metabolic acidification in a 24-hour period ranged from 0.1 to 0.4 *p*H unit, depending on the specific cell and the population density. The development of additional buffers with a pK_a of 7.5 to 8.0 would further limit that acid shift. A slow alkaline shift in media at *p*H 6.2 to 6.8, due to loss of CO_2, can be reduced by decreasing the concentration of bicarbonate in that range, from 24 m*M* to, for example, 5 m*M*.

A special problem in *p*H control is introduced when cells must be grown in containers open to the air (such as petri dishes) in a CO_2 incubator. With ordinary bicarbonate-buffered media, the final *p*H is determined by the concentrations of $NaHCO_3$ in the medium and of CO_2 in the atmosphere, subject only to a time lag in CO_2 diffusion. The *p*H cannot be flexibly adjusted with nonvolatile buffers, since the final *p*H will then be intermediate between the initial *p*H of the medium and that imposed by the CO_2-$NaHCO_3$ system; nor can the $NaHCO_3$ be eliminated, since added bicarbonate is an essential metabolite for many cell lines (*12*). However, the two buffer systems can be made mutually supportive by adjusting the concentration of $NaHCO_3$ to that level which in conjunction with a fixed atmospheric CO_2 concentration gives approximately the desired *p*H (*1, 13*). The combination of buffers shown in Table 2 can then be added in order to enhance the buffering capacity of the medium.

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References and Notes

1. C. G. Mackenzie, J. B. Mackenzie, P. Beck, *J. Biophys. Biochem. Cytol.* **9**, 141 (1961).
2. C. Ceccarini and H. Eagle, *Proc. Nat. Acad. Sci. U.S.* **68**, 299 (1971).
3. E. M. Levine, Y. Becker, C. F. Boone, H. Eagle, *ibid.* **53**, 350 (1965); H. Eagle, E. M. Levine, H. Koprowski, *Nature* **220**, 266 (1968).
4. H. Eagle and E. M. Levine, *Nature* **213**, 1102 (1967).
5. H. Eagle and C. Ceccarini, *ibid.*, in press.
6. Unpublished results on the effects of *p*H variation on the production of collagen, globulin, interferon, and viruses and on the efficiency of virus-mediated transformation.
7. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, R. M. M. Singh, *Biochemistry* **5**, 467 (1966).
8. H. Eagle, *Science* **130**, 432 (1959).
9. ———, G. E. Foley, H. Koprowski, H. Lazarus, E. M. Levine, R. A. Adams, *J. Exp. Med.* **131**, 863 (1970).
10. V. I. Oyama and H. Eagle, *Proc. Soc. Exp. Biol. Med.* **91**, 305 (1956).
11. H. Eagle, *Arch. Biochem. Biophys.* **61**, 356 (1956).
12. H. E. Swim and R. F. Parker, *J. Biophys. Biochem. Cytol.* **4**, 525 (1958); R. P. Geyer and R. S. Chang, *Arch. Biochem. Biophys.* **73**, 500 (1958).
13. A. C. Taylor, *J. Cell Biol.* **15**, 201 (1962).
14. Supported by PHS research grant AI-4153 and grants from the American Cancer Society and the National Science Foundation.

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Juvenile Hormone Induces Vitellogenin Synthesis in the Monarch Butterfly

Abstract. *The vitellogenin (yolk protein) of the monarch butterfly has been identified by electrophoretic and immunochemical techniques. In freshly emerged female adults ligature of the neck prevents appearance of this protein in the hemolymph; it prevents yolk deposition and egg maturation as well. These processes are restored by injection of juvenile hormone; the restoration involves induction of vitellogenin synthesis, as shown by incorporation of* [^3H]*leucine.*

Yolk formation in insects entails the selective uptake of vitellogenins ("female proteins") from the blood into the oocytes (*1*). Vitellogenin is synthesized by the fat-body cells, and in insects of several orders other than Lepidoptera (moths and butterflies) this synthesis is induced by juvenile hormone secreted by the corpora allata (*2*). Although earlier studies on Lepidoptera (chiefly with silkmoths) showed that allatectomy failed to prevent yolk formation (*3*), recent reports on other members of this order clearly demonstrate dependence on the corpora allata (*4*). Until now, however, nothing has been reported on the mode of action of the juvenile hormone in controlling egg maturation in any lepidopteran. To investigate this, we selected the monarch butterfly *(Danaus plexippus)* because the occurrence of both a reproductive phase and a sexually dormant migratory phase in its long-lived adults suggested the existence of hormonal regulation.

