Properties of β-Lipovitellin as Influenced by Enzymic and Other Treatments.* (32357)

ROBERT B. SAMUELS† AND TOSHIRO NISHIDA (Introduced by F. A. Kummerow)
Burnsides Research Laboratory, University of Illinois, Urbana

Dissociation into two ultracentrifugally indistinguishable monomer subunits in alkaline media is a characteristic feature of α- and β-lipovitellin(1). The dissociation has been shown to be reversible and to decrease with increasing ionic strengths(2). The treatment of lipovitellin with 4 M urea also caused dissociation, which was partly reversible upon dilution of the solvent(3). Both lipovitellins contain about 80% protein and 20% lipids(4), and the molecular weights of their dimer and monomer forms were found to be approximately 4.5×10^5 and 2.3×10^5, respectively(3,5,6).

Although the nature of association-dissociation phenomenon is not yet understood, the sulphydryl groups and the protein phosphate groups of lipovitellin have not been shown to be directly involved in the association of lipovitellin monomer units(7). The present communication describes the successful use of various proteolytic enzymes with differing substrate specificities, venom phospholipase A, and surface-active agents in elucidating some aspects of the β-lipovitellin association-dissociation phenomenon.

Materials and methods. Yolk granules were prepared from fresh hen's eggs according to the method of Burley and Cook(8). β-Lipovitellin was isolated from the high density protein fraction of granules by column chromatography on TEAE-cellulose according to the method of Radomski and Cook(4). The lipid and protein contents of the lipoprotein were determined by gravimetric methods(9). A representative sample of β-lipovitellin used in the present study had a sedimentation coefficient ($S^{o}_{20,w}$) of 10.2 and contained 78.3% proteins and 21.7% lipids on a dry weight basis. In carbonate buffer of pH 9.0 with ionic strength 0.3, this sample at a concentration of 0.8 mg per ml produced 71.9% dissociation as determined by ultracentrifugal analysis(2). These values were in agreement with those previously reported(2,4).

Three-times crystallized α-chymotrypsin, twice-crystallized trypsin, and twice-crystallized papain were obtained from Worthington Biochemical Corp. Lyophilized venom from *C. adamanteus* was purchased from Ross Allen's Reptile Institute as a source of phospholipase A. Surface active agents used were Triton X-100 (Rohm and Haas) and Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Co.). Unless otherwise noted, 1 M sodium chloride containing 0.037 M potassium phosphate buffer at a final pH 6.8 was used as the incubation media; its relatively low pH and high ionic strength allowed predominant distribution of the dimer form in native β-lipovitellin(2), hence the formation of monomer units by specific treatments was easily detected. The concentration of monomer lipovitellin was approximated by ultracentrifugal analysis of monomer-dimer mixtures, since the proportions of the 2 ultracentrifugal components seem not to be appreciably altered during centrifugation(2,6).

Although inhibitors were considered to terminate the enzymic reactions, preliminary studies indicated that they caused extensive denaturation of β-lipovitellin. Since the present study involved observation of changes in distribution of β-lipovitellin molecular species, the incubations were conducted at enzyme concentrations which gave slow reaction rates, and subsequent ultracentrifugal analyses of aliquots were carried out immediately after incubation.

The degree of proteolysis in the incubation media was determined by analyzing the trichloroacetic acid (5%) insoluble material for protein content according to the method of

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† Present address: Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

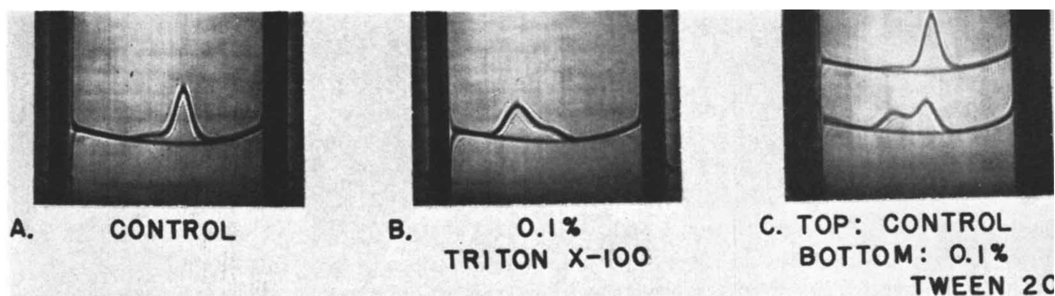

FIG. 1. Sedimentation patterns of β-lipovitellin stored with surface-active agents. Each solution contained 0.8 mg β-lipovitellin per ml and surface-active agents at final concentrations indicated in the Figure. In each case the picture was taken at a bar angle of 65°, 91 min. after a speed of 52,640 r. p. m. with an acceleration time of 5 min. and 20 sec. was obtained. The total Schlieren peak area of the β-lipovitellin samples treated with surface-active agents did not significantly change as compared to the peak area of control samples. Increase in percentage of slow-sedimenting component in the samples corresponding to pictures B and C (bottom) was 60.9% and 27.4% respectively, in comparison with control samples.

