conversion of tyramine to octopamine are stimulated by extracts from cockroach ganglia and hemolymph (*7*), and that octopamine itself is present in high concentrations in the thoracic nerve cord of the lobster (*6*). Unfortunately, the absolute levels of octopamine are not known for the thoracic ganglia of the cockroach. In addition, although it has been suggested that octopamine might function as a primary neurotransmitter in those invertebrate species in which it is found in high concentrations (*6*), there have been as yet no reported investigations of possible electrophysiological effects of octopamine on invertebrate nerve cells. It is known, however, that norepinephrine and dopamine, both of which increased ganglionic cyclic AMP in our experiments, are excitatory when applied to neurons of the cockroach abdominal ganglia (*15*). Because octopamine also increased cyclic AMP in intact ganglia, it will be important to determine whether this phenolic amine has any effects on the electrical activity of nerve cells in this or similar ganglia. In this regard, it is of interest that octopamine-stimulated adenylate cyclase activity, in our experiments, was 2.2-fold higher in cockroach thoracic ganglia themselves than in the interganglionic nerve connectives (data not shown), suggesting that the enzyme may be localized more in nerve cell bodies and synaptic areas than in axons (of which the connectives are composed).

It has been shown (*8*) that octopamine can activate phosphorylase in cockroach nerve cord, and it was suggested that this glycogenolytic effect of octopamine in insects might be mediated through an increase of cyclic AMP. If the activation of phosphorylase by octopamine in the cockroach is, in fact, mediated through an increase in cyclic AMP, a possibility supported by our results, this would not rule out a possible role (*6*) of octopamine as a neurotransmitter. Indeed, our demonstration in invertebrate ganglia of an adenylate cyclase specifically sensitive to low concentrations of octopamine provides a possible mechanism by which this phenolic amine could be involved both in the regulation of carbohydrate metabolism as well as in the physiology of synaptic transmission.

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9. Pro-, meso-, and metathoracic ganglia were dissected from adult male cockroaches (Ward Scientific), cleaned of adhering fat, and, in some cases, desheathed. The ganglia were kept until use in a cold, oxygenated insect Ringer solution that contained (in millimoles/liter): NaCl, 214; $CaCl_2$, 9; KCl, 3.1; $MgSO_4$, 1.2; $KH_2PO_4$, 0.4; $NaHCO_3$, 25; and D-glucose, 10. This buffer had been equilibrated with a mixture of 95 percent $O_2$ and 5 percent $CO_2$ and had a *p*H of 7.4 at 23°C. For broken cell studies, the tissue was washed once in calcium-free Ringer solution and then homogenized (20 mg/ml) in 6 m*M* tris-maleate buffer (*p*H 7.4) containing 2 m*M* EGTA [ethylene glycol bis(aminoethyl ether)tetraacetate]. Adenylate cyclase activity was measured in an assay system containing (in millimoles per liter): tris-maleate (*p*H 7.4), 80; theophylline, 10; $MgSO_4$, 2; EGTA, 0.5; adenosine triphosphate (ATP), 0.5; and tissue homogenate (0.5 mg wet weight), plus test substances as indicated, in a final volume of 0.2 ml. The standard incubation was for 3 minutes at 30°C in a shaken water bath. The reaction was initiated by the addition of ATP, terminated by boiling for 90 seconds, and then centrifuged at low speed to remove insoluble material. Cyclic AMP in the supernatant was measured by the method of B. L. Brown, R. P. Elkins, J. D. M. Albano [*Advan. Cyclic Nucleotide Res.* **2**, 25 (1972)]. Under the experimental conditions used, enzyme activity was linear with respect to time and enzyme concentration.
10. For measurement of phosphodiesterase activity, assay conditions were identical to those described above (*9*), except that 3 pmole of cyclic AMP was used in place of ATP, theophylline was sometimes omitted, and the incubation time varied from 1 to 15 minutes. Under these conditions, control activity (rate of disappearance of added cyclic AMP) per milligram of protein was $15 \pm 1$ pmole/min. Activity in the presence of either 250 $\mu M$ *dl*-octopamine, dopamine, serotonin, or L-norepinephrine was not significantly different from control.
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13. To measure the accumulation of cyclic AMP in intact tissue, ganglia were hemisected into left and right halves and given preliminary incubation in oxygenated insect Ringer's for 20 minutes at 23°C. Then 8 to 12 half-ganglia were transferred to each incubation tube, which contained 1.0 ml of insect Ringer solution (*9*) and the appropriate test agent; the tubes were incubated for 10 minutes at 23°C with continuous oxygenation; the insect Ringer's was then aspirated, and 0.65 ml of cold 98 percent ethanol–0.2*N* HCl was added, and the tissue was immediately homogenized. The homogenate was centrifuged, the supernatant was removed and evaporated to dryness, and cyclic AMP was assayed (*9*). The precipitate was dissolved in 0.8 ml of 1.1*N* NaOH and assayed for protein [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)] with bovine serum albumin as the standard.
14. The additivity studies (Table 1), dose response relationships (Fig. 1A), and use of homogenates make it most unlikely that the observed effects of octopamine on adenylate cyclase activity were due to release of endogenous dopamine, norepinephrine, or serotonin from stores within the thoracic ganglia.
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29 December 1972