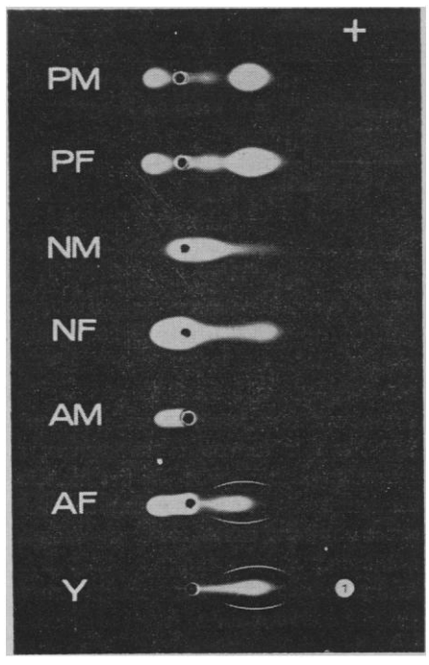
Initially, we looked for the presence of the vitellogenin in this species. Larvae were reared in the laboratory on either milkweed leaves or an artificial diet under 16 hours of illumination per day at 25°C; adult butterflies were fed twice daily on diluted honey (*5*). Antiserum against vitellogenin was obtained from a rabbit immunized with the proteins of mature eggs (*6*). Hemolymph was collected from animals at various developmental stages and analyzed by immunoelectrophoresis (Fig. 1). The only sample which produced a major precipitin line identical to that from yolk and representing vitellogenin was the blood of the female adult in which vitellogenesis was in progress. Neither males at any stage of development nor pupal or newly emerged adult females contained this protein. These results were further confirmed by results of the immunodiffusion tests of Ouchterlony and Oudin in which antiserum absorbed with male blood was used (*6*), and by disc electrophoresis on acrylamide gel.

To test hormonal control over yolk formation, we fed seven newly emerged adult females once and then ligated four of them at the neck, thus cutting off the brain and associated neuroendocrine glands from the rest of the body. The remaining three served as controls. All were then maintained for 5 days by a daily injection of glucose solution into the hemocoel. Hemolymph and ovaries were then examined. The results (Table 1, experiment 1) were clear-cut, and indicated that an endocrine center in the head of the butterfly is required for the appearance of vitellogenin, and therefore for the deposition of yolk and egg maturation.

A second experiment was designed to test whether control over yolk production was a function of juvenile hormone, a product of the corpora allata,

Table 1. Effects of ligation and treatment with juvenile hormone on vitellogenin production and egg development in the monarch butterfly. Female butterflies, within 12 or 24 hours after emergence, were fed on 30 percent honey and immediately ligated at the neck (except for the intact controls of experiment 1). In experiment 2, juvenile hormone (JH) or oil was injected. The animals were then kept at 25°C and 60 percent relative humidity, and maintained by daily intraabdominal injection of 50 to 80 μl of 2 or 5 percent glucose solution. Finally, hemolymph samples were collected, and the abdomens were dissected and examined. Vitellogenin in the hemolymph was determined by the Oudin immunodiffusion test and other techniques as described in the text (+, present; −, absent). Vitellogenesis in the oocytes was determined by visual examination (+, oocyte growth and presence of abundant opaque yolk; −, no more than traces of opaque material). Newly emerged butterflies also had only traces of opaque material. The juvenile hormone used in experiment 2 was *dl*-JH (*dl*-methyl *t,t,c*10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate), kindly given by Dr. H. Röller. It was dissolved in olive oil (1 μg/μl) and 4 μl were injected; controls received 4 μl of olive oil.

Treatment	Animals (No.)		Vitellogenin	Vitellogenesis	Mature eggs
		Experiment 1			
Control	3	5	+	+	+
Ligated	4	5	−	−	−
		Experiment 2			
Olive-oil	3	2.5	−	−	−
JH	2	2.5	+	+	−
JH	1	4.5	+	+	+



