Table 2. Formation of LAL in proteins by heating under nonalkaline conditions.

Protein	Concentration (% by volume)	Temperature (°C)	Time (hours)	*p*H	LAL (micrograms per gram of protein)
Bovine serum albumin	1	120	1	6.0	3500
Casein	1	120	1	6.0	1700
Lysozyme	1	120	1	2.0	None
Lysozyme	1	120	1	4.0	275
Lysozyme	1	120	1	6.0	1000
Ovalbumin	1	120	1	2.0	150
Ovalbumin	1	120	1	3.0	250
Ovalbumin	1	120	1	4.6	310
Ovalbumin	1	120	1	6.0	570
Soya globulin	6	120	16	2	<40
Soya globulin	6	120	16	3	<40
Soya globulin	6	120	16	4	80
Soya globulin	6	120	16	5	120
Soya globulin	6	120	16	6	130
Soya globulin	6	120	16	7	180
Soya globulin	6	100	1	6.5	<40
Soya globulin	6	100	2	6.5	80
Soya globulin	6	100	3	6.5	130

air-evacuated sealed ampuls with 6*N* HCl at 120°C for determinations by ion-exchange chromatography with an automated amino acid analyzer (Technicon) (*2*) and for 24 hours at 110°C for determinations by thin-layer chromatography (TLC) (*11*).

Most samples were analyzed by both methods except those that gave an interference with the LAL peak on the ion-exchange chromatographs. The identity of LAL determined only by TLC was confirmed by preparative separation on paper chromatography with the TLC solvent system, elution of the LAL area with water, and determination of LAL by ion-exchange chromatography.

The results of the determinations (Table 1) reveal the widespread occurrence of LAL in home-cooked and commercial foods and ingredients that had not been exposed to alkali. Particularly significant is the finding of LAL in condensed milk, acid casein, cooked chicken thigh, and sirloin steak pan scrapings, none of which were exposed at any time to an alkaline medium. Therefore, we hypothesize that LAL does form in proteins under conditions that do not entail alkali treatment.

Soya globulin, ovalbumin, lysozyme, casein, and bovine serum albumin (*12*), all proteins occurring in food systems, were heated under nonalkaline conditions. All formed variable amounts of LAL in the range of *p*H, temperature, and time commonly used in home cooking and commercial processing (Table 2).

In light of the experiments connecting the presence of LAL in proteins with reduced nutritional availability and possible toxic effects, our finding of LAL in the structure of heated proteins not necessarily subjected to alkali treatment may be a factor to be considered in explaining the reduction of nutritional value by heating. The formation of LAL in proteins by heating at *p*H values considerably lower than those obtained during alkali treatment, as reported in (*5*), suggests the ubiquity of LAL in cooked foods and that humans have long been exposed to proteins containing LAL.

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11. Determinations of LAL in protein hydrolyzates by TLC were done on cellulose sheets (Eastman catalog number 6064), developed with a solvent system consisting of a mixture of 1-butanol, formic acid, and water in the volume ratio 23 : 4 : 5, and made visible by spraying with 0.3 percent ninhydrin solution in 1-butanol containing 3 percent acetic acid (by volume). The intensity of the LAL spots was read in a spectrodensitometer (Schoeffel model SD 3000), the peak areas measured with a planimeter, and the concentration of the unknown determined from a graph of LAL controls. Details of the procedure are given in M. Sternberg, C. Y. Kim, R. A. Plunket, *J. Food Sci.* **40**, 1168 (1975).
12. Soya globulin was obtained by precipitating at a *p*H of 4.5 a water extract of defatted soya flour, washing with water, and freeze-drying. Bovine serum albumin and lysozyme were commercial preparations from Miles Laboratories, Research Products Department. Casein was prepared by the method described in B. H. Webb and E. O. Whittier, *By-Products from Milk* (Avi, Westport, Conn., ed. 2, 1970), p. 333. Ovalbumin was prepared by the method described in *Merck Index* (Merck, Rahway, N.J., ed. 8, 1968), p. 29. All commercial samples of crystallized ovalbumin contained LAL.
13. We thank R. B. Kocher for discussions and suggestions and C. J. O'Donovan for reviewing the manuscript.

