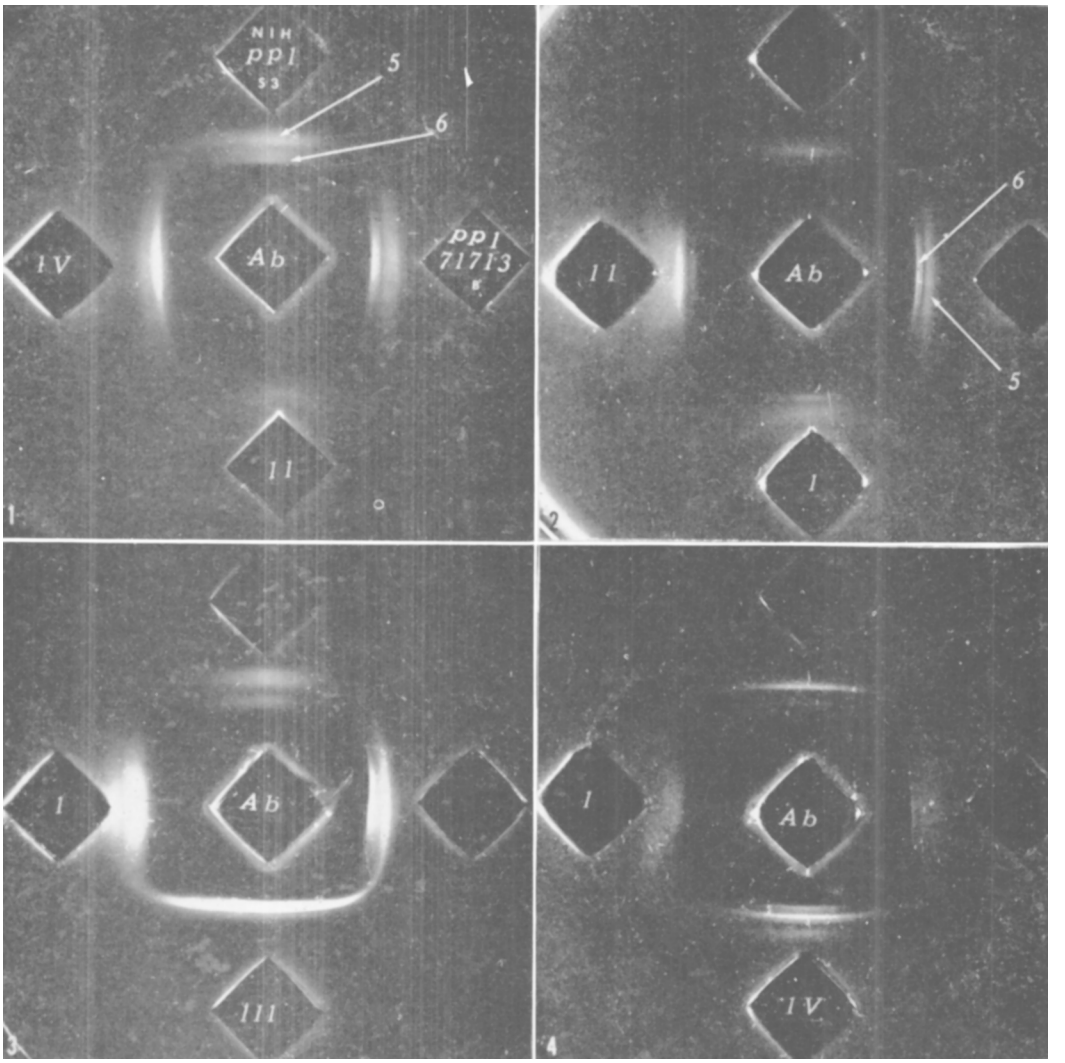


FIG. 8. Comparison of precipitin reactions in agar gel of ovine prolactin (PPL - NIH-PS-3, top cups) prolactin (71713, right cups); and fractions of I, II, III and IV filtered through Sephadex in Petri dishes 1, 2, 3 and 4. Antibody (Ab) in center cup. Fraction IV (4) shows tendency of Sephadex fractions to aggregate into multiple components. Continuity of lines between fractions I and III (3) and IV (1) and unfractionated prolactin indicates failure to separate fast and slowly diffusing components. Photographed at 14 days.

single band, 6 mm from the antigen cup, usually within 24 hours. As precipitation increased in intensity, the second band or "5 mm band" made its appearance, usually 24 hours later. These precipitin lines increased in length until by approximately 7 days they were continuous with bands of adjacent cups, if these contained homologous antigenic components. Frequently, a faint, diffuse third band could be seen at 4 mm from the antigen cup, but this was usually too faint to appear in the photographs. The time of separation and sharpness of definition of the bands varied slightly in different experiments, but their relative position from the antigen cup was constant. Since they exhibited a loss in sharpness as time progressed, photographs were made within one to 2 weeks after the beginning of the experiments.

