

ERYTHROID CELL DIFFERENTIATION AND THE INHIBITION OF CYTOKINESIS BY CYTOCHALASIN

LINDA W. REYNOLDS and VERNON M. INGRAM

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

Cytochalasin B produces multinucleated erythroid cells in tissue cultures of very young chick blastoderms. There is no apparent qualitative interference with differentiation and maturation of erythroid cells, but the amounts produced are reduced 4- and 10-fold. These effects of cytochalasin are readily reversible.

The cytochalasins, a group of mold metabolites, will inhibit movement and cytoplasmic cleavage of cells in culture, as shown by Carter (1967). For example, cytochalasin B will increase the apparent adhesiveness of fibroblasts, L cells, to substrate and will thereby prevent locomotion of these cells at concentrations as low as 0.5 $\mu\text{g}/\text{ml}$. Cytoplasmic cleavage of fibroblasts is inhibited by preventing the first step of this process, the development of the cleavage furrow. Carter found that division of the cell nucleus is not inhibited so that multinucleate cells are produced. Curiously enough, not all the nuclei divide at the same time so that multinucleate cells form which have 2, 3, 4, 5, etc. nuclei. Cytochalasin B acts rapidly, its effects are readily reversible by washing the cells, and viability is unimpaired for "many days." In a recent paper, Wessels et al. (1971) come to the conclusion that cytochalasin B reversibly inhibits the contractile microfilament machinery of cells, such as, for example, those microfilaments supposedly located underneath the cleavage furrow (Schroeder, 1969) and whose normal function it is to pinch the dividing cell into halves. Thus cytochalasin prevents cytoplasmic division. The consequent unexpected type of nuclear division is presumably a secondary manifestation.

It seemed of interest to test the inhibition of cytokinesis by cytochalasin in a differentiating

system and to see whether this inhibition would stop or delay the specific maturation of erythroid cells in organ culture. In addition, we wanted to find out whether cytochalasin would produce multinucleate cells in unattached cells, such as our erythroid cells, in contrast with the fibroblasts used by Carter.

MATERIALS AND METHODS

Fertilized eggs of the White Leghorn breed were obtained from Thompson's Farm, Andover, Mass. They were incubated for 24 or 36 hr, yielding embryos at the head fold and the six to nine somite stage, respectively. Blastoderms were prepared in sterile chick Ringer solution under sterile conditions (Hagopian and Ingram, 1971). Usually the whole blastoderm was cultured, dorsal side up, on cellulose acetate discs ($1\frac{5}{16}$ inches diameter, 0.45 μ pore size; Schleicher & Schuell, Inc., Keene, N. H., or Matheson-Higgins Co., Cambridge, Mass.) which rested on the inner well of sterile plastic organ culture dishes (Falcon No. 3010, Falcon Plastics, Div. of Bioquest, Oxnard, Calif.). The cellulose acetate discs were sterilized under UV light and soaked in sterile chick Ringer solution overnight before use. Medium 199 with Hanks' salts was purchased from Grand Island Biological Co., Grand Island, N. Y. The culture medium, 1.2 ml, filled the center well of the culture dish up to the support disc. The outer well was moistened with sterile chick Ringer solution and the cultures were incubated at 37°C in air at 100%

humidity. The medium was replaced every day. Since cytochalasin B had to be added as a solution in dimethyl sulfoxide, the latter was itself added to the control cultures at the appropriate level, between 0.2 and 1%.

The red cells were usually harvested in 20% chick serum in medium 199 by tearing the tissue and allowing the cells to pour out (Hagopian and Ingram, 1971). Sometimes the tissue had to be teased to free the red blood cells. This method gave very good yields of red cells, almost quantitative, as judged by the release of hemoglobin-containing cells. The cells, which had been dispersed by handling them in a Pasteur pipette, were sedimented by gentle centrifugation and washed three times with buffered saline. Cells were counted in a hemocytometer, and slides were prepared in a Shandon Cyto centrifuge (Shandon Scientific Co., Inc., Sewickley, Pa.) using 0.2 ml of a suspension containing 10^6 cells per ml for each slide. The slides were stained with benzidine stain, followed by May-Grünwald-Giemsa stain.

For the determination of hemoglobin production or for electrophoresis, the cells were lysed in water in the presence of toluene and carbon tetrachloride, as described elsewhere (Hagopian and Ingram, 1971); hemoglobin was estimated by the Drabkin procedure (1935). Hemoglobin electrophoresis was carried out by the acrylamide gel technique described by Moss and Ingram (1968).