Lowry *et al*(10). Free fatty acids released from the phospholipid components of β-lipovitellin by treatment with *C. adamanteus* venom were determined according to the procedure of Dole(11). All ultracentrifugal analyses were performed on a Spinco Model E analytical ultracentrifuge utilizing Schlieren optics at 20°. Peak areas were measured with a planimeter on tracings of 10× (linear) enlargements on the photographic plates.

Results. Storage of β-lipovitellin solution in 1 M sodium chloride-0.037 M potassium phosphate buffer (pH 6.8) containing 0.1% Triton X-100 or Tween 20 for 48 hours at 4° increased the area of slow-sedimenting peak of lipovitellin monomer by approximately 61% and 27%, respectively (Fig. 1). This observation indicated that the molecular surface region of β-lipovitellin involved in the association-dissociation phenomenon may be predominantly hydrophobic in nature.

Incubation of β-lipovitellin with *C. adamanteus* venom for 24 hours at 37° in 1 M sodium chloride-0.001 M calcium chloride (pH 6.8) revealed a more than 3-fold increase in the area of the slow-sedimenting peak (Table I). Microtitration of the fatty acids indicated that this treated lipovitellin sample contained 16 times more free fatty acids than an appropriate control. Measurements of absorbancy (at 280 mμ) of trichloroacetic acid-soluble material and of total Schlieren pattern peak areas revealed negligible differences between treated and control samples. These findings indicated that the formation of slow-sedimenting material was due to the action of venom phospholipase A on the lipid moiety of lipovitellin. This view was further sub-

TABLE I. Properties of β-Lipovitellin as Influenced by Treatment with *C. adamanteus* Venom.

Analyses conducted	Venom-treated sample	Control sample
Slow sedimenting component, %	42.1	12.2
Free fatty acid, μmoles per ml	1.69	.10
TCA-soluble material, absorbance at 280 mμ	.139	.143
Relative total peak area, %	108.0	100.0
Apparent sedimentation coefficients		
Fast moving peak	7.1	7.3
Slow moving peak (approximate values)	4.8	5.2

The incubation mixtures, in a final volume of 4 ml, contained 8 mg β-lipovitellin per ml with and without the addition of 0.5 mg *C. adamanteus* venom per ml in the medium of 1 M sodium chloride - 0.001 M calcium chloride, pH 6.8. Incubations were conducted for 24 hr at 37° in nitrogen. Absorbance of TCA-soluble material was determined for the filtrate obtained from the mixture containing one volume of incubation mixture and 5 volumes of 6% trichloroacetic acid.