Prolactin preparations S-89 and 71713, in agar gel exhibit precipitin bands which are continuous with the "5 and 6 mm" bands of

prolactin (N.I.H.-PS-3), indicating the similarity of the 3 preparations. When preparation 71713 was used, the 6 mm band separated into 2 components lying close together (Fig. 8—1 and 2, right cups) and occasionally a fourth, faint band was observed.

Antibody binding with fractions of prolactin. Fractionation of bovine prolactin (Bates, S-89) by countercurrent distribution yielded 4 fractions (Fig. 1), the first 3 in descending order of activity (Table I); the fourth without measurable activity, was not used for precipitin studies. In the test tube fractions I and II showed low capacity to bind antibody, while fraction III composed largely of the slowly diffusing "5 mm" component gave precipitins with nitrogen values almost as high as the original material (Fig. 4). All fractions of S-89 show the presence of several components which precipitate with antiserum (Fig. 4 and 5).

In agar gel, fractions separated by countercurrent distribution show the isolation of components having different rates of diffusion. Fraction I, when precipitated by antibody, was found to contain only the fast diffusing component of the 6 mm band (Fig. 7—1, right cup). Fraction II contained part of the fast component of band 6 but more of the slow component of band 5 (Fig. 7—2 and 3, right cups). Fraction III was composed almost entirely of the band 5 component with only a trace of the rapidly diffusing component represented in band 6 (Fig. 7—3, cup 3, bottom). The continuity of the precipitin bands in adjacent cups indicates the presence or absence of components in the various fractions (Fig. 7).

Fractionation of prolactin (S-89) by chromatography on DEAE-C column yielded fractions A, B, C and D (Fig. 2). Fractions A and C composing the major part of the extracted material, showed strong capacity to bind antibody (Fig. 7). Fractions B showed 2 low but distinct zones of precipitation in the test tube (Fig. 5). Fraction D gave low but measurable precipitation in the test tube but failed to show precipitation in agar gel (Fig. 7—2 and 3).

The continuity of the precipitin bands in gel, which formed between homologous antibody serum and prolactin (N.I.H.-PS-3) and fractions prepared by the two methods, indicate that by countercurrent distribution, the fast diffusing component, represented by band 6, is isolated from the slowly diffusing component of the 5 mm band (Fig. 7—1 and 3). Furthermore, the slowly diffusing antigenic component of band 5 (Fig. 7—1, C) appears to be the same as the dominant component isolated in fraction A by chromatography on DEAE-C column (Fig. 7—1, A) and in fraction III prepared by countercurrent distribution (Fig. 7—3, III, bottom cup).

Fractionation of prolactin (71713-B) by filtration through Sephadex G-100 produced 4 fractions with the major part of the hormone in the fourth fraction (Table I). Although all fractions precipitated with antisera, fraction IV produced the highest concentration of the precipitin complex (Fig. 6). In agar gel, fraction IV produced 2 to 4 precipitin bands with antiserum (Fig. 8—4, IV). Filtration through Sephadex G-100 did not separate the fast and slowly diffusing components as did the countercurrent distribution or elution from a DEAE-C column.

Activity of prolactin in relation to precipitation with antibody. Assayed immediately after preparation, prolactin preparations S-89 and 71713 (Bates) possessed 20 and 22 I.U. per mg respectively. These preparations, which were prepared 12 years ago, when recently reassayed were found to be still more active than the prolactin designated as N.I.H.-PS-3, which possessed 15 I.U. per mg.