RESULTS

Production of Multinucleate Cells

De-embryonated blastoderms from embryos with six to nine somites (day 1.5) were cultured on medium 199:egg white (3:1) in the presence of 1 or 10 $\mu\text{g/ml}$ of cytochalasin B added as a 0.1% solution in dimethyl sulfoxide. The solvent itself had no effect on our cultures at the concentration used in our experiments. Fig. 1 displays the histograms of the frequencies of cells with one, two, or more nuclei from samples taken after treatment. Medium was changed every day. The cultures on 10 $\mu\text{g/ml}$ of cytochalasin B did not survive to day 8.5.

The lower concentration of cytochalasin shows no effect until after 2 days of culture, day 3.5

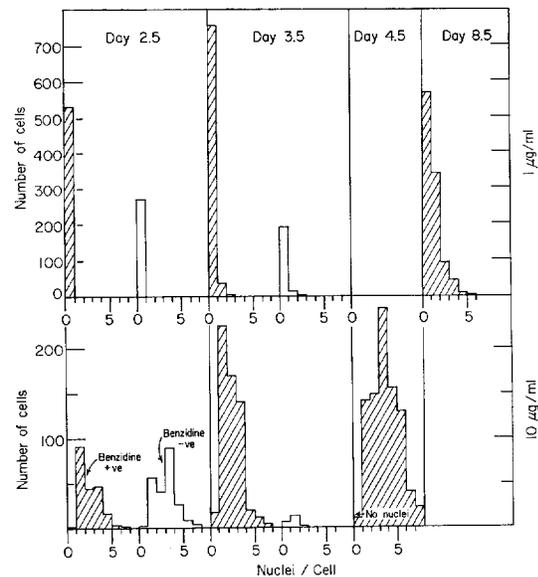


FIGURE 1 Effect of cytochalasin B on the production of multinucleated cells in culture at 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, using de-embryonated blastoderms with six to nine somites. On days 2.5 and 3.5 many benzidine-negative (immature) cells can still be seen. Benzidine-positive cells, cross-hatched; benzidine negative cells, no cross-hatching.

(Fig. 1). Even then, only very few bi- and trinucleate cells are found. However, on day 8.5, after 7 days of culture, a large proportion of the cells, 47%, have two or more nuclei.

At 10 $\mu\text{g/ml}$, on the other hand, cytochalasin produces about 100% of multinucleate cells after only 24 hr of treatment. The average number of nuclei per cell rises from a little less than three nuclei per cell on days 2.5 and 3.5 to four nuclei per cell on day 4.5. At that time, a few cells have lost their nuclei altogether, as described by Carter (1967), and the proportion of cells with abnormal or fragmented nuclei, hitherto very low, has risen to about 12%. Such cultures do not survive to day 8.5.

The effect of 1 $\mu\text{g/ml}$ of cytochalasin on whole head fold blastoderms in culture is similar to the effect of that concentration of the drug on older

FIGURE 2 Effect of cytochalasin B on erythroid cell production in whole blastoderms at head fold stage in culture; 2 $\mu\text{g/ml}$ of cytochalasin. (A) Average of two closely similar experiments. (B) Slide preparation of erythroid cells on day 3 and day 4. In the presence of cytochalasin, cells are usually misshapen, as reported by Carter (1967). Stained with benzidine—May-Grünwald-Giemsa; magnification $\times 1180$.