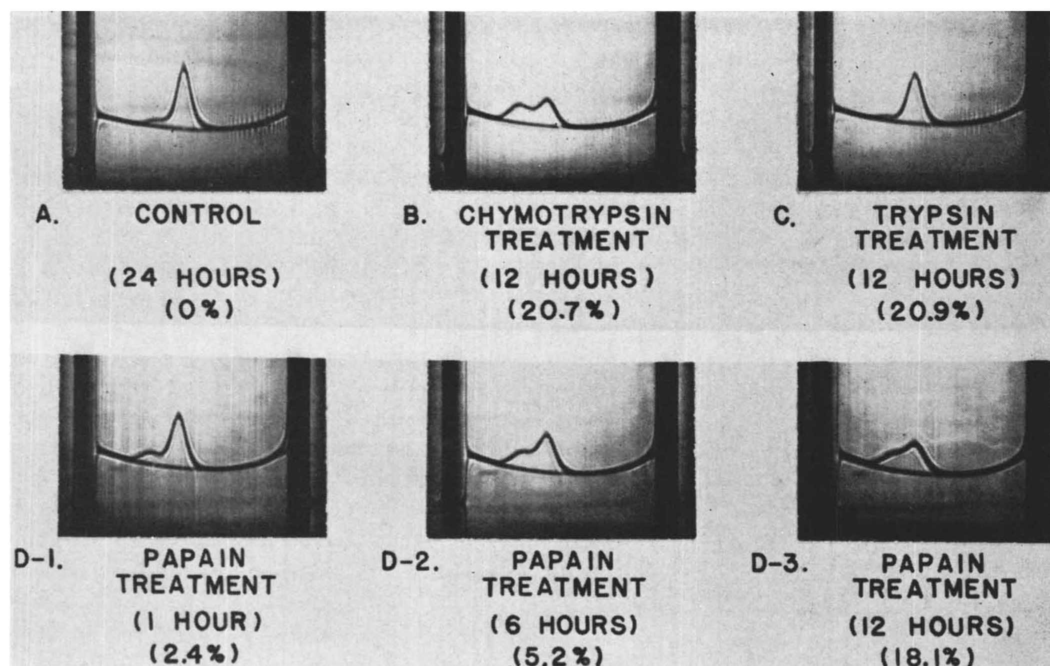

FIG. 2. Sedimentation patterns of β-lipovitellin treated with proteolytic enzymes. All incubation mixtures contained 0.8 mg β-lipovitellin per ml in 1 M sodium chloride-0.037 M potassium phosphate buffer (pH 6.8). For the treatment with proteolytic enzymes 0.028 mg (183 units) chymotrypsin, 0.014 mg (262 units) trypsin, or 0.006 mg (0.09 units) papain per ml was added. Incubation mixtures for papain treatment also contained 0.005 M cysteine and 0.005 M EDTA. Incubation time for each sample is indicated in parentheses. Percentage decreases in the apparent area under the total Schlieren peak, an indication of the degree of proteolysis, are also given in parentheses. Pictures were taken at a bar angle of 65°, 75 min after reaching maximum speed of 52,640 r.p.m. with an acceleration time of 5 min and 20 sec. Apparent sedimentation coefficients of the fast-sedimenting component in pictures A, B, C, D-1, D-2, and D-3 were 7.1, 6.9, 6.8, 6.7, 6.5, and 6.2, respectively, and those of the slow sedimenting component in pictures B, D-1, D-2, and D-3 were 4.3, 4.5, 5.7, and 4.9, respectively.

stantiated by our observation that agents which activate or inhibit phospholipase A similarly affected the formation of slow-sedimenting components. The elimination of 0.001 M calcium chloride from, or the addition of 0.05% EDTA to the incubation medium reduced the release of free fatty acids from the lipovitellin by 51% and 92%, respectively, and subsequently decreased the formation of the slow-sedimenting component by 54% and 100%, respectively.

The formation of β-lipovitellin half-molecules equivalent to monomer units was also found to occur after treatment with specific proteolytic enzymes. Ultracentrifugal analysis of β-lipovitellin incubated with chymotrypsin in 1 M sodium chloride-0.037 M potassium phosphate buffer (pH 6.8) resulted in an increase in the area of the slow-sedimenting peak, accompanied by a decrease in the area of the fast-sedimenting peak (Fig. 2, B). The increase in percentage of slow-sedimenting component almost reached a maximum when approximately 12% of the lipovitellin originally present had been subjected to proteolysis. Further proteolysis did not appreciably alter the percentage of slow-sedimenting component. Unlike chymotrypsin treatment, proteolysis by trypsin did not produce any substantial change in the percentage of slow-sedimenting component (Fig. 2, C). Although papain treatment caused an initial increase in the area of the slow-sedimenting component (Fig. 2, D-1, D-2), as papain degradation proceeded, the two separate peaks gradually became less distinct (Fig. 2, D-3) and finally merged into one rather heterogeneous peak primarily as a result of pro-

gressive decrease in the apparent sedimentation constant of fast-sedimenting component.

Discussion. The dissociation of β-lipovitellin or the formation of its half-molecules equivalent to monomer units was found to be promoted by specific enzymic alteration of either the protein or lipid moiety, as well as by exposure to common surface-active agents. The increased percentages of monomer β-lipovitellin observed in solutions of the lipoprotein stored with Triton X-100 or Tween 20 may indicate that the hydrophobic region of a monomer β-lipovitellin could be competing with the surface-active agent for the non-polar region of another monomer lipoprotein molecule. Shielding of the hydrophobic region by surface-active agents would increase the compatibility of the monomer with its aqueous environment, essentially by increasing the net surface polar quality of the lipoprotein.

A study of the effect of *C. adamanteus* venom phospholipase A on β-lipovitellin indicated the possible involvement of bound phospholipids in the association phenomenon. Dissociation of the dimer lipoprotein following treatment with phospholipase A might be promoted by exposure of the newly formed lysophospholipid free hydroxyl groups, which would increase the polar nature of the association site and, hence, the probability of solute-solvent interaction. Carboxyl groups of the released free fatty acids, still bound to β-lipovitellin molecules, might also have promoted the solute-solvent interaction.

Increased dissociation of β-lipovitellin, observed from the outset of incubation with chymotrypsin, might be caused by initial hydrolysis of peptide bonds near, or at, the association site on the molecular surface. The formation of new amino and carboxyl groups or, for that matter, any increase in polarity of the predominantly non-polar association site by treatment with chymotrypsin will greatly reduce the hydrophobic nature of this region. On the other hand, tryptic hydrolysis may not occur near the association region in particular. The action of papain on β-lipovitellin may be explained in terms of the broad substrate requirements of this enzyme. Initial proteolysis of the peptide region, which may be involved in the association-dissociation phenomenon, seemed to be accompanied by a rapid proteolysis of the other peptide regions, thus producing a series of protein fractions with varying molecular sizes.

The present results, obtained by treatments of β-lipovitellin with various agents, may suggest the presence on the surface of the monomer an effectively non-polar region which is directly responsible for the intermolecular association. However, the non-polar cohesion between monomer β-lipovitellin molecules is by no means excessive, for it seemed that any treatment of β-lipovitellin which increases the polar character near, or at, the molecular association site will inherently promote dissociation of the dimer molecule.

Summary. The effects of certain enzymic and other treatments of β-lipovitellin of egg yolk were studied in a medium of 1 M sodium chloride-0.037 M potassium phosphate (pH 6.8), which allowed predominant distribution of dimer form in native β-lipovitellin. Ultracentrifugal analysis of β-lipovitellin incubated with the surface-active agents, Triton X-100 and Tween 20, and with snake venom phospholipase A resulted in an increase in the percentage of slow-sedimenting component corresponding to monomer β-lipovitellin. Although treatment of β-lipovitellin with α-chymotrypsin also caused a sizable decrease in the proportion of associated lipovitellin, a similar treatment of β-lipovitellin with trypsin caused no appreciable change in the relative amounts of monomer and dimer components. Exposure to papain resulted in a series of overall changes in Schlieren sedimentation pattern of the lipoprotein with incubation time.

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Anterior Pituitary Levels of FSH, LH, ACTH and Prolactin After Mating in Female Rabbits.* (32358)

C. DESJARDINS,† K. T. KIRTON,‡ AND H. D. HAFS
Animal Reproduction Laboratory, Department of Dairy, Michigan State University, East Lansing

Copulation induces ovulation in rabbits presumably by stimulating release of hypothalamic neurohumor which causes the anterior pituitary to release ovulating hormone(1). Although the timing of ovulating hormone release from the anterior pituitary gland has been extensively studied in female rabbits (2,3,4), few studies have explored the possibility that copulation may stimulate release of other anterior pituitary tropins. Saxton and Greene(5) provided indirect evidence that copulation altered pituitary level of FSH, LH, ACTH, and TSH in female rabbits. Friedgood and Dawson(6) reported a marked increase in the number of acidophilic carmine cells in rabbit pituitaries 0.5 hour after copulation and rapid degranulation of these cells 3 hours after copulation. These cells are currently believed to be the origin of prolactin(7,8). Based upon this evidence, experiments were designed to test directly the hypothesis that copulation alters pituitary FSH, LH, ACTH and prolactin levels before ovulation.

Materials and methods. Mature female rabbits (8-12 months old) were isolated for at least 35 days in an animal room maintained at 18°C and provided with a 14-hour photoperiod daily. Female rabbits were placed with one of 5 males, allowed to mate, and immediately returned to their home cages. Eight were killed by decapitation at each of the following intervals after mating: 0, 0.25, 0.75, 3, 7, or 11 hours. Anterior pituitaries were removed within 3 minutes after slaughter and immediately trimmed, weighed, and placed in a glass vial directly on Dry Ice.

The 8 pituitaries within each interval after mating were pooled and homogenized in 0.85% sodium chloride in pyrogen free distilled water at 5°C with a glass grinding vessel and Teflon pestle. Homogenates of anterior pituitary tissue were centrifuged at 800 *g* for 10 minutes at 5°C and aliquots of supernatant fluid were stored at —20°C until assayed for hormonal activity. When the first 6 pools of 8 pituitaries had been assayed, the complete experiment was replicated once.

The potencies of FSH, LH, and prolactin were estimated as previously described(9) and modified as follows. FSH potency (ovarian weight augmentation assay(10)) of each pool of pituitaries was measured at 5 and 10 mg-equivalents of fresh anterior pituitary tissue and these were compared with 50 and 100 μg NIH-FSH-S2 and with 30 IU of HCG alone in 21-day rats. LH potency (ovarian ascorbic acid depletion assay(11)) was measured at 0.2 and 0.8 mg-equivalents fresh anterior pituitary tissue and these were compared with 0.4 and 1.6 μg NIH-LH-B2. Five rats were used at each dose level of each unknown and reference preparation for

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† Present address: Jackson Laboratory, Hamilton Station, Bar Harbor, Maine.

‡ NIH Predoctoral fellow. Present address: Upjohn Co., Kalamazoo, Mich.