Fractions of S-89 prepared by countercurrent distribution and by elution from DEAE-C column were assayed immediately after preparation and the primary fractions 1 and A found to have potencies equal to the starting material. The other fractions were of successively lower activity (Table I). Unlike the stable starting prolactin, lyophilized fractions prepared by countercurrent distribution and DEAE-C column were found to be unstable. By the time precipitin studies were undertaken, the countercurrent fractions I,

II and III were 7 years old and had potencies of only 1 I.U. per mg. The DEAE-C fractions were 5 years old and had potencies of 1 to 2 I.U. per mg except for C which possessed 5 I.U. per mg. However, precipitation with anti-prolactin rabbit serum was readily demonstrable both in the test tube (Fig. 4 and 5) and in agar gel (Fig. 7). It seems clear, therefore, that precipitability with antisera is retained even when more than 90% of the biological activity has been lost.

Fractions of prolactin (71713) recently isolated by filtration through Sephadex G-100 were biologically active at the time the precipitin studies were carried out. Most of the active material (Fraction IV—23 I.U. per mg), gave strong precipitation in the test tube and in agar gel. Fraction I, however, which possessed only 3.1 I.U. per mg also gave strong precipitation in the test tube (Fig. 6), showing little correlation between activity and ability to precipitate with antisera (Table I).

Antibody precipitation in agar gel of countercurrent distribution and DEAE-C column fractions have demonstrated immunochemically the separation of slow and fast diffusing components of prolactin (Fig. 7). Similar results were not achieved by filtration through Sephadex G-100; however, fractions prepared by this method tended to give multiple components which precipitated with antibody (Fig. 8—4, IV).

Discussion. Earlier studies previously cited, have demonstrated that prolactin is inhomogeneous and that by various methods it can be fractionated to obtain components having diverse physical properties, and, when freshly prepared, possessing biological activity characteristic of prolactin. Squire, Starman and Li(7) have shown that the "monomer fraction of the highly purified lactogenic hormone, isolated by exclusion chromatography, displays several components when analyzed by starch gel electrophoresis." They have attributed this polydispersity to the presence of aggregation or "association complexes" in the hormone rather than to contamination by components which do not have prolactin activity.

This association phenomenon is not unique with prolactin. Craig, King and Stracher(13) studying numerous highly purified substances by means of electrodialysis have presented evidence to show that various protein substances may appear as monomers, or associate to form dimers and trimers which retain their respective degrees of biological activity. Condliffe, Bates, Garrison and Howard(14) have shown that thyrotropin also exists in multiple forms but these are not aggregates. Grumbach and Kaplan(15) report that in simple diffusion of human growth hormone with rabbit antisera the antigen diffuses as a single unit. Hayashida obtained single precipitin bands with sheep prolactin and rabbit antisera both in Ouchterlony plates as well as in electrophoresis in gels(9). We also obtained single precipitin bands with sheep prolactin following electrophoresis in Noble agar and in .05 M barbital buffer at pH 8.6. If, however, the agar was changed to Ionagar and .05 M barbital acetate buffer adjusted to pH 8.6 with .15 M sodium phosphate, the prolactin separated into several components following electrophoresis for 6 hours. Recent studies by Pierce and Free(16) using electrodialysis through membranes, have shown that at pH 5.0 three components of sheep prolactin differing in size migrate toward the cathode, but at pH 9.5 all 3 components migrate toward the anode.

The data presented above on the immunochemical binding with several lots of prolactin suggest either that multiple binding sites are present or that antibody is capable of binding subunits of prolactin. Precipitin reaction in gel with fractions of prolactin isolated by countercurrent distribution and on DEAE-C columns has demonstrated that fractions of prolactin can be separated into components which show, by the position of the precipitin bands, differences in rate of diffusion as well as the capacity to bind antibody. Since differences in the gel environment as well as the shape of the molecule irrespective of its size or combination with other proteins may affect the diffusion rate in gel(17), any interpretation of the observed precipitin bands must await further

studies on the physical properties of prolactin.

In a recent discussion on the nature of the inhomogeneity of prolactin, Grumbach has raised the question as to whether during immunoelectrophoresis of prolactin, the multiple precipitin bands may not be the result of antibody binding with denatured antigen(18). The data from simple diffusion experiments in agar gel have shown that antibody is capable of precipitating with active as well as inactive prolactin fractions (Table I). Moreover, Reisfeld *et al*(6) have shown that the 3 fractions isolated by chromatography on DEAE-C column, fraction I contained most of the active prolactin while components II and III were altered forms of this component. Furthermore, they have shown that fraction I may be readily converted into fractions II and III by standing in 2% aqueous NH_4OH. The data shown in Table I indicate that in our experiments fractions A, B and C obtained by DEAE-C column fractionation yielded originally components with a similar sequence of activity. The results of Reisfeld *et al*(6) as well as our own suggest that a relationship exists between these fractions and the stable unfractionated prolactin. As shown by the immunochemical binding with antibody (Fig. 7, A, B and C), there exists not only a continuity of the precipitin band between the unfractionated active prolactin and inactive fraction A (Fig. 7, 4), which contained both the 5 and 6 mm components, but also with B and C which contained the slowly diffusing 5 mm component.

