Table 2. Incorporation of radioactive nucleic acid precursors into the KOH-insoluble residue (DNA). (5-FU, 5-fluorouracil; U, uracil.)

Precursor	Control (count/min)	5-FU* (20 μg/ml)	5-FU + U* (20 μg/ml + 18 μg/ml)	Control + U* (18 μg/ml)
Adenine-8-C^{14}	(100)	28	14	102
Guanine-2-C^{14}	(100)	35	17	99
Hypoxanthine-8-C^{14}	(100)	21	9	109
Sodium formate-C^{14}	(100)	46	23	103

* Values expressed as a percentage of the control culture. Actual control values ranged from 500 to 30,000 count/min. Each value is the average of two to five determinations.

tent is not caused by a reduction in cell number but represents a real difference in the average DNA content per cell. The viable cell count, however, was reduced after growth in 5-fluorouracil and was not restored to normal by the addition of uracil.

Incorporation of radioactive purine precursors into DNA again confirmed the reduced DNA synthesis in the presence of 5-fluorouracil (Table 2). When uracil was added at the same time, at a uracil to 5-fluorouracil ratio of 1, results were produced which implied an even greater depression of uptake into DNA than did 5-fluorouracil alone. It must be realized, however, that the isotopic method of analysis measured only radioactivity in newly formed DNA whereas the total DNA was analyzed by the Burton method. The DNA synthesized before the addition of 5-fluorouracil, or 5-fluorouracil plus uracil was stable during subsequent growth, as measured by incubation studies with isotopes (*7*). Protein and RNA synthesis, measured colorimetrically and by isotope incorporation, were not affected by the inhibition of growth and of DNA synthesis produced by 5-fluorouracil. Other experiments (*8*) have shown that thymidine, if added with the drug at zero time and every 15 minutes thereafter, can maintain the DNA content at a control level but can not prevent the inhibition of growth.

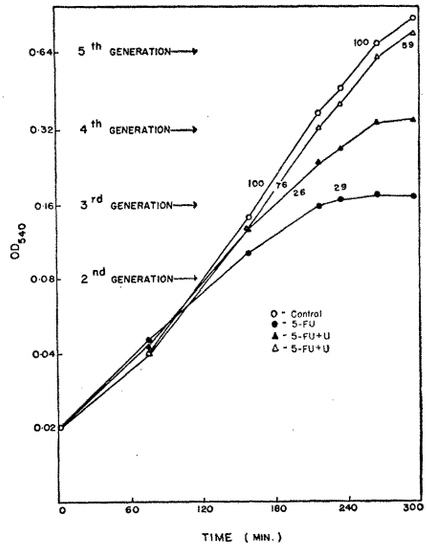

Fig. 1. Growth of *B. cereus*. Solid circles, 5-fluorouracil (5-FU) (0.1 μg/ml); solid triangles, 5-FU (0.1 μg/ml) plus uracil (0.9 μg/ml); open triangles, 5-FU (0.1 μg/ml) plus uracil (9 μg/ml). Numbers refer to DNA content, expressed as a percentage of the control value which is equal to 100.

At a drug concentration of 0.1 μg/ml (initial OD_{540} 0.02) there was a complete cessation of growth after three generations (Fig. 1). At a uracil to 5-fluorouracil ratio of 10, growth was inhibited (after three generations) when the DNA content, as measured by adenine-8-C^{14} incorporation, was reduced to 26 percent of the control, but did not terminate until after the fourth generation. A ratio of 100 was able to maintain a normal rate of growth for five generations although the cells contained only 60 percent of the DNA content of the controls at a comparable turbidity.

These results indicate that the inhibitory action of 5-fluorouracil on DNA synthesis is not reflected in the growth rate until the DNA content is diluted by growth, in this case after three generations, to a minimum quantity equal to 20 to 30 percent of the control value. Thus, about 75 percent of the normal DNA content of *B. cereus* may not be essential for growth, protein, or RNA synthesis. These results are perhaps related to those reported by Allfrey and Mirsky (*9*) who reported that up to 80 percent of the DNA in thymus nuclei could be removed without impairing protein or RNA synthesis. They concluded that most of the DNA was inactive and existed in a repressed state.

In *B. cereus* the presence of excess, inactive DNA may be associated with the multinucleate nature of the cell. Actively growing cells may contain as many as four separate chromatin bodies containing deoxyribonucleoprotein (*10*).

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27 February 1964

Hemoglobin Synthesis in Marrow Cell Culture: The Effect of Rat Plasma on Rat Cells

Abstract. *In cultures of rat marrow cells essentially all of the labeled iron which was incorporated into heme was shown to be in hemoglobin. Iron bound to rat plasma was incorporated into hemoglobin to a greater extent than was iron bound to fetal calf serum. Incorporation of iron bound to rat plasma was greater when some fetal calf serum was present than when rat plasma provided the total protein. These data suggest preferential use of homologous iron-binding proteins of the plasma.*

In a recent paper (*1*) we showed that continued heme synthesis by cultures of bone marrow cells from the rat for periods longer than about 12 hours was dependent upon the presence of erythropoietin in the culture medium. The utility of marrow cell cultures for the assay of erythropoietin, and for the investigation of the chemical mode of action of the hormone as a model for the study of differentiation, has been the subject of further experimentation with this system.