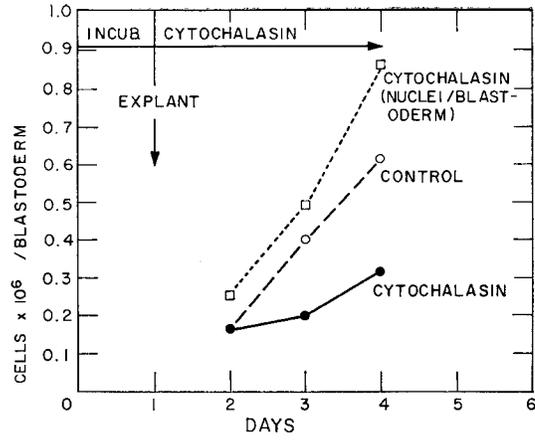
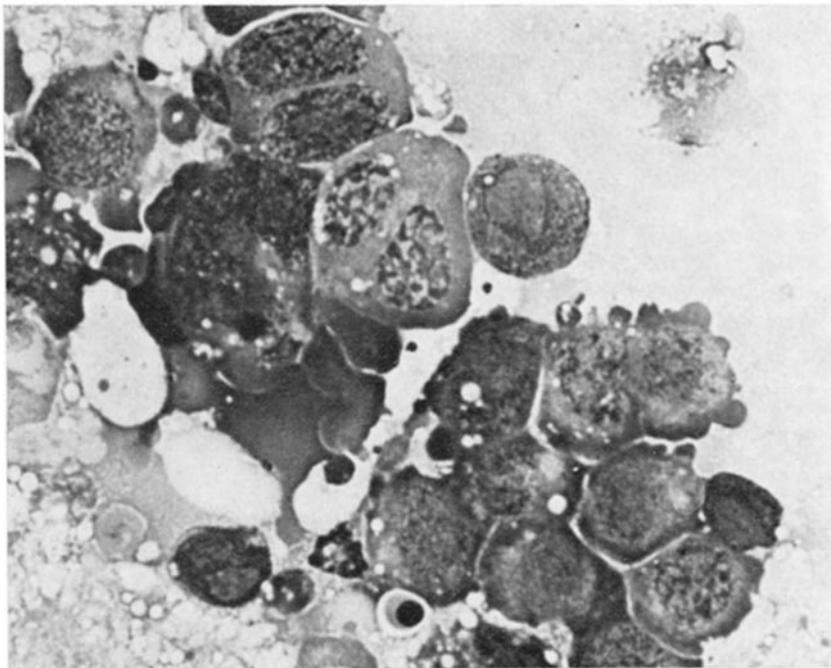
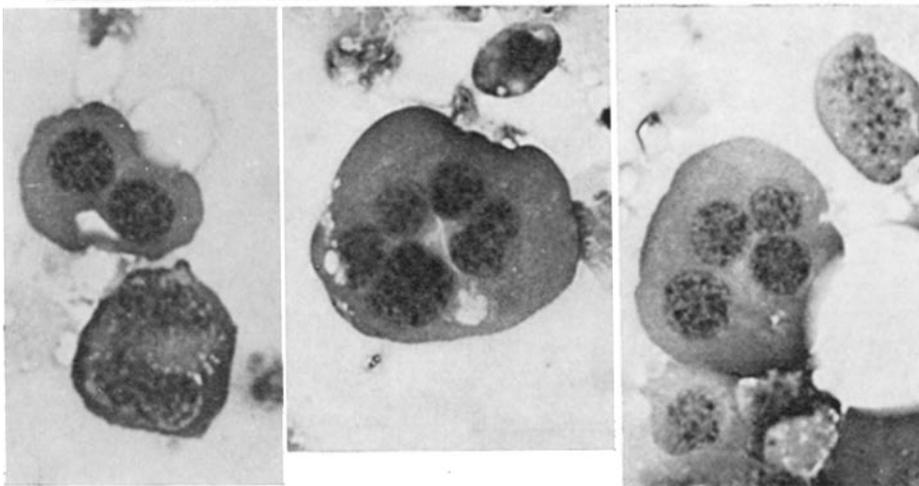


FIGURE 2 A



DAY 3



DAY 4

FIGURE 2 B

blastoderms, although after only 1 day in culture 20% of the cells contain two or more nuclei.

It is very evident that the presence of cytochalasin B does not prevent differentiation of precursor cells to the erythroid cell line nor does it prevent the morphological maturation of these cells. On day 4, i.e. after 3 days of cytochalasin treatment, the erythroid cells are mostly orthochromatic or late polychromatic erythroblasts, with fewer immature cells than the controls. Basophilic staining has disappeared in the cells from test cultures, the nuclear to cytoplasmic ratio is low, and the nuclei are condensed and granular in appearance. The cytoplasm stains for hemoglobin with the benzidine stain (Fig. 3), but the intensity of staining is much less than in control cells of comparable morphological maturity. The cells are, however, misshapen, as if they were made more adhesive than the control cells. Qualitatively, the cells in the test cultures have matured well.

