able amounts of free rabbit ^{14}C-β-chains. Additional studies showed that 90 percent of the ^{14}C radioactivity in the pure rabbit hemoglobin band of Fig. 2 was located in the β-chain. This observation implies that during protein synthesis unlabeled rabbit α-chains combine with newly synthesized ^{14}C-β-chains to form completed rabbit hemoglobin. Presumably, these rabbit α-chains are endogenous to the ribosome and enzyme fractions of the incubation mixture.

We suggest that, in rabbit hemoglobin synthesis, growing α-chains are released from the polyribosomes to the solution when their primary-sequence synthesis is completed. Synthesized β-chains are brought into the molecule finally by complementation with these α-chains. Complementation may occur with the β-chains still growing on the polysome. Colombo and Baglioni have shown that polysomes isolated from reticulocytes contain about one complete α-chain for every 5.5 being synthesized (*12*). Others have suggested that soluble α-chains might form complexes with growing β-chains on the polysome and aid in their completion and release (*13*). Therefore, in our experiments addition of human β-chains to the cell-free system during active protein synthesis might indirectly inhibit release of rabbit β-chains by depleting the supply of rabbit α-chains through α_2^{RAB} β_2^{A} hybrid formation. However, there also exist alternative explanations for the observed effect of human β-chains on the release of rabbit β-chains.

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4. The rabbit reticulocyte cell-free system was reconstituted according to E. H. Allen and R. S. Schweet [*J. Biol. Chem.* **237**, 760 (1962)]. The usual mixture contained 2.4 mg of ribosomes, receiving a single centrifugation and 3 mg of a soluble enzyme fraction purified by ammonium sulfate precipitation at *p*H 6.5 (*5*) in a final volume of 0.84 ml. Each assay contained 0.025 μmole of L-^{14}C-leucine (231 mc/mmole) and 0.025 μmole of L-^{14}C-valine (208.5 mc/mmole); incubation at 37°C for 60 minutes.
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9. Chain separation of the globin from the incubation without added human chains revealed that 29 percent of the radioactivity was in the α-chain peak, and 71 percent was in the β-chain peak. The reason for this disparity in the radioactivity of the soluble chains of the cell free system is not yet clear.
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15. For electrophoresis, 5 μl of the sample was applied to a cellulose acetate strip (2.5 × 17.1 cm) (Sepraphore III, Gelman Instrument Co.) wet with the electrophoresis buffer (tris-barbital-sodium barbital, *p*H 8.8; ionic strength = 0.025). The electrophoresis was run at 300 volts (approximately 1.5 ma per strip) and 4°C for 90 minutes. The strips were stained with Ponceau S in 5-percent trichloroacetic acid, washed with 5-percent acetic acid, and allowed to dry. The strips were cut up in sections (0.20 cm wide) for liquid-scintillation counting (*14*). In certain experiments, globin was eluted from unstained strips.
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"Blastokinin": Inducer and Regulator of Blastocyst Development in the Rabbit Uterus

Abstract. *A protein fraction that induces blastulation of rabbit morula and stimulates blastocyst development has been isolated from the uterus of the rabbit at early pregnancy. Partial purification of this fraction indicates that the activity is restricted to a single homogeneous protein component. The term "blastokinin" is proposed to describe the compound.*

Development of mammalian ova in vitro is arrested by the early blastocyst stage (*1, 2*). Even under the most favorable culture conditions known, only initially cavitated blastocysts have been obtained from fertilized rabbit ova or any intermediate cleavage stage in vitro. Rarely any progress much beyond this stage has been noticed. Typically, 3-day rabbit morulae will grow to the stage of initial cavitation in 12 to 24 hours in media supplemented with serum; thereafter they cease to grow, or they expand very slowly over a period of several days. However, somewhat older embryos (5- to 6-day blastocysts) can be grown in vitro for several days but at a growth rate that continues to decline relative to that occurring in vivo (*3, 4*). Although slightly better growth rates have been obtained with each new modification of the medium, it has not yet been possible to get continued growth of blastocysts, nor growth of cleavage-stage embryos beyond initial cavitation, comparable to the normal.

Obviously, the uterine environment in some way provides for the regulation of these phenomena. This regulation may be brought about by a single component or may be the result of proper balancing in the uterus of other, more common components of relatively small molecular size, inadequately provided for in the culture media. So far as is known, all evidence tends to exclude the latter possibility. The inference then follows that, should there be such a regulator, it is probably a macromolecular component. We present evidence that such a component occurring at the time of blastulation and blastocyst development can induce and regulate these processes.

Table 1. Effect of supplementation of Ham's F10 with "blastokinin" on growth and development of 3-day rabbit morulae into blastocysts, compared to that of supplementation with other proteins.