## Activation of Hemoglobin C Synthesis in Sheep Marrow Culture

Abstract. *Erythropoietin preferentially stimulates hemoglobin C synthesis in suspension cultures of marrow cells from sheep homozygous for hemoglobin A; the amount of synthesis is dependent on the dose of erythropoietin and is blocked by antiserum to erythropoietin. The results provide the first in vitro evidence that erythropoietin mediates the hemoglobin A → C "switch" in sheep and indicate that bone marrow cultures may be used to investigate the mechanisms involved in the preferential gene activation characteristic of the hemoglobin A → C system.*

Goats and sheep possess the unusual property of synthesizing a new hemoglobin under the influence of a variety of erythropoietic stimuli (*1*). Thus, when sheep homozygous for hemoglobin A (Hb A) are made anemic, their hemoglobin type switches to C (Hb C) because of the selective synthesis of $\beta^C$ globin chains (*2*). No such alteration is seen in animals homozygous for hemoglobin B (Hb B). These alterations in hemoglobin synthesis, under the influence of external factors, are of particular interest in that they provide a model for study of the mechanisms involved in differential gene activation in higher organisms. While several lines of in vivo study have provisionally identified the switching factor as erythropoietin (ESF), investigation into the mechanisms of the switch has been limited by the size of the animal and the length of time for the effect to be seen. We report that the hemoglobin A → C switch may be induced in vitro in suspension cultures of sheep marrow cells from animals with Hb A.