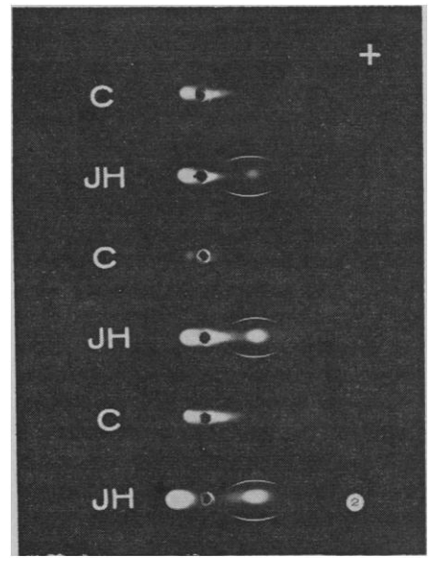


Fig. 1 (left). Identification of vitellogenin in the monarch butterfly. Immunoelectrophoresis was performed with 1.5-μl samples on 7.5 by 10 cm glass plates coated with 0.6 percent agarose made up in a composite running buffer of 50 m*M* tris, 10 m*M* sodium phosphate, 5 m*M* citric acid, and 5 m*M* disodium ethylenediaminetetraacetate, *p*H 8.2. Separation was run at 4°C at 7 v/cm for 3 hours. Duplicate plates were run simultaneously. After each run, one plate was fixed in 7 percent acetic acid, dried, and stained for protein with 1 percent thiazine red R in acid. The other plate was treated with antiserum in the troughs, allowed to develop for 24 hours in the cold, washed with cold buffer (as above plus 0.08*M* NaCl) to remove unreacted proteins, and then dried and stained for the precipitin lines. The two plates were then superimposed and photographed. *PM*, male pupa; *PF*, female pupa; *NM*, newly emerged male adult; *NF*, newly emerged female adult; *AM*, male adult 5 days old; *AF*, female adult 5 days old; *Y*, yolk extract. Fig. 2 (right). Regulation of vitellogenin production by juvenile hormone, demonstrated by immunoelectrophoresis. Hemolymph from the animals of experiment 2, Table 1, was analyzed as in Fig. 1. *C*, control; *JH*, treated with juvenile hormone. The results for six animals are shown individually, the lowest being that exposed to hormone for 4½ days.

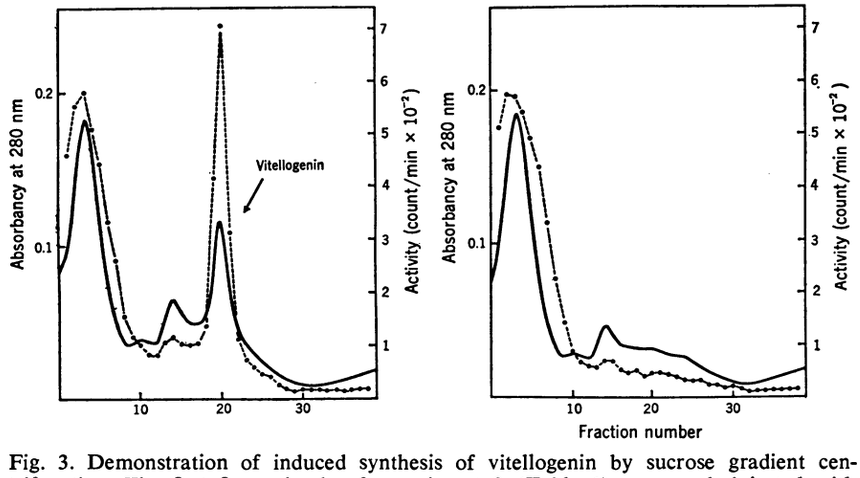


Fig. 3. Demonstration of induced synthesis of vitellogenin by sucrose gradient centrifugation. The first five animals of experiment 2 (Table 1) were each injected with 5 μc of [^{3}H]leucine (45 c/mole, Schwarz) 6 hours before they were killed. Hemolymph plasma (25 μl) was diluted with 0.1 ml of buffer (0.2*M* NaCl, 5 m*M* disodium EDTA, 50 m*M* tris, *p*H 7.5) and dialyzed for 6 hours in the cold against the same buffer. The sample was then layered on a 12-ml 5 to 25 percent linear sucrose gradient made up in the same buffer, and centrifuged at 40,000 rev/min in an SB-283 rotor in the B-60 International centrifuge at 0°C for 15½ hours. Gradients were fractionated and analyzed in an ultraviolet analyzer (Instrumentation Specialties). Then 0.2 ml of each fraction was mixed with 10 ml of Kennedy's scintillation fluid (*10*) and counted in a Packard scintillation counter. The top of the gradient is at the left. Upper diagram: animals treated with juvenile hormone. Lower diagram: controls treated with olive oil. Solid line, absorbancy at 280 nm; broken line, radioactivity.