Our earlier paper contained indirect evidence that a simple procedure in which the extraction of heme from an acidified lysate of marrow cells into butanone (*2*) followed by the measurement of heme radioactivity is adequate for the determination of hemoglobin synthesis. Since one well-defined end product of stem-cell differentiation is hemoglobin, it was important to demonstrate directly that the as-

Table 1. Effect of iron-binding component in plasma upon incorporation of Fe^{59} into hemoglobin. All culture media contained 52.4 percent of medium 109 supplemented with 0.024 mg of glutamine and 80 units of penicillin per milliliter. Each plastic petri dish (60 × 15 mm) contained 10×10^6 nucleated cells derived from marrow of tibias and fibulas of male Sprague-Dawley rats fasted for 72 hours, and 1.0 μc of $Fe^{59}Cl_3$ bound to protein in a total volume of 2.16 ml. The cells were incubated for 24 hours at 37°C in an atmosphere of 95 percent air and 5 percent CO_2.

Expt.	Contents of medium (%)		Fe^{59} content (%)		Fe^{59} incorporated* (count/min)	% standard conditions (experiment A)
	Rat plasma	Fetal calf serum	Rat plasma	Fetal calf serum		
A	43.8	0	3.8	0	3689	100
B	0	43.8	0	3.8	1745	47
C	43.8	0	0	3.8	617	17
D	0	43.8	3.8	0	12113	330

* Net counts per minute of Fe^{59} incorporated into hemoglobin in 24 hours.

sumption of equivalence between heme formation and hemoglobin synthesis was justified. The experiments described in this report indicate that essentially all of the heme found in the butanone extract is, in fact, derived from hemoglobin. In addition, we have found a modification of the culture medium which can effect a substantial increase in the amount of hemoglobin synthesized.

The assumption regarding the correspondence between heme synthesis and hemoglobin formation by this system is shown to be warranted by the data obtained in the experiment described in Fig. 1. Approximately 5 percent of the total heme counts were lost in washing the hemoglobin crystals obtained from both cultures. The hemoglobin isolated after crystallization and chromatography on diethylaminoethyl cellulose represented about 93 percent of the original Fe^{59} extractable by the acid-butanone method. The chromatograms shown in Fig. 1 demonstrate that in both cases the hemoglobin had a constant specific activity and that there were no other components containing Fe^{59} present.

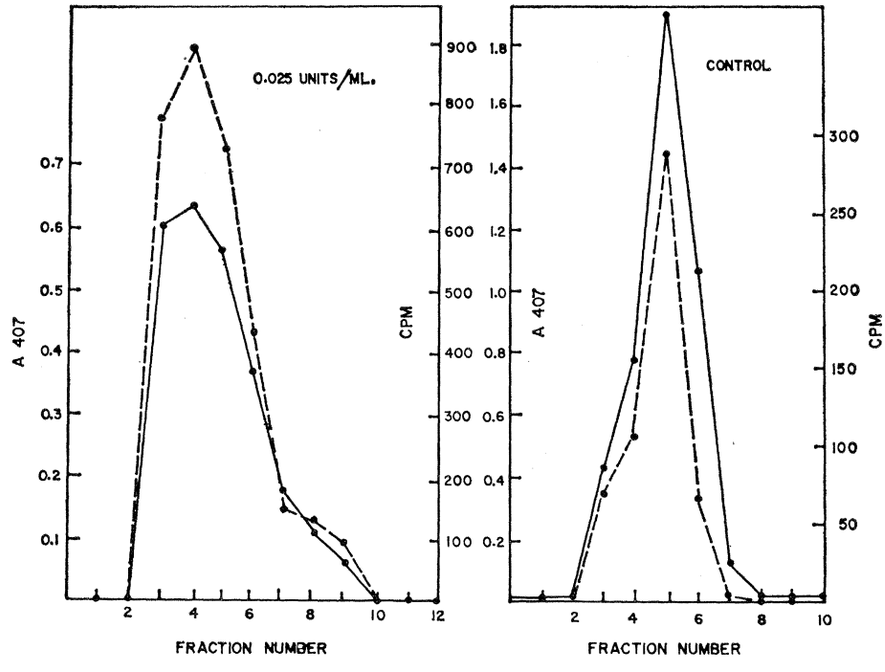

Fig. 1. (Left) Pool of cells (7.5×10^6) cultured in the presence fo 0.025 units of erythropoietin per milliliter in a total volume of 2.4 ml. After the cells were washed and lysed the stroma was removed and a solution containing 0.4 mg of crystalline rat hemoglobin was added to the lysate. (Right) Pool of cells (8.4×10^6) cultured in the absence of erythropoietin, treated in the same manner as above with 0.6 mg of carrier crystalline hemoglobin added. To each lysate was added an equal volume of Drabkin's solution (*3*); cyanmethemoglobin was crystallized by addition of ammonium sulfate to one-half saturation and allowing the solutions to stand at 4°C for 20 days. The crystals were collected, washed with saturated ammonium sulfate and dissolved in 2.0 ml of water. Chromatography was done on a column (3 cm × 3 cm^2) of DEAE-cellulose at 4°C. The cyanmethemoglobin was eluted with 0.01*M* tris buffer, *p*H 7.4, saturated with 10 percent ammonium sulfate. All Fe^{59} determinations were done in a well-type scintillation counter. The solid lines refer to absorbancy at 407 mμ and the broken lines to net count rate.