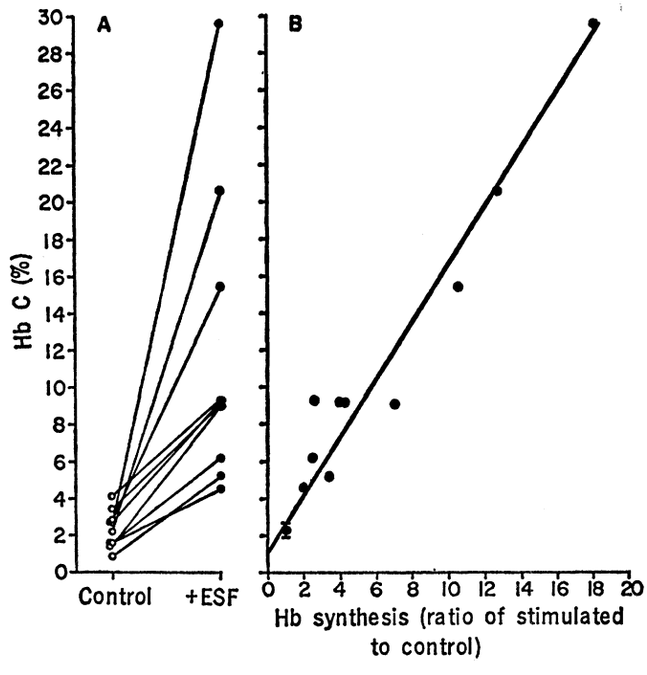
Five healthy and hematologically

normal sheep, shown to have only Hb A by hemoglobin electrophoresis, were studied. Cell preparation and culture techniques were modified from those of Krantz *et al.* (*3*). Bone marrow cells were aspirated under sterile conditions from the posterior iliac crest of unanesthetized animals, and the aspirate was placed immediately into 5 to 10 ml of cold Hanks balanced salt solution (BSS) (Grand Island Biologicals) containing heparin (10 unit/ml). The cells were centrifuged at 4°C for 10 minutes at 200*g*, washed twice with additional BSS, and suspended at a final concentration of $5 \times 10^6$ trypan blue dye–excluding cells per milliliter of NCTC-109 (Microbiological Associates) containing 10 percent autologous serum. A 1-ml portion of cell suspension was added to each culture dish (10 by 35 mm) (Falcon Plastics); one or two culture dishes were used for each data point. Human ESF of high potency (*4*) was added to appropriate cultures. The suspensions were incubated for 36 hours in a mixture of 95 percent air and 5 percent $CO_2$ at 100 percent humidity in a tissue culture incubator at 37°C. At that time, either homologous transferrin labeled with 0.5 to 1.0 $\mu$c of $^{59}$Fe (10 to 25 mc/mg; New England Nuclear) or 10 $\mu$c of [$^{14}$C]-leucine (23.3 mc/mmole; Amersham/Searle) was added to label heme or globin, respectively. The incubation was then continued for an additional 12 hours. The cells were harvested and washed twice with phosphate-buffered saline (*p*H 7.35). Lysates prepared from cultured cells were chromatographed on diethylaminoethyl Sephadex (A-50) (*5*) in the presence of unlabeled carrier Hb A and Hb C. The eluted hemoglobin peaks were concentrated, and the relative synthesis of Hb C was estimated from the radioactivity in the Hb A and Hb C peaks. Globin chains were separated by carboxymethyl (CM-) cellulose chromatography by the method of Clegg *et al.* (*6*).

The proportion of newly synthesized Hb C in the control (no ESF) cultures, was 2.34 ± 0.32 percent (standard error of mean) as determined by heme labeling in ten experiments, and was 5.60 ± 1.25 percent as determined by globin labeling in four experiments. In the $^{59}$Fe experiments, a 2- to 18-fold stimulation of total hemoglobin synthesis was achieved with ESF (*7*), with a preferential synthesis of Hb C (Fig. 1A). In addition, the content of Hb C was directly related to the overall degree of in vitro response (Fig. 1B). Thus, the amount of Hb C increased as much as 200-fold during the culture period, while that of Hb A increased 13-fold (*8*).

Carboxymethyl cellulose chromatography of globin chains from control cultures after [$^{14}$C]leucine labeling demonstrated that no discernible radioactivity was in the $\beta^C$ peak. With ESF stimulation, however, specific and approximately equal labeling was observed in separated $\beta^C$ and $\alpha^C$ peaks, results confirming new $\beta^C$ chain synthesis (Fig. 2).