rather than some other hormone from the head. Six fresh female butterflies were fed and ligated; immediately thereafter three were injected with pure synthetic juvenile hormone dissolved in olive oil, and three, which served as controls, were injected with olive oil. They were maintained for 2.5 to 4.5 days, after which the blood was analyzed and the ovaries were examined. All animals which received juvenile hormone had initiated yolk deposition and had vitellogenin in their blood, as revealed by immunoelectrophoresis (Table 1, experiment 2; Fig. 2) and the Oudin test, whereas neither vitellogenin nor yolk deposition was detectable in the controls. Because of the limited amount of material, no attempt was made to quantitate these results.

To demonstrate further that the appearance of vitellogenin under the influence of juvenile hormone was the result of induced synthesis de novo, we injected [^{3}H]leucine into the first five of the animals used in experiment 2, 6 hours before termination. Blood was collected, centrifuged to remove the cells, dialyzed to remove unincorporated leucine and other small molecules, and fractionated by sucrose gradient centrifugation. The fractions were counted for radioactivity, and the identity of the vitellogenin was established by Oudin's immunodiffusion test. Results from control and experimental animals (Fig. 3) not only confirm the immunoelectrophoretic analysis for the presence of vitellogenin, but also demonstrate that this protein had efficiently incorporated leucine, an indication that its synthesis was induced by juvenile hormone. Incorporation into the other hemolymph proteins (which are scarcely resolved by this technique) differed little, if at all, between experimental and control groups.

Our data show that juvenile hormone induces synthesis of vitellogenin essential for egg formation in a lepidopteran insect. These findings are consistent with those of Herman and Barker (*7*), who independently began experiments with the monarch butterfly and have demonstrated stimulation of oogenesis by analogs of juvenile hormone. The effects of different times of treatment and amounts of hormone remain to be explored; comparison with other systems stimulated by juvenile hormone suggests that doses lower than that we used would be effective (*8*). Also unknown is whether the hormone has an additional function in regulating pro-

tein uptake into the oocytes, as has recently been demonstrated in a cockroach (*9*). Thus, vitellogenin synthesis which is dependent on juvenile hormone appears as an integrated part of insect oogenesis, and the lack of hormonal control exhibited by certain Lepidoptera may be regarded as an adaptive exception to the more general pattern.

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References and Notes

1. W. H. Telfer and D. S. Smith, in *Insect Ultrastructure*, A. C. Neville, Ed. (Blackwell, Oxford, 1970), p. 117.
2. W. J. Bell, *J. Insect Physiol.* **15**, 1279 (1969); V. J. Brookes, *Develop. Biol.* **20**, 459 (1969); F. Engelmann, *Science* **165**, 407 (1969); and other papers. For review, see G. R. Wyatt, in *Biochemical Actions of Hormones*, G. Litwack, Ed. (Academic Press, New York, in press), vol. 2.
3. W. H. Telfer, *Annu. Rev. Entomol.* **10**, 161 (1965). Allatectomy on early 5th instar cecropia silkmoth larvae does not prevent the appearance of vitellogenin in the blood of this species at the normal time after spinning, nor does allatectomy of diapausing pupae alter the pattern of [^{3}H]leucine incorporation into this protein during late pharate adult development (M. L. Pan, unpublished observations).
4. T. Wu and F. Quo, *Acta Entomol. Sinica* **12**, 411 (1963); S. Fukuda and S. Kondo, *Zool. Mag.* **74**, 393 (1965); A. Karlinsky, *C. R. Acad. Sci. (Paris)* **264**, 1735 (1967); K. Endo, *Develop. Growth Differ.* **11**, 297 (1970); G. Benz, *Experientia* **26**, 1012 (1970); M. T. El-Ibrashy and I. Z. Boctor, *Z. Vergl. Physiol.* **68**, 111 (1970).
5. We thank Dr. Lincoln P. Brower of Amherst College for providing us with rearing stock and milkweed.
6. W. H. Telfer, *J. Gen. Physiol.* **37**, 539 (1954).
7. W. S. Herman and J. F. Barker, personal communication. We thank them for communicating their results to us.
8. See (*2*). Also, in *Blattella germanica* 0.16 to 0.66 μg of juvenile hormone induces vitellogenin synthesis (J. G. Kunkel and G. R. Wyatt, in preparation).
9. W. J. Bell and R. H. Barth, *Nature* **230**, 220 (1971).
10. J. F. Kennedy, *Experientia* **25**, 1120 (1969).
11. Supported by grant HD-02176 from the U.S. Public Health Service and by Whitehall Foundation.