Effect on Cell Numbers

Most of the experiments from now on were done with 2 $\mu\text{g}/\text{ml}$ of cytochalasin B which produces a high proportion of multinucleate cells (see later, Figs. 4 and 5). By using 10 blastoderms at the head fold stage for each time point, the yield of cells in the presence of cytochalasin B and in control cultures can be compared (Fig. 2 A). There is clearly a drastic reduction of total cell number by cytochalasin, as would be expected from its known effect on cell division. At this con-

centration of cytochalasin, there is, however, still a slow increase in cell numbers, so that cytokinesis has not been completely suppressed. When allowance is made for the many multinucleated cells, the number of nuclei per blastoderm counted in stained slide preparations from the cultures treated with cytochalasin is appreciably greater than the number of nuclei or cells in the control cultures. This effect has been a consistent finding in other experiments.

Hemoglobin Production

In the same cultures of head fold blastoderms used in Figs. 2 A and B, the amount of hemoglobin per blastoderm, or per 10^6 cells, was measured. Although hemoglobin is being produced in the presence of cytochalasin B during the days 2-4, the amount made is greatly reduced (Fig. 3 A). Plotted as hemoglobin per 10^6 cells in this figure, the reduction per 10^6 cells is reduced by factor of nearly 4, and the production per 10^6 nuclei is one-tenth.

In the presence of cytochalasin, differentiation and nuclear division continue. A simple histological benzidine stain for hemoglobin (Fig. 3 B) shows that conversion of cells to hemoglobin production occurs at the same rate as it does in the control series, mostly between day 2 and day 3. This finding, which at first sight is in conflict with the effect on hemoglobin production (Fig. 2 A), is understandable when it is realized that the benzidine stain is very sensitive and that a cell with a small amount of hemoglobin will be scored

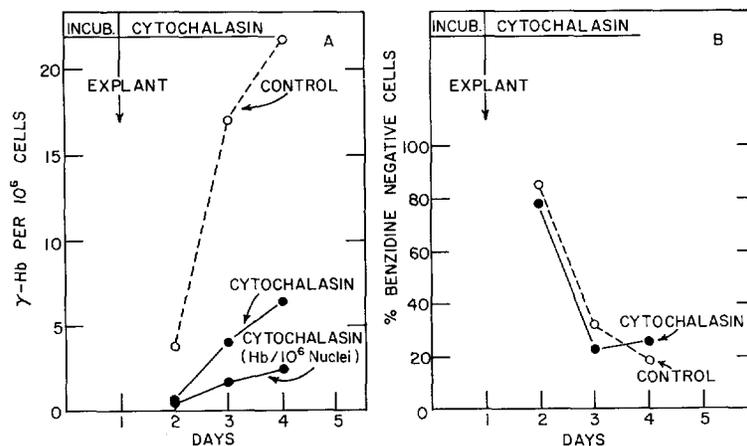


FIGURE 3 Hemoglobin production (A) and erythroid cell maturation (B) in head fold blastoderms in culture with cytochalasin B, 2 $\mu\text{g}/\text{ml}$. Average of two closely similar experiments.

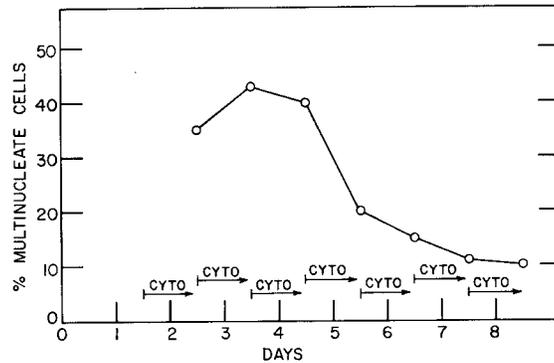


FIGURE 4 Sensitivity of de-embryonated blastoderms from embryos with six to nine somites to the effect of cytochalasin B which leads to multinucleated cells; $2 \mu\text{g/ml}$ of cytochalasin B.

as benzidine positive, as will a cell rich in hemoglobin. Cells from cultures with cytochalasin showed a benzidine reaction which was much weaker than the reaction of control cells. Thus qualitatively, differentiation to hemoglobin formation is unimpaired by the presence of cytochalasin, but the amounts of hemoglobin produced are greatly depressed. It should be noted that since 80% of all cells are benzidine positive by day 4 (see Fig. 3 B), as are the control cells, it is more likely that all the cells are differentiating, rather than that the low hemoglobin yield is due to the maturing of only a few cells. Electrophoresis acrylamide gels of the hemoglobin produced in these cultures show the presence of the two expected early embryonic hemoglobins (Bruns, 1971) on day 4.