In several simultaneously established



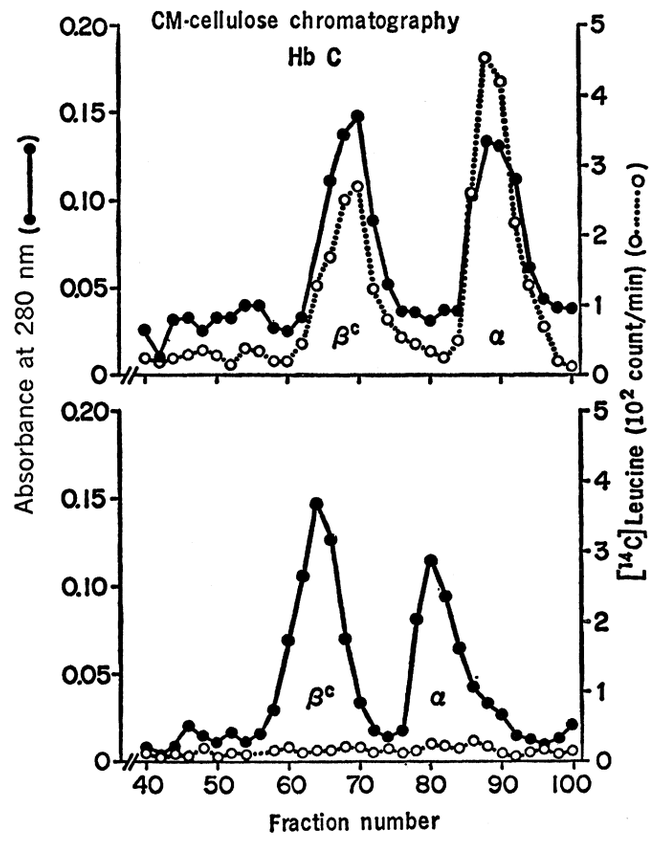




Fig. 1 (left). Hemoglobin C synthesis in suspension cultures of sheep bone marrow. The graphs show data for $^{59}$Fe heme radioactivity in chromatographically separated Hb A and Hb C peaks and illustrate the preferential stimulation of Hb C synthesis by ESF. (A) The effect of ESF on the proportion of Hb C in ten separate experiments is shown. Differences in responsiveness may be attributed to differences in culture condition as well as to variations in the quantity of added ESF. (B) The relation between the increase in total hemoglobin synthesis (expressed as ratio of labeled hemoglobin in stimulated cultures to that in control cultures) and the percentage of Hb C in response to ESF is shown. Basal Hb C synthesis (mean ± S.E.M.) for ten studies is indicated (ratio = 1). The correlation coefficient (*r*) is .94, and the origin cannot be separated statistically from the line of regression. Fig. 2 (right). CM-cellulose chromatography of globin chains from isolated Hb C. The absorbance (solid circles) and radioactivity of [$^{14}$C]leucine (open circles) are shown for control cultures (bottom) and ESF-stimulated cultures (top). A concentration gradient of 0.005*M* to 0.03*M* $Na_2HPO_4$ was used to elute chains.

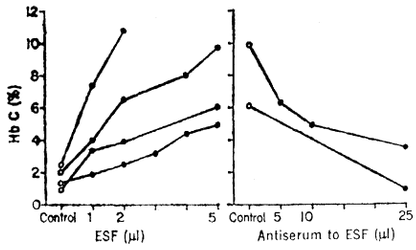



Fig. 3. Relation between ESF dose and percentage of Hb C synthesis in culture and the blocking effect of antiserum to ESF.

cultures, different doses of ESF were added to determine the dose-response relation of ESF to Hb C synthesis. As shown (Fig. 3), there was a direct dose dependence between ESF and Hb C synthesis, which was completely blocked by the simultaneous addition of antiserum capable of neutralizing the biological activity of ESF (*9*).

These studies demonstrate that the switch from Hb A to Hb C synthesis can be induced in suspension cultures of marrow from nonanemic sheep and provide the first in vitro evidence that the switching substance is ESF. Preferential Hb C synthesis resulted from and depended on the dose of added human ESF, an effect that was completely blocked by the addition of neutralizing antiserum. Similar to results for rat marrow cultures, the ESF response in this system is dependent on cell proliferation. Thus, ionizing irradiation in low doses blocks ESF-dependent hemoglobin synthesis.

The high correlation between the percentage of Hb C synthesized in culture and total hemoglobin synthesis in response to ESF (Fig. 1B) points up an important feature that may apply in the intact animal. The marrow response in this system is totally ESF-dependent; since total hemoglobin synthesis and Hb C content are linearly related, the amount of Hb C synthesized in the nonanemic animal may be due to endogenous concentrations of ESF. Thus, any increase above normal in total hemoglobin synthesis predictably should be accompanied by a preferential increase in Hb C synthesis. While these data do not offer information as to the mechanism by which ESF exerts its differential effect, the relatively simple method may lend itself to the elucidation of these mechanisms.