In our original method the marrow cells were incubated in 60 percent medium 109 and 40 percent rat plasma (*1*). The Fe^{59} was added to rat plasma and incubated at 37°C for 12 hours or more, before addition to the cells, to allow sufficient time for equilibration. To avoid having to collect rat plasma we attempted to substitute commercially available fetal calf serum in the medium. This change in conditions resulted in a decrease of hemoglobin radioactivity by about 50 percent. When the iron was bound to the fetal calf serum, while the remainder of the protein part of the medium was rat plasma, the amount of Fe^{59} in the hemoglobin was reduced to about one-sixth that found when the medium was all rat plasma. On the other hand, when the main part of the protein consisted of fetal calf serum and the iron was bound to rat plasma, the label in hemoglobin was increased somewhat more than three-fold. These data are summarized in Table 1.

We interpret these data as indicating that the marrow cells in culture can synthesize hemoglobin at an accelerated rate when provided with some factor, possibly nutritional, in fetal calf serum. This factor would appear to be different from the "sticking factor" for which fetal calf serum is usually employed, since the marrow cells did not attach to the dish during the relatively short time of incubation used. In addition, rat plasma must contain an iron-binding factor (probably transferrin) which is important for optimal hemoglobin synthesis by rat marrow cells. The observation that the iron-binding material in fetal calf serum is much less effective than that in the isologous plasma points to a type of specificity which enables cells to utilize only a limited range of iron-binding materials for maximal hemoglobin synthesis.

Although the total iron was not determined in these experiments, other independent measurements indicate that fetal calf serum contains at most about 40 percent more iron than rat plasma and that the transferrin contents of the two are approximately the same. These data and those in Table 1 clearly rule

out any effect of iron concentration being responsible for the differences seen with the various mixtures.

These observations provide a basis for increasing the sensitivity of the marrow-cell culture method for the study of hemoglobin synthesis, for the assay of erythropoietin in vitro, and for investigations into the mechanism of action of the hormone.

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3. Drabkin's solution: 1 g of $NaHCO_3$, 52 mg of KCN, and 198 mg of $K_3Fe(CN)_6$ per liter.
4. We gratefully acknowledge the capable assistance of Mrs. Sandra Shin and Mr. Edward Morris.

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11 June 1964

Mutant Gene That Changes Protein Composition and Increases Lysine Content of Maize Endosperm

Abstract. *Preliminary tests have shown that the endosperms of maize seeds homozygous for the* opaque-2 *mutant gene have a higher lysine content than normal kernels. As a critical test, a backcross progeny was divided into* opaque-2 *and normal kernels, the endosperms separated, and the amino acids determined. The* opaque-2 *endosperms had a different amino acid pattern and 69 percent more lysine than the normal seeds. The major reason for these changes is the synthesis of proteins with a greater content of basic amino acids in the acid-soluble fraction of the mutant endosperm. This is accompanied by a reduction in the ratio of zein to glutelin.*

Since the development of the copper extraction-fractionation method for separating maize proteins (*1–4*), we have been searching for maize with a lower zein and a higher lysine content. The importance of examining the separated endosperms has been stressed (*5*). Preliminary tests showed that a strain homozygous for the recessive mutant gene, *opaque-2* (o_2), had in the endosperm a lysine content (4 percent of the protein) which was twice that found in typical hybrid corn (*7*). Copper fractionation (*2*) of protein from *opaque-2* endosperm revealed 15.7 percent zein and 42.3 percent glutelin based on total protein (*7*). Endosperms from normal North American and Guatemalan maize lines (*4*) contained 41 to 52 percent zein and 17 to 28 percent glutelin on the same basis. Thus, there is a reversal in the ratio of zein to glutelin in the *opaque-2* endosperm when compared with normal maize lines.

We have now determined the lysine content of endosperms from two additional strains containing the *opaque-2* gene in different genetic backgrounds from each other, and from the *opaque-2* strain first tested. In both strains, the lysine content (3.3 to 4.0 percent) was more than twice that of endosperms from a normal strain used as a control (1.3 percent lysine).

To make a critical test of the hypothesis that the *opaque-2* mutant is responsible for the increased lysine content the normal and *opaque-2* kernels from a single backcross ear ($+/o_2 \times o_2/o_2$) were separated. The endosperms were isolated and defatted as described previously (*1*), and 5 mg of endosperm protein was hydrolyzed at 110°C for 24 hours by refluxing with 20 ml of 6*N* HCl. One-milligram portions of hydrolyzed protein were placed on the