have employed—that is, whether only the discard breeders are susceptible (*3*).

If this is the case it focuses attention on the stress of repeated pregnancy in these animals as a sensitizing factor in the subsequent development of arteriosclerosis. Preliminary experiments we have performed on the effect of exposure to cold and heat suggest that stressful situations other than exogenous administration of ACTH will also induce arteriosclerosis in these discard breeders.

Although unilateral nephrectomy is not essential to the production of arteriosclerosis, it does appear to intensify the lesions. However, the age of the rats is clearly a critical factor. Experiments are in progress to ascertain the youngest age at which rat arteriosclerosis can be initiated by ACTH injections. In addition, the effects of smaller amounts of ACTH and of less frequent injections are being studied in an effort to detect the earliest lesions of arteriosclerosis.

Because so much attention has been centered on the role of fat in the genesis of human arteriosclerosis, it should be emphasized that our rats were fed a normal diet not supplemented with fats. Furthermore, the serum cholesterol levels of our animals did not rise during the development of vascular disease. This does not, however, exclude the possibility that fat metabolism in the arterial wall itself is altered, and that this alteration is responsible for the changes observed in the rats.

The characteristic lesions observed in male rats may also provide some interesting experimental tools. The deep, penetrating peptic ulcers and the multiple renal calculi produced by ACTH administration may prove to be extremely useful in the study of peptic ulcer and kidney stones in man. The apparent speeding up of the aging process, observed in both male and female rats, also suggests research applications.

It is of interest to note that the dosage of ACTH we have administered (0.333 of a unit per 100 g body weight) is the equivalent of approximately 200 units in an average-sized human being. This is close enough to therapeutic levels to make one question the possible toxic effects of ACTH on the human vascular system in susceptible patients. Indeed, when one surveys the spectrum of lesions obtained in both male and female rats —arteriosclerosis, hypertension, senescent changes, renal stones, and gastric ulcerations—it is tempting to compare these changes with those seen in Cushing's disease. The possibility that the severe arteriosclerosis observed in our rats may actually result from hyperfunctioning of the adrenal glands is suggested by the fact that we have found these glands to be enlarged and the thymus glands to be involuted.

If further experiments prove that the arteriosclerotic lesions in the rat closely resemble those in human beings, the effects of an overactive pituitary-adrenal axis on arteriosclerosis in man will demand evaluation. If, as Selye believes, the stresses of life are channeled through the pituitary-adrenal system (*4*), stress may conceivably be an important determinant in human arteriosclerosis.

BERNARD C. WEXLER*
BENJAMIN F. MILLER
*May Institute for Medical Research of the Jewish Hospital Association of Cincinnati* and *Departments of Pathology and Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio*

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* William S. Merrell scholar in gerontology.

25 November 1957

## Use of Arginine to Eliminate Medium Changes in Tissue Culture Systems

Conventional tissue-culture techniques require that the nutrient medium be changed at frequent intervals. This methodology presents no difficulty in the instance of the small volumes handled in flask or test-tube type cultures. On the other hand, it presents a major obstacle to successful and continuous cultivation of mammalian cells in fluid agitated suspension (submerged culture), in volumes of 3 to 20 liters, as commonly employed in our laboratories (*1–3*). This report presents the results obtained when L-arginine is employed as a means of circumventing the necessity for making frequent changes of medium in such submerged culture systems. This is important not only from the standpoint of economy or ease of manipulation but also in relation to certain types of biochemical and biological experiments wherein it is desirable to maintain, as closely as possible, a somewhat constant cellular environment.

On the basis of the studies of Westfall *et al.* (*4*) it was known that, in certain tissue culture systems, histidine, arginine, isoleucine, methionine, and phenylalanine were rapidly depleted. It occurred to us that these substrates might be limiting and, as such, might be used to maintain active rates of cellular proliferation without renewal of media. Subsequent experiments (*5*) have demonstrated the validity of this assumption and, further, the fact that use of arginine alone is sufficient for this purpose.

In the basic investigations, Earle's L cell was used in Spinner culture (*1*). The nutrient medium routinely used was a modified Eagle's mixture (*3*). Rates of cellular proliferation were appraised by direct enumeration in a hemocytometer. Cell viability was determined through use of the vital stain trypan blue (*1*).