From these results as well as from fractions obtained by countercurrent distribution it appears that components differing in diffusion rates may be isolated from prolactin and however changed or inactivated, are still capable of precipitating with specific antibody.

Summary. Quantitative measurements of antibody-prolactin precipitins formed in the test tube have demonstrated 3 zones of precipitation, decreasing in intensity with dilution of the antigen. The inhomogeneity of 3 preparations of purified prolactin has been demonstrated immunochemically by the formation of multiple precipitin bands in agar gel. Prolactin fractions were prepared by countercurrent distribution, by chromatography on DEAE-C column and by filtration through Sephadex G-100. These fractions differed in biological activity. The fractions obtained in countercurrent distribution and DEAE-C fractions were found to be unstable, losing 90 to 95% of their activity, while the unfractionated prolactin remained stable. Precipitation by antibody occurred whether prolactin fractions were fully potent or had lost activity. Fractionation by countercurrent distribution and by chromatography on DEAE-C column separated components which diffused at different rates and precipitated with antibody to form 2 distinct bands in agar gel. The continuity of these bands with those of the original material identified them with the original source material.

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Inhibition of Viruses by Secretions from the Female Genital Tract.* (28789)

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For several years an extensive search has been made in this laboratory for viruses in human vaginal and cervical secretions. This work was directed toward establishing a baseline of the viral flora of the normal vagina and cervix as a prelude to studies on the possible role of these agents in the etiology of cervical cancer. A total of 229 washings and swabbings taken from adult women all proved negative for viruses as determined in tissue culture. These findings could be interpreted either as meaning that viruses are not normally present in the female genital tract or that if present they are masked or restrained by some type of inhibitor. The present report relates the demonstration of such an inhibitor.

Materials and methods. Volunteers providing secretions. The volunteers were principally patients at the Jackson Memorial Hospital in Miami, Fla. and at University Hospital, University of the West Indies in Jamaica. Economically and socially they cut across most strata of the population, but the highest number were women of the lower social and economic status.

Cervicovaginal secretions were obtained by using Tassettes,† which are rubber cups normally used to collect menstrual secretions. The Tassette was kept in the vagina for 6–24 hours. The secretions obtained were very small in quantity (0.5–1 ml), usually ranged in color from gray to light green and had a pH of 3–4. They were highly tenacious and required dilution approximately 5-fold with Hanks' balanced salt solution to facilitate removal from the Tassettes. The Hanks' solution contained 1,000 units penicillin, 0.5 mg streptomycin and 0.025 mg fungizone per ml. The secretions were frozen at –20°C until used. In the initial experiments, and in all experiments with Rous sarcoma virus, the individual secretions were tested separately. However, no single specimen sufficed for more than one test. Two or 3 secretions were therefore pooled in several instances to obtain a workable quantity of material. All secretions were brought to a pH of 7.0 to 7.6 at time of the test.

Tissue culture. HeLa or KB cell lines cultivated and maintained in Eagle's medium supplemented with calf serum (10% for growth; 5% for maintenance) were used. After inoculation, cultures were examined daily for cytopathogenic effect (CPE) and experiments were terminated after 7 days of observation.

Viruses. Rous sarcoma virus was supplied by Dr. W. Ray Bryan, NIH. Herpes simplex strain GCA3 was received from Dr. T. F. McNair Scott, Children's Hospital, Philadelphia. Poliovirus type I, Mahoney strain, was obtained from The Connaught Laboratories. Serial passages in tissue culture of both herpes and poliovirus were made in this laboratory.

Attenuated trivalent oral polio vaccine was provided by Dr. Herald Cox, Lederle Laboratories. The initial titer of this vaccine was greater than $10^{6.0}$ $TCID_{50}$ per ml, but after

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† Obtained from Tassette, Inc., Stamford, Conn.