Even at $1 \mu\text{g/ml}$ of cytochalasin B, blastoderms of head fold embryos show a hemoglobin production reduced to 15% of control.

Timing of Sensitivity of Cytochalasin

With the use of the older six to nine somite de-embryonated blastoderms, a test was made to see for how long during culture the maturing red cells remain sensitive to that effect of cytochalasin which leads to the formation of multinucleate cells. The de-embryonated blastoderms were cultured on medium 199:egg white as usual and were exposed to $2 \mu\text{g/ml}$ of cytochalasin B for 24 hr before harvesting (Fig. 4). The proportion of multinucleate cells falls off rapidly after day 4.5, as does total hemoglobin production. However, some sensitivity to cytochalasin remains up to day 5.5, and possibly longer. Here again, the number

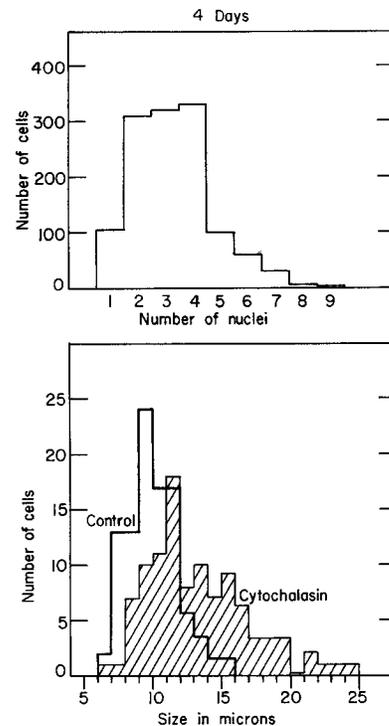


FIGURE 5 Effect of cytochalasin B on cell size. Cultures of head fold blastoderms in $2 \mu\text{g/ml}$ of cytochalasin.

of nuclei per blastoderm have increased beyond the control values (Hagopian and Ingram, 1971).

Effect on Cell Size

Multinucleate cells are larger than ordinary cells. Fig. 5 shows the results of incubating head

fold blastoderms for 3 days with 2 $\mu\text{g}/\text{ml}$ of cytochalasin B and measuring the diameter of cells on a cytocentrifuge slide preparation with a Vickers split-image micrometer (Vickers Instruments, Inc., Malden, Mass.). This preparation had on the average three nuclei per cell (Fig. 5), with equal numbers of bi- and tetranucleated cells and few cells with single nuclei. The histogram of apparent cell diameters is clearly displaced towards higher values when compared with the control. Whereas the control diameters seemed to group around a mean of 10 μ , the cytochalasin-treated cells formed at least two groups at 11 and 15 μ or more, probably a good many different subgroups. Undoubtedly, problems of cell geometry make inadvisable a direct interpretation of these numbers in terms of volumes, but we are clearly thinking here of a several fold increase in cell volume.

Reversal

The effect of a 24 hr treatment of head fold blastoderms with 2 $\mu\text{g}/\text{ml}$ of cytochalasin B, which has the effect shown in Figs. 2 and 3, can be

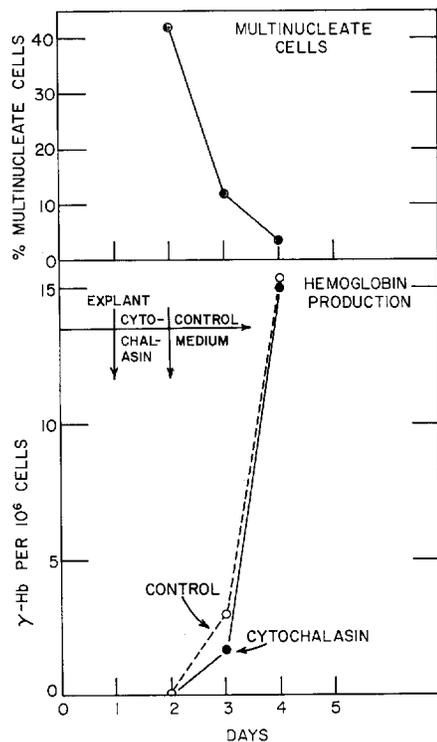


FIGURE 6 Reversal of cytochalasin effect, using whole head fold blastoderms and 2 $\mu\text{g}/\text{ml}$ of the drug.