Protein concentration (mg/ml)	Embryos used (No.)	Cavitated in 24 hr (No.)	Expanded in 24 hr (No.)
Control (Ham's F10)			
	24	12	(1?)
"Blastokinin"			
0.05	10	10	0
.10	10	10 (2?)	2 (1?)
.20	14	13	10
.40	10	10	6
0.60	11	6	4 (1?)
1.00	10	5	3
2.00	6	0	0
10.00	6	0	0
Complete uterine-fluid protein, 5 days after coitus			
0.30	10	5	0
.50	14	12	11
1.00	12	4	2
3.00	8	5	0
10.00	11	8	0
Maternal serum proteins			
0.30	10	8	0
3.00	12	10	(2?)

We collected uterine fluids either by flushing the isolated uteri with Ham's F10 solution (*5*) or by gently absorbing the fluid contents of the lumen with small filter-paper disks, taking care to avoid contamination by blood or tissue debris. In the latter case, the filter-paper disks were then liberally extracted with 0.33*M* NaCl at 4°C. Since there was essentially no difference between the two methods with respect to the proteins and other macromolecules, the latter was adopted routinely for qualitative studies, while the former was the method of choice in obtaining large amounts of uterine fluids for preparative purposes.

The extracts of uterine fluid were then lyophilized, and the protein components were separated by gel filtration through Sephadex G-200 in 0.02*M* phosphate-citrate buffer, *p*H 7.4, and determined quantitatively according to Lowry's procedure (*6*). A comparison of the gel-filtration profiles thus obtained (Fig. 1) shows the onset of occurrence of a new protein (indicated by the arrows) at a time just before blastulation of the embryo in vivo. The quantity of this protein in the fluid increases rapidly, reaching a maximum at approximately 5 days after coitus; it then declines to near zero by the 9th day (Fig. 1A).

A similar analysis of blastocoelic fluid taken from 6-day blastocysts revealed that it is one of the major protein components in the embryonic fluid (Fig. 1B). Nonetheless, it is difficult to conceive that such large quantities of the particular protein, as are present in the uterine fluids, represent the metabolic product of these embryos. This is further substantiated by the fact that uterine fluid collected from pseudopregnant rabbits 7 days after the injection of chorionic gonadotropin (Follutein, Squibb) contained significant amounts of the protein comparable to that of normal rabbits 7 days after coitus. Rather strikingly, however, it is totally absent in maternal serum, fetal serum, fetal amniotic fluid (24 days), or even in the uterine fluid accumulated in the uterus by ligation during 3 to 10 days of gestation (Fig. 1B). The implication is thus very strong that the uterus, in the early phase of normal or simulated pregnancy, secretes into its lumen a certain protein which is, in some way, associated with controlled and continuous development of the embryo before implantation.

To determine whether the same protein fraction would ensure the orderly development and growth of early rabbit embryos in an otherwise unfavorable environment in vitro, we isolated a quantity of the protein by gel filtration of uterine fluids through Sephadex G-200 as previously. Subsequent chromatography of the isolated protein fraction through Sephadex G-75 in 0.02*M* phosphate-citrate buffer (*p*H 7.4) and acrylamide-gel disk electrophoresis in tris(hydroxymethyl)aminomethane-glycine buffer (*p*H 8.6) indicated predominantly a single homogeneous component. Its molecular weight is estimated by gel-filtration techniques to be approximately 27,000. The biological activity was tested in the following manner.

Morulae were flushed from the oviducts (or uteri) of rabbits 3 days after coitus. They were transferred to small watch glasses (10 mm diameter) containing 0.1 ml of culture medium (Ham's F10) and held inside petri dishes adequately humidified with moist, sterile cotton. The rest of the culture procedure was essentially similar to that described elsewhere (*4*). Protein supplementations were made to the basal culture medium as indicated in Table 1. The cultures were observed after 24 hours of incubation and scored for the number of embryos that had cavitated and exhibited expansion and growth as blastocysts.