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Stage-Specific Switches in Histone Synthesis During Embryogenesis of the Sea Urchin

Abstract. *Histones H2A and H2B of the sea urchin embryo have been resolved by new methods into components that are synthesized at different stages of development. One form of H2A and one form of H2B are synthesized only during the period from fertilization to the blastula stage. Subsequently, two other types of H2A and H2B molecules are synthesized. In addition, a histonelike protein was detected which is synthesized only from fertilization until the 16-cell stage when the synthesis of still another H2A-like protein begins. None of the late-appearing forms are derived from histone polypeptide chains synthesized earlier in development. Since the early components do not disappear after their synthesis stops, these modulations of histone synthesis lead to an increase in histone multiplicity, concomitant with the beginning of cell diversification and a decrease in cell division rate.*

Histones are the principal proteins of eukaryotic chromosomes. Although they have many properties that make them suited for long-term gene regulation (*1*), the failure of conventional methods to demonstrate extensive histone diversity or tissue-specific variability has discouraged serious consideration of a regulatory role for these proteins. However, over the last few years one of the five major histone types, H1 (*2*), has been shown through the application of improved methods to be a family of related protein components that exhibit considerable tissue-specific variation (*3*). In the sea urchin the complexity of H1 histones increases from a single species in morulae to three species in gastrulae (*4, 5*).

Histones H2A, H2B, and H3 are difficult to resolve by standard electrophoretic techniques because of their similarity in molecular size and ionic charge. Their resolution can be improved by performing the

electrophoresis in the presence of a nonionic detergent such as Triton X-100, which decreases the mobility of different proteins to different degrees (*6*). With this method it was possible to resolve mammalian histones H2A, H2B, and H3 into small families of variants. Previous studies with conventional electrophoretic techniques have suggested changes in the synthesis of non-H1 histones during the development of the sea urchin embryo (*4, 5, 7*). In our study, we have used nonionic detergent electrophoresis to explore this question.

When embryos of the sea urchin *Strongylocentrotus purpuratus* are labeled at the prism stage with [^{3}H]leucine and the isolated histone fraction is resolved by electrophoresis in the absence of detergent (*8*), the radioactivity and stain profiles do not correspond (Fig. 1a), indicating that not all histones are being synthesized at this stage. If the same histone preparation is resolved in a gel containing 8*M* urea and 6m*M* Triton X-100, the electrophoretic mobility of H2A histone is preferentially decreased (*6*), and a number of previously overlapping components are resolved (Fig. 1b). We have tentatively identified the various newly resolved forms as H2A or H2B on the basis of Triton affinity (*6*), staining properties (*9*), solubility in organic solvents (*10*), and leucine incorporation [H2A in a leucine-rich histone (*11*)]. A comparison of radioactivity and stain profiles in these gels indicates that the major stained components in both the H2A and the H2B regions (designated α in each case), are not synthesized during the prism stage.

To study the synthesis of different components in detail, embryos were exposed to [^{3}H]leucine at different times after fertilization for a period of about 4 hours; the labeling was then stopped and the embryos were allowed to develop until the end of the blastula stage or later. This procedure was chosen in order to determine whether any histones were derived from previously synthesized polypeptide chains. In addition, it allowed the embryos to be harvested at stages most favorable for histone isolation. Fluorography of the labeled histones after electrophoresis in a gel containing 8*M* urea and 6 m*M* Triton X-100 (Fig. 2) leads to the following conclusions. (i) During early cleavage (1 to 16 cells) the α forms of both H2A and H2B are synthesized. Within each α component, two or three bands can be discerned, probably because of post-translational modifications of H2A and H2B such as those known to occur in other organisms (*12*). (ii) A component (X), resembling H2A in Triton affinity but not in staining color, is synthesized only until about 5 hours after fertilization (1 to 16 cells), at which time one of the later H2A-