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Anemia in Sleep-Deprived Rats Receiving Anticoagulants

Abstract. *Independent groups of rats were deprived of sleep and treated with the anticoagulant drugs phenylindanedione or dicoumarol for 1 to 8 days. These animals developed an extremely severe anemia which was accelerated by p-chlorophenylalanine. The red cell count and amount of hemoglobin decreased to half of normal values. No decrease occurred in animals subjected to any one single treatment. Histological examination indicated hemolysis, hypoplasia of hemopoietic organs, slight hemorrhage, but no evidence of stress. The severity of the anemia was inversely related to the amount of sleep permitted during sleep deprivation. This new syndrome demonstrates marked effects of sleep deprivation on both maturation and destruction of red blood cells. Depletion of serotonin by injection of parachlorophenylalanine blocked the increase in amount of brain waves of the type commonly seen in slow wave sleep but did not eliminate the production of these waves. This result is at variance with the theory that serotonin is the neurochemical responsible for the "priming" of slow wave sleep.*

Prolonged wakefulness produces widespread effects, but very few other than those on brain and behavior have been studied. In experiments designed to test the effect of sleep deprivation combined with anticoagulant drugs, it was discovered that rats receiving anticoagulants of the indirect type, when deprived of sleep for 8 days, developed an extremely severe anemia. A number of rats were then subjected to these treatments for various lengths of time (*1*). On each day, ten animals for each treatment were killed, and blood and tissue samples were obtained (*2*). Some rats received the anticoagulants phenindione or dicoumarol for this period, some were deprived of sleep, and others were deprived of sleep and treated with anticoagulant. Others received no treatment and served as control animals. There was no change in the mean amount of hemoglobin (Fig. 1) in animals given single treatments or in control animals. However, there was a marked decrease in the mean amount of hemoglobin in those groups receiving combined treatments on days 7 and 8, with the decrease beginning on day 6. These values were significantly low, relative to the values for the groups that received a single or no treatment [$P = .001$ (*3*)]. Since the chemical structure and side effects of dicoumarol and phenindione are quite different, the fact that anemia develops similarly in animals deprived of sleep and treated with these drugs suggests that the contribution of these drugs to the phenomenon must be due to their anticoagulant properties. Additional groups of rats received an intraperitoneal injection of *p*-chlorophenylalanine (PCPA, 316 mg/kg) every 72 hours. The first injection was given 24 hours before the start of sleep deprivation and treatment with anticoagulant. Treatment with PCPA in addition to sleep deprivation and treatment with anticoagulant had an accelerating effect on the development of anemia; the anemia began to develop by day 3 (Fig. 1).

The anemia developed in these animals was very severe and could easily be seen on gross examination. The tissues such as the liver, kidneys, skin, and lungs were extremely pale. At times it was even possible to detect an anemic rat before its internal organs were inspected. The ears were very pale, the eyes were barely pink, and their feet seemed to have lost circulation. Symptoms of hemorrhage such as those observed in other studies (*4*) were minimal. Hence, the anemia could not be accounted for in terms of hemorrhagic disturbance. The hematologic examination revealed a decline in hemoglobin to 50 percent of normal values (Fig. 1), with a proportionate decline in red cell count and hematocrit values, indicating a normocytic anemia.

All animals were weighed just before the beginning and again at the end of their respective experimental program. Most animals had lost weight. However, the anemia could not be attributed to loss of weight, since weight loss was similar in anemic and nonanemic rats. Weighing of food showed that the animals were eating the same amount of food regardless of treatment group. Prothrombin times demonstrated that all rats fed anticoagulant were hypoprothrombinemic.

To determine the contribution of sleep to the appearance of anemia, we altered conditions to increase sleeping time. Ten rats deprived of sleep and treated with phenindione were fed in their home cages for eight continuous hours instead of three times a day for 2 hours, 40 minutes each time. This group, which could sleep a certain amount of time, had hemoglobin values almost within normal limits. Ten rats were placed for 8 days in the same containers with a larger platform (11 cm diameter) and fed phenindione in the usual schedule. The blood tests in this group were totally normal. One animal had slight hemorrhage around the testicular area. These experiments support the conclusion that the deprivation