In the presence of the protein fraction in question in concentrations up to 0.4 mg/ml nearly all the embryos underwent cavitation; at 0.2 mg/ml, 72 percent of the embryos expanded and began growth as blastocysts (Table 1). Similarly, the complete protein component of the uterine fluid at a con-

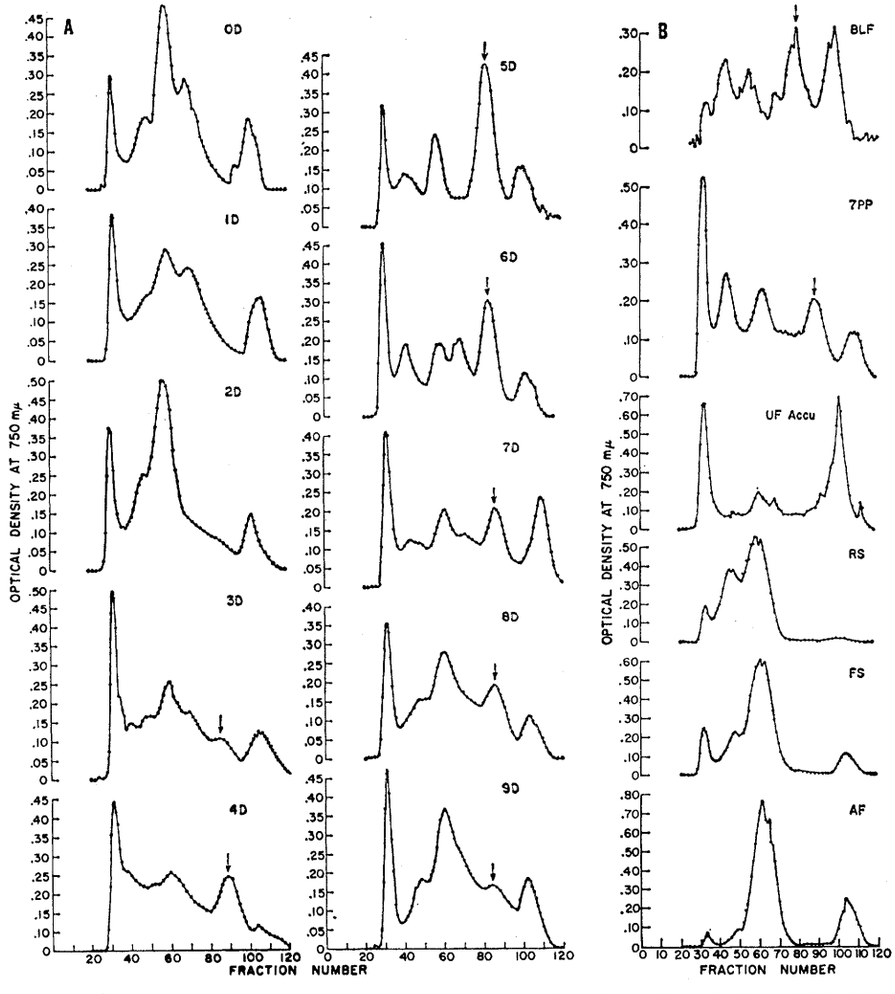

Fig. 1. (A) Gel filtration of rabbit uterine-fluid proteins, from 0 to 9 days (*OD–9D*) of pregnancy, through Sephadex G-200 in 0.02*M* phosphate-citrate buffer (*p*H 7.4). The patterns are normalized to 1 mg of total protein in each case. Arrows indicate the central protein of this study. (B) Gel filtration through Sephadex G-200 of blastocoelic fluid (*BLF*), uterine fluid from 7-day pseudopregnant rabbit (*7PP*), uterine fluid accumulated by ligation (*UF Accu*), rabbit (maternal) serum (*RS*), fetal serum (*FS*), and fetal amniotic fluid (*AF*). Arrows indicate the same protein as in Fig. 1A.