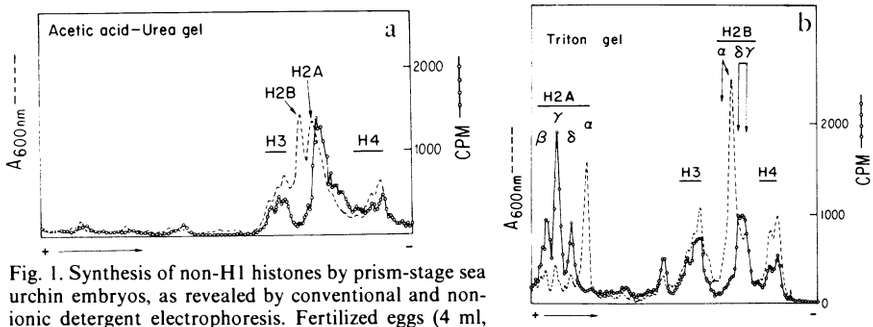


Fig. 1. Synthesis of non-H1 histones by prism-stage sea urchin embryos, as revealed by conventional and nonionic detergent electrophoresis. Fertilized eggs (4 ml, packed volume) of *Strongylocentrotus purpuratus* (Pacific Biomarine Supply) were cultured in artificial seawater (*21*) at 17°C. At 42 hours after fertilization, embryos were incubated for 2 hours in 400 ml of seawater containing [^{3}H]leucine (2.5 μc/ml) (Schwarz/Mann; specific activity 52 c/mmole). Embryos were then washed twice in seawater, twice in 0.8*M* dextrose containing a mixture of 0.1*M* NaCl, once in 0.2*M* dextrose and 0.15*M* NaCl, and then lysed by blending in a solution of 30 ml of 50 m*M* glycine, 10 m*M* potassium-trismaleate buffer (*p*H 7.3), 10 m*M* mercaptoethanol, and 5 m*M* $MgCl_2$ at 45 volts (low setting) for 1 to 2 minutes, until most but not all of the embryos were disrupted. The homogenate was then filtered through nylon filters (Nitex) with 48- and 20-μm pore sizes. Nuclei were isolated and washed with Triton X-100 and with saline EDTA, and histones were extracted, as described (*6*). The non-H1 histones were prepared by precipitation with 5 percent $HClO_4$ (*22*). Electrophoresis was performed in polyacrylamide gels (4 mm in diameter by 130 mm long). Gels were stained with amido black, scanned with a Gilford spectrophotometer, and sliced into 0.5-mm slices with a Joyce-Loebl gel slicer. (a) Electrophoresis in a 15 percent polyacrylamide gel containing 0.9*M* acetic acid and 2.5*M* urea (*8*). (b) Nonionic detergent electrophoresis, in a 12 percent polyacrylamide gel containing 0.9*M* acetic acid, 8*M* urea, and 6 m*M* Triton X-100 (*6*).

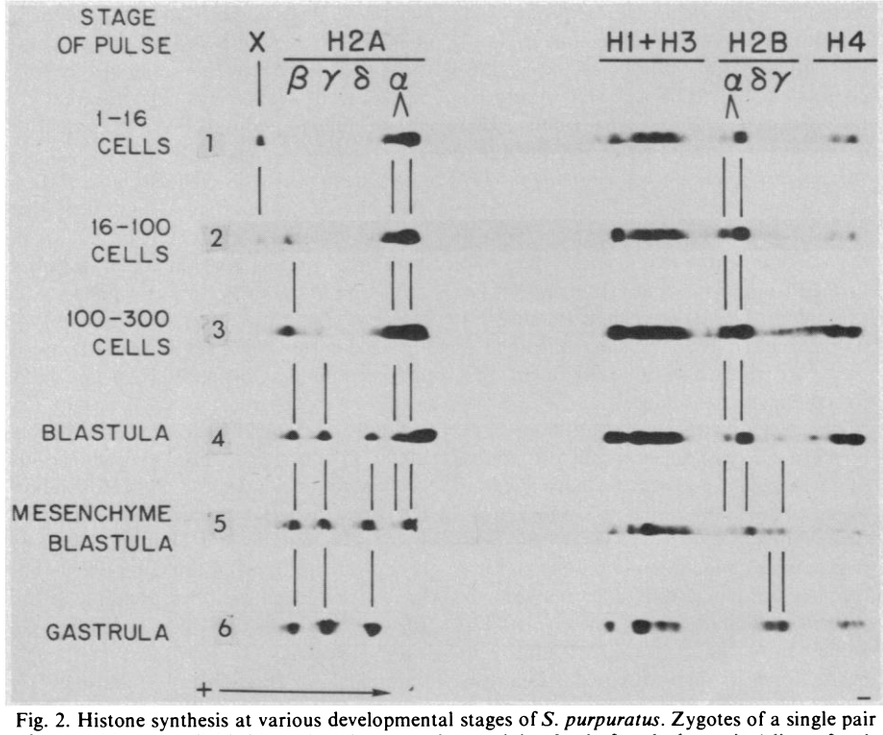
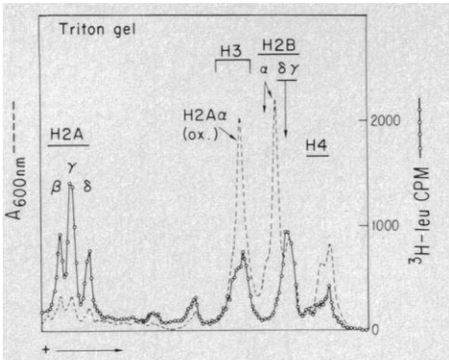