Submerged-culture cells of the L strain procured from a New Brunswick fermentor were centrifuged and resuspended in equal volumes of nutrient media in three Spinner vessels. Thereafter, the three Spinner cultures received additions of substrate every other day as follows: the control culture received saline; the second vessel received 21 μg of L-arginine per milliliter of culture; 42 μg of L-arginine per milliliter was introduced into the third vessel.

In this typical experiment there were uniform rates of cellular proliferation in all three Spinners during the first 2 days after initiation of the test (Fig. 1). After the 4th day, the cell concentration of the control culture decreased rapidly; this was followed by a loss in cell viability of from 98 percent on the 6th day to 30 percent by the 11th day.

Cells in the Spinner vessel that had received 21 μg of arginine per milliliter of culture continued to proliferate until the 4th or 5th day, when stabilization of the cell population occurred; this was followed by a gradual drop in cell concentration. The cell viability of this culture was maintained at a level above 90 percent through the 11th day. Active cellular proliferation in the 42 μg/ml culture was maintained, in this experiment, until the 8th or 9th day. Viability of this culture was maintained at a level above 95 percent throughout the 11th day (Fig. 1).

Addition of concentrations of 84 to

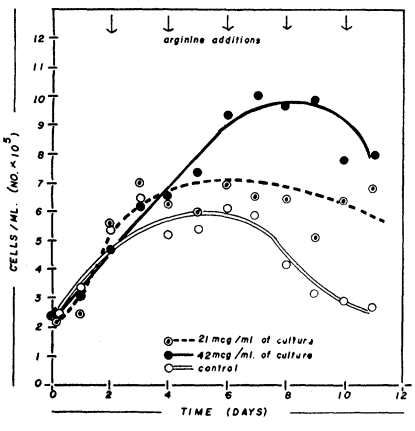


Fig. 1. The effect of arginine on the proliferation of L cells in submerged culture.

336 μg of arginine per milliliter of culture has resulted in greater early stimulation of cellular proliferation, although the final cell populations did not approach that achieved with the 42 μg/ml level. No manifestations of toxicity were apparent at the higher concentrations.

Addition of arginine has been found to be useful for the maintenance of strain HeLa or Westwood's ERK/KD cell line (*6*). In addition, arginine has been used effectively as a supplement in a serum-free medium employed for maintenance of the ERK/KD cells in submerged culture (20-liter) during the experimental production of viral antigens (*7*). In some instances, active cellular proliferation has been maintained for periods up to 20 days through the addition of arginine and new media.

Several possibilities relating to the mechanisms of this action of arginine may be mentioned, such as one similar to the mechanism described for the protection, by arginine, of rats against ammonia toxicity (*8*). Again, arginine may be incorporated directly into tissue protein, as was demonstrated by Bloch (*9*). We have found that citrulline (but not ornithine) will operate in the same fashion and to the same degree of efficacy as arginine in cultures of L cells. The mechanism of action of arginine in cultures of mammalian cells has not been determined as yet, and further comment on the subject must await more detailed studies.

WILLIAM J. THOMAS
DONALD W. ZIEGLER
SAUL A. SCHEPARTZ
WILLIAM F. MCLIMANS
*Wistar Institute of Anatomy and Biology, University of Pennsylvania, Philadelphia*

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9 December 1957

## Oxygen Equilibrium of Phascolosoma agassizii Hemerythrin

Hemerythrin is the respiratory pigment of the phylum Sipunculida, whose members are small-to-medium-sized worms frequently found buried in an oxygen-deficient substratum. Hemerythrin is reddish-purple when oxygenated, colorless when deoxygenated; this is in contrast to the other two iron-containing respiratory pigments, hemoglobin and chlorocruorin. Hemocyanin, the oxygen-binding copper protein of arthropods and molluscs, resembles hemerythrin in being colorless when deoxygenated. On the basis of chemical and thermodynamic evidence, Klotz and Klotz (*1*) have concluded that oxygenation of hemerythrin and hemocyanin differs from that of heme respiratory pigments in that the metal undergoes a valence change upon the reversible combination with $O_2$. Klotz and Klotz have reported that the heat of oxygenation of *Golfingia* [formerly *Phascolosoma* (*2*)] *gouldii* is about twice that of hemoglobin and have utilized this fact as evidence for the acceptance of two electrons by the oxygen molecule in oxyhemerythrin—in contrast to oxyhemoglobin, where the ligand does not gain electrons.