reversed by placing the cultures on normal medium and changing this medium three times at hourly intervals. In this series of experiments, the yield of hemoglobin on day 2 was extremely low, for no obvious reason, in both the controls and the treated cultures. Hemoglobin production per cell (Fig. 6) recovers completely. The same is true when hemoglobin production per blastoderm is calculated. In addition, the proportion of multinucleated cells, which was quite high at 42%, returns to almost zero. At the same time there is a 150% increase in the number of nuclei per blastoderm not shown in the figure. During recovery from cytochalasin in these cultures there must be not only nuclear division but also additional cytokinesis, as mentioned by Carter (1967) for his experiments with fibroblasts.

DISCUSSION

These experiments confirm Carter's observations (1967), which pertained to fibroblasts, in an erythroid system, that is to say, in cells which progressively are neither attached to surfaces nor show locomotion. This is itself interesting, because Carter (1967) believes that cytochalasin acts by increasing the adhesiveness of the cell surface of his fibroblasts, yet we find that it can interact with the nonadhesive erythroid cells. Cytochalasin B produced multinucleated cells in our cells and these occur as cells with 1, 2, 3, 4, 5, etc. nuclei, which suggests that nuclear divisions were not always effective and that a binucleate cell might have only one of its nuclei dividing into two daughter nuclei. The effect of cytochalasin B is concentration dependent and is reversible after 24 hr, the only time point tested for reversibility. Our cells in these organ cultures are largely in the terminal series of cell division and maturation. Yet they remain sensitive to cytochalasin up to day 5 of culture. Such cells are normally dividing during these days. One suspects that cytochalasin will act whenever these cells try to undergo cytoplasmic division, no matter what stage of maturation they have reached. At the high concentration of cytochalasin B (10 $\mu\text{g}/\text{ml}$) the tissue did not survive much beyond day 4.5.

The events of differentiation in our cells, i.e. the maturation to the production both of early and of late hemoglobins and to morphological cell type, are not impaired qualitatively, and therefore do not depend on cytoplasmic division. Yet hemoglobin production is quantitatively greatly

reduced. The mechanism for this is unknown. On the other hand, more nuclei per blastoderm are produced than in control cultures; in both these cases, one can ask whether changes in pool size of controlling intermediates are involved.

Cytochalasin B at 2 $\mu\text{g}/\text{ml}$ does not interfere with the production of late adult (A) and definitive (D) hemoglobin, and by day 8 these are easily recognizable in gel electrophoresis. The quantities of hemoglobins A and D made in cytochalasin are low, as expected. Hemoglobin samples from cultures exposed to cytochalasin B at 2 $\mu\text{g}/\text{ml}$ for 1 or 2 days show, in addition to the expected early embryonic and primitive hemoglobins, also small quantities of the late hemoglobins A and D. This was an unexpected finding, not seen in control cultures, which points to either facilitation or selective depression by cytochalasin of the amounts of various hemoglobins made.

A low degree of multinucleate cell formation is seen in some relatively long-term normal organ cultures. The long delay before 1 $\mu\text{g}/\text{ml}$ of cytochalasin acts on young embryos (Fig. 1) is hard to understand, but might just be the natural slow accumulation of normally occurring multinucleate cells. Again, the mechanism for this phenomenon is not known.

Cytochalasin B in these experiments has produced interesting and puzzling effects in erythroid cultures which deserve further study.

Cytochalasin B was supplied by Dr. S. B. Carter, to whom we are indebted for this gift.

This work was supported by the National Science Foundation (GB 5181) and by the National Institutes of Health (AM 13945).

Received for publication 15 March 1971, in revised form 6 May 1971.

REFERENCES

- BRUNS, G. P. 1971. Erythropoiesis in the developing chick embryo. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, Mass.
- CARTER, S. B. 1967. *Nature (London)*. **213**:261.
- DRAKIN, D. L., and J. H. AUSTIN. 1935. *J. Biol. Chem.* **112**:51.
- HAGOPIAN, H. K., and V. M. INGRAM. 1971. *J. Cell Biol.* **51**:440.
- MOSS, B., and V. M. INGRAM. 1968. *J. Mol. Biol.* **32**:481.
- SCHROEDER, T. E. 1969. *Biol. Bull. (Woods Hole)*. **137**:413.
- WESSELS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WRENN, and K. M. YAMADA. 1971. *Science (Washington)*. **171**:135.