Fig. 2. Histone synthesis at various developmental stages of *S. purpuratus*. Zygotes of a single pair of sea urchins were divided into six cultures, each containing 2 ml of packed eggs in 1 liter of artificial seawater (*21*). Embryos were labeled with [^{3}H]leucine (43 c/mmole) during the following stages and for the time periods indicated after fertilization: (culture 1) 1 to 16 cells, 0.5 to 4.5 hours; (culture 2) 16 to 100 cells, 5 to 10 hours; (culture 3) 100 to 300 cells, 10 to 16 hours; (culture 4) blastula stage, 15 to 19 hours; (culture 5) mesenchyme blastula stage, 20 to 24 hours; (culture 6) during gastrulation, 25 to 40 hours. Isotope concentrations for individual cultures were: culture 1, 7 c/ml; cultures 2 and 3, 3.5 c/ml; and cultures 4 to 6, 1.5 c/ml. At the end of the labeling period, unlabeled leucine (final concentration 20 μ*M*) was added, and the embryos were concentrated and resuspended in seawater containing 2.5 μ*M* unlabeled leucine. Histones were isolated from the various cultures at the following times after fertilization: cultures 1 to 4 at 19 hours, culture 5 at 25 hours, and culture 6 at 40 hours. Cell numbers were estimated from embryo age (*14*), and a slight adjustment was made for incubation temperature (here 17°C). Electrophoresis was carried out as in Fig. 1b in gels 1.8 mm in diameter and 200 mm long. The gels were then infiltrated with 2,5-diphenyloxazole for fluorography (*23*).



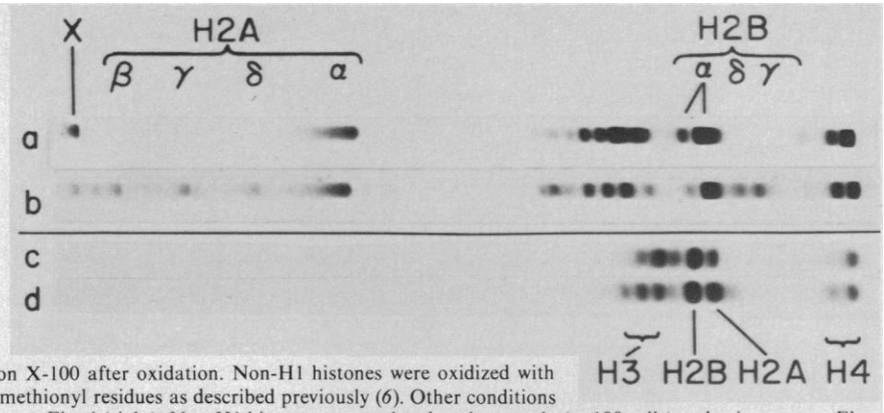


Fig. 3 (left). Decreased affinity of H2A for Triton X-100 after oxidation. Non-H1 histones were oxidized with hydrogen peroxide under conditions that oxidize methionyl residues as described previously (*6*). Other conditions and procedures were the same as those in Fig. 1b. Fig. 4 (right). Non-H1 histones accumulated at the morula (~ 100 cells) and prism stages. Electrophoretic conditions were as in Fig. 1, but in gels 1.8 mm in diameter. (a) Nonionic detergent electrophoresis of morula histones; (b) nonionic detergent electrophoresis of prism-stage histones; (c) acetic acid–urea electrophoresis of morula histones; and (d) acetic acid–urea electrophoresis of prism-stage histones.