However, two other workers have observed that the effect of temperature on the oxygen equilibrium of the hemerythrin of *Sipunculus nudus* (*3*) and of a species of *Golfingia* (*4*) was very similar to its effect on the oxygen-hemoglobin equilibrium. This, coupled with the small amount of published information on the oxygen-hemerythrin equilibrium, has warranted a study of the hemerythrin of the "peanut worm," *Phascolosoma* (*Physcosoma*) *agassizii* Keferstein (*5*).

Hemerythrin was prepared by distilled water hemolysis of clotted coelomic cells, followed by centrifugation and filtration. Forty specimens of this small sipunculid yield approximately 1 ml of a 3 percent hemerythrin solution. Oxygen dissociation curves were determined by spectrophotometric analysis ($\lambda = 580$ to 600 mμ) of the mixture of "reduced" and oxygenated hemerythrin formed upon equilibration with known partial pressures of oxygen. The partial pressure of oxygen was varied by injection of known quantities of air into a confined space with the respiratory pigment, as in the technique of Riggs (*6*).

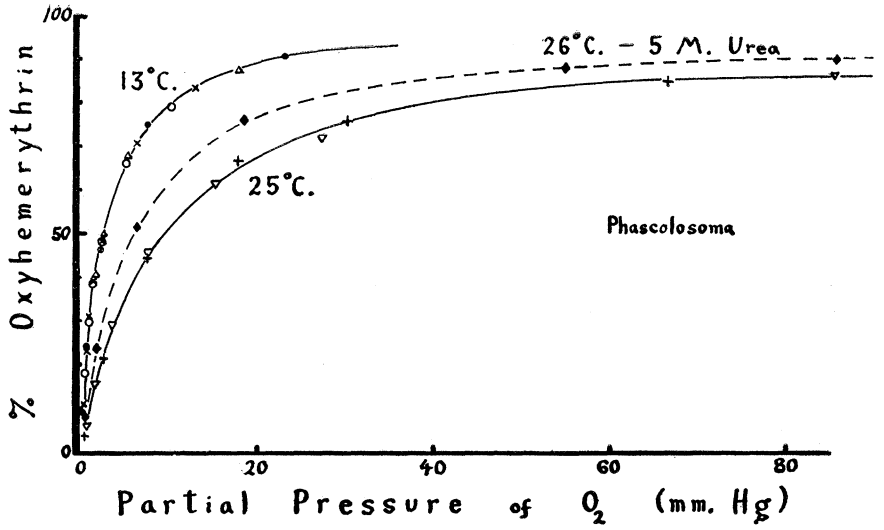


Fig. 1. Oxygen equilibrium of *Phascolosoma agassizii* hemerythrin. Two percent hemerythrin in potassium phosphate buffer, $\Gamma/2 = 0.2$. Solid lines: no urea present; experiments at 13°C performed at *p*H's of 7.90, 7.23, 6.79, and 5.75; experiments at 25°C performed at *p*H's of 7.74 and 7.27. Dashed line: 5*M* urea present; experiment performed at 26°C and a *p*H of 8.08.

The result of experiments on the effect of *p*H, temperature, and urea on the oxygen-hemerythrin equilibrium is shown in Fig. 1. Since the position of the oxygen dissociation curve is invariant to *p*H change, this particular hemerythrin lacks a Bohr effect, as do other sipunculid hemerythrins (*3*, *4*). Although a slightly sigmoid oxygen dissociation curve has been observed for *Sipunculus* hemerythrin (*3*), *Phascolosoma agassizii* hemerythrin possesses a hyperbolic curve.

From the data (Fig. 1) on *Phascolosoma agassizii* hemerythrin at two different temperatures, it is possible to calculate (*7*) a heat of oxygenation ($\Delta H°$) in terms of a standard state of 1-atm pressure of dissolved oxygen gas; the following integrated form of the van't Hoff equation was used:

$$\ln\left[\frac{p_{50}(T_1)}{p_{50}(T_2)}\right] = \frac{\Delta H°}{R}\left[\frac{T_2 - T_1}{T_1 T_2}\right],$$

where $p_{50}(T)$ represents the partial pressure of $O_2$ (in atmospheres) at which, at the specified temperature, there is exactly 50 percent of the respiratory pigment in an oxygenated condition. When the oxygen dissociation curve is hyperbolic, the equilibrium constant is the reciprocal of $p_{50}(T)$. For this particular hemerythrin, a value of $\Delta H° = -17$ kcal was observed; this is