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4. Human urinary ESF was obtained from a patient with aplastic anemia and prepared in this laboratory. Specific activity of the dialyzed lyophilized preparation is 50 to 100 units per milligram of protein. The material was solubilized in BSS and added in microliter quantities corresponding to 0.1 to 0.25 unit per culture dish.
5. Approximately 20 mg of hemoglobin were applied to 2 by 50 cm columns and eluted with 0.05*M* tris(hydroxymethyl)aminomethane hydrochoride buffer (*p*H gradient, 8.2 to 7.3). Radioactivity was confined completely to the hemoglobin peaks.
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8. Radioactivity of Hb C in basal cultures ranged from 8 to 74 count/min, while that of Hb A ranged from 431 to 2041 count/min. With ESF, Hb C radioactivity was 116 to 1665 count/min, and that of Hb A was 963 to 7192 count/min. The percentage of Hb C was calculated as 100 times the radioactivity in Hb C divided by the sum of radioactivity in Hb C and Hb A.
9. Rabbit antiserum against human urinary ESF was prepared by the method of J. Schooley and J. Garcia [*Blood* **25**, 204 (1965)].
10. Supported by NIH research grants HE-06242 and GM-15253, NIH contract 70-2221, and by an NIH (NIAMDD) research career development award to J.W.A. Excellent technical assistance was provided by S. Hornung, E. Lee, N. Lin, and D. Marsh.

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## Somatomedin: Inhibition of Adenylate Cyclase Activity in Subcellular Membranes of Various Tissues

Abstract. *Somatomedin in concentrations between 3 and 20 units per milliliter significantly inhibits the basal activity of adenylate cyclase in crude membrane preparations obtained from homogenates of fat cells, liver, and spleen lymphocytes of the rat, and from chondrocytes and cartilage of chick embryos. The enzyme activity measured in the presence of stimulating hormones (epinephrine, prostaglandin $PGE_1$, parathyroid hormone) is also inhibited in these preparations by somatomedin. These observations may be relevant in a general way to the mechanism of action of growth-promoting substances and to the processes which normally regulate cell growth.*

Somatomedin is the term which has been proposed (*1*) to designate an apparently unique plasma hormone which is responsible for the activities of "sulfation factor" and "thymidine factor," and which is similar to if not identical with the acid ethanol–soluble component of serum which elicits nonsuppressible insulin-like activity (*2*). Although the mechanism of production and the precise physiological significance of somatomedin are not known, it appears to play an important role in mediating the effects of growth hormone on skeletal as well as nonskeletal tissues. This hormone, whose activity may be attributed to a neutral peptide with a molecular weight of about 8000 (*3*), enhances the rate of incorporation of thymidine into DNA (*4*) and uridine into RNA (*5*), and it stimulates the conversion of proline to hydroxyproline in cartilage (*6*). In addition, somatomedin at physiologic concentrations exhibits striking insulin-like effects (*7*) in adipose tissue which have recently been correlated with specific interactions of this hormone with insulin receptors of plasma membranes. Physiological concentrations of somatomedin effectively compete with [$^{125}$I]insulin for receptor sites on isolated fat cells, liver membranes, and isolated chondrocytes (*8*). Since the final purification of somatomedin has not yet been achieved, it is not known whether this biological activity can be attributed to a single peptide or to multiple species of closely related peptides. The possible relationship of somatomedin to other insulin-like pleiotypic peptides has been pointed out previously (*8*).

The present report describes a biochemical effect of a partially purified preparation of somatomedin in a subcellular system—the inhibition of adenylate cyclase activity in crude membrane preparations of various tissues. Similar inhibition of adenylate cyclase activity has been described for insulin in membrane preparations of fat cells (*9*) and liver (*9*), in fat cell ghosts (*10*), and in membranes obtained from *Neurospora crassa* (*11*).