like components (β) first begins to be synthesized. (iii) Between 15 and 24 hours after fertilization (blastula and mesenchyme blastula) four components begin to be synthesized for the first time, two with H2A and two with H2B characteristics (γ and δ). At the same time, the synthesis of the α components decreases until, by about 25 hours, it is no longer detectable. (iv) In each case, synthesis of the γ component starts before that of the δ component. The synthesis of the γ and δ components of H2A appears to precede the synthesis of the corresponding H2B components. This, however, may be due to the much higher leucine content of H2A histones and thus to a difference in sensitivity of detection of synthesis. (v) None of the components synthesized late are formed from those synthesized earlier. For example, it can be seen that, in culture 1, no radioactivity was transferred from the α forms to the late synthesized forms after the labeling was stopped.

The fact that the various histone components are synthesized at different stages of development and are not interconverted suggests that they differ in primary structure rather than as a result of post-translational modification. Support for a primary structural difference between the α form and the β, γ, and δ forms of H2A comes from the fact (Fig. 3) that only $H2A_\alpha$ loses its high affinity for detergent upon oxidation with hydrogen peroxide under conditions for methionine oxidation (*6*). Data from our laboratory on the isolation and analysis of this protein has confirmed the presence of one methionyl residue. In this respect $H2A_\alpha$ resembles drosophila H2A (*6*) and a minor variant (observed in our laboratory) of mammalian H2A, whereas the β, γ, and δ forms behave like the major H2A histone of vertebrates, which lacks methionine (*11*).

There is no indication from our data of similar stage-specific forms of histones H3 or H4. There are several additional unidentified minor components of the histone fraction of sea urchin embryos; these are synthesized throughout the embryogenesis period covered by our experiments.

Examination of stained gels (Fig. 4) shows that early in development (10 hours) (Fig. 4a) the embryo does not contain detectable amounts of the late synthesized forms of H2A or H2B and that the early synthesized protein X is a prominent component. It is evident that none of the early synthesized forms has disappeared by prism stage (42 hours) (Fig. 4b), long after their synthesis has virtually ceased, in agreement with the frequent observation that histones other than H1 are degraded slowly, if at all (*5, 13*). The relatively small amount of protein X at the prism stage is a reflection of the early time at which its synthesis is shut off (~ 16 cells). The late synthesized components (β, γ, and δ) are not the predominant forms of H2A and H2B at 42 hours despite the fact that they are virtually the only forms synthesized after about 25 hours, probably a consequence of the reduction in the rate of cell division (*14*) after the onset of their synthesis.

In gels that do not contain detergent (Fig. 4, c and d) all of the components behave like typical histones, running in the region containing H3, H2A, and H2B.

Our findings suggest that, although chromatin at all stages of embryogenesis contains histones with the molecular characteristics of histones H2A and H2B, different forms of these histones are synthesized at different stages. The synthesis of one form during cleavage and of two different forms after the blastula stage is similar to the observed changes in histone H1 synthesis (*4, 5*). In separate experiments, we have found that the synthesis of the late H1 histones begins substantially before that of the γ and δ forms of H2A and H2B. Since all histone variants persist even after they cease to be synthesized, the complexity of the histone complement increases substantially as development proceeds. This complexity cannot be ascribed to genetic polymorphism since the number and type of components as well as the developmental sequence in which they appear is reproducible in different matings.

Histones H2A and H2B appear to have been more conserved in structure during evolution than most proteins other than histones H3 and H4 (*6, 15*). Our observations, moreover, raise the possibility that even the previously observed variation may not be solely phylogenetic but may stem in part from differences in the tissues and developmental stages compared. This evolutionary stability indicates an unusually strict dependence of function on structure, encouraging the view that the observed changes during development have functional consequences.

The existence of multiple forms of H2A and H2B is of special interest because these histones, together with H3 and H4, are thought to play a role in the architecture of the basic unit of chromatin structure, the ν body or nucleosome (*16, 17*). *Thus there must exist multiple forms of nucleosomes.* The number of nucleosome types resulting from relatively few forms of each histone class could be quite large if different forms of a given histone class are present in the same nucleosome, because there appear to be more than one molecule of each class per nucleosome (*17*). Furthermore the observation that the complement of histones differs greatly at different stages implies that nucleosome composition is related to the stage of development. The fact that the synthesis of the late histone forms is accompanied by the virtual cessation of synthesis of the early (α) components indicates that the late forms are distributed through the subsequently synthesized chromatin, possibly affecting some general functional property of chromatin important in development.