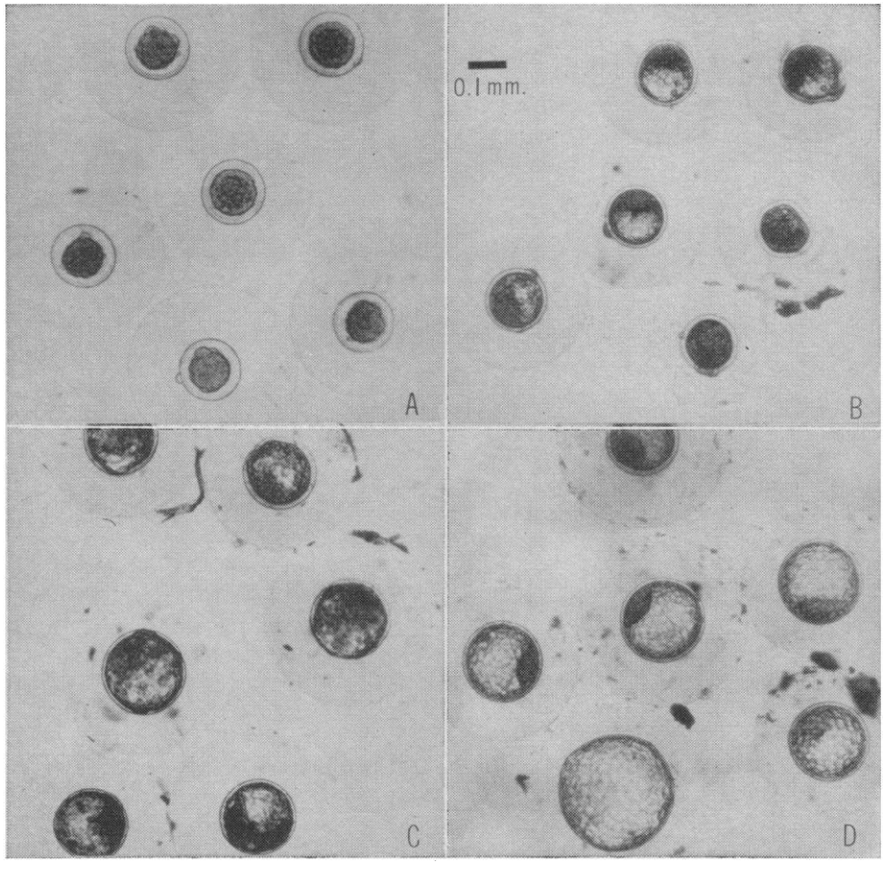

Fig. 2. Photomicrograph of 3-day rabbit morulae cultured in vitro for 24 hours in Ham's F10. (A) Normal 3-day morulae; (B) cultured in the presence of 0.3 mg of rabbit serum proteins per milliliter; (C) cultured in the presence of 0.2 mg of "blastokinin" per milliliter; (D) normal 4-day blastocysts. All pictures were taken at the same magnification.

centration of 0.5 mg/ml caused 78 percent of the embryos to expand. On the contrary, maternal serum proteins did not promote comparable expansion of embryos of these stages. This finding rules out the possibility that expansion and continued growth of blastocysts in vitro is a mere protein effect, thereby suggesting that early rabbit embryos cultured in vitro require a specific protein to continue their controlled development and growth. When one considers the absence of the specific protein from maternal serum, the ineffectiveness of supplementation with serum protein is not surprising.

Embryos cultured in vitro in the presence of the specific protein (see Fig. 2) are not exactly comparable in appearance to normal 4-day blastocysts. The trophoblast appears to be composed of extra layers of cells; nevertheless the size of the blastocysts is comparable to those grown in vivo. Although the cells have been replicating, total expansion appears limited, possibly by a tight zona pellucida. This assumption seems reasonable since cultured rabbit embryos can more easily expand when the zona pellucida is removed or weakened by the use of pronase (*2*).

The demonstration that a specific protein fraction isolated from the maternal uteri can induce and regulate blastocyst development in embryos of the same species strongly suggests the existence of endogenous substances that control blastulation. When one considers the effectiveness of appropriate concentrations of the complete uterine-fluid protein component in promoting cavitation and expansion of blastocysts, it becomes apparent that the substance (or substances) is a normal rather than a fortuitous agent. We propose that the term "blastokinin" be used to describe those substances which appear to function as endogenous regulators of blastocyst development.

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Intracellular Pool of Unhydroxylated Polypeptide Precursors of Collagen

Abstract. *The hydroxyproline and hydroxylysine in collagen are synthesized by an apparently unique pathway in which proline and lysine are hydroxylated after they are incorporated into a large polypeptide precursor of collagen called protocollagen. When the hydroxylation of protocollagen in isolated tissues is intermittently interrupted, hydroxylation can occur after complete polypeptides are released from ribosomal complexes. Cartilage from chick embryos was incubated with the iron chelator α,α'-dipyridyl for 2 hours to inhibit protocollagen hydroxylase, and then the inhibition was reversed by transferring the tissues to medium containing ferrous iron and no α,α'-dipyridyl. "Pulse labeling" of the tissues during these two periods indicated that both the accumulated protocollagen and the polypeptides synthesized after reversal of the inhibition were hydroxylated at the same rate. Even when no measures are taken to inhibit the hydroxylation of protocollagen, most of the hydroxyproline in collagen is probably synthesized after complete protocollagen polypeptides are released from ribosomes.*

Hydroxyproline (*1*) and hydroxylysine (*2*) in collagen are synthesized by an apparently unique pathway in which proline and lysine are hydroxylated after they have been incorporated into a large polypeptide precursor of collagen called protocollagen. The enzyme protocollagen hydroxylase hydroxylates the proline and probably the lysine in protocollagen (*3*), and both hydroxylations require ascorbate, α-ketoglutarate, atmospheric oxygen, and ferrous iron (*1*, *3*, *4*). When tissues synthesizing collagen are incubated under anaerobic conditions or