The time of development during which these changes occur is one of cell diversification, including differential decreases in the mitotic rates of different cell types. The relation between the histone changes and these events may be clearer when we know whether specific late histone forms are confined to specific cell types or specific regions of the genome (or both). The persistence of the early histone forms after the synthesis ceases raises the question of where these forms reside in subsequently synthesized chromatin. The answer to this question would reveal much about the assembly of chromatin during DNA replication.

There are few clues to the mechanisms of these developmental modulations in histone synthesis. Although messenger RNA (mRNA) that codes for histones begins to be synthesized within a few cell divisions after fertilization (*18*), actinomycin D prevents neither histone synthesis nor morphogenesis until the blastula stage (*5, 19*). In the presence of actinomycin, therefore, histone synthesis represents translation of a store of histone mRNA in the unfertilized egg (*19, 20*). Since at least two of the switches in histone synthesis (X-off and $H2A_{\beta}$-on) occur well before the stage at which development becomes sensitive to actinomycin, these particular switches either are under translational control or do not occur in the presence of actinomycin and are not needed to reach a morphologically normal blastula stage. In contrast, the synthesis of the γ and δ forms of H2A and H2B does not begin until a stage when mRNA synthesis is required for further development. Whether the synthesis of these forms depends on new transcription remains to be determined.

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Prostaglandins in Rabbit Blastocysts

Abstract. *Rabbit blastocysts recovered at 144 hours post coitum contained the prostaglandins F and E-A. We suggest that one or more of these prostaglandins act as mediators in blastocyst steroidogenesis. (In another study we have demonstrated steroidogenesis in rabbit blastocysts.)*

There is strong evidence that steroid hormones are synthesized by morulae and blastocysts of rats (*1*), mice (*2*), hamsters (*3*), and rabbits (*4, 5*). Various approaches could be used to obtain more information about steroidogenesis in preimplantation embryos. Since it has been suggested by a number of authors (*6*) that prostaglandins (PG's) act as mediators in steroidogenesis, the present study was made to determine whether rabbit blastocysts contain PG's.

Sexually mature female New Zealand rabbits (Langshore Rabbitry, Augusta, Mich.) were mated and immediately thereafter injected intravenously with 50 international units (I.U.) of human chorionic gonadotropin (hCG) to ensure induction of ovulation. At 144 hours post coitum, the rabbits were killed and their uteri excised and flushed with 5 ml of saline (0.9 percent NaCl) in order to recover blastocysts. The saline contained indomethacin (10 μg/ml) to inhibit possible PG synthesis while handling blastocysts. The blastocysts were washed three times in saline-indomethacin and were then homogenized in saline-indomethacin, extracted with a mixture of ethyl acetate, isopropanol, and 0.1*N* HCl (3 : 3 : 1) according to Orczyk and Behrman (*7*), and radioimmunoassayed without chromatographing the extracts. For the assay of PGF an antibody previously described (*8*) was used. A second antibody used reacts equally with PGE and PGA, but does not cross-react with PGF; thus the PG measured in this assay is referred to as PGE-A.

In addition to blastocysts, uterine flushings from rabbits that were 144 hours pregnant or 144 hours pseudopregnant (injected with 50 I.U. of hCG to induce ovulation) were similarly extracted and assayed.

Table 1. Prostaglandins (PG's) F and E-A in groups of rabbit blastocysts recovered at 144 hours post coitum.

Sample No.	Rabbit No.	PG's per sample (ng)		Blastocysts per sample (No.)
		F	E-A	
1	1, 1a	8.6	7.3	15
2	2	8.6	8.7	10
3	3	9.7	7.0	11
4	4	7.3	3.8	7
5	5	5.8	6.7	5
6	6	1.3	2.7	8

Table 2. Prostaglandins (PG's) F and E-A in uterine flushings of pregnant (P) and pseudopregnant (PSP) rabbits at 144 hours after an ovulation-inducing injection.

Rabbit No.	PG's from both uteri (ng)	
	F	E-A
4 (P)	10.8	5.6
5 (P)	7.0	5.9
6 (P)	2.6	2.6
7 (PSP)	3.9	3.8
8 (PSP)	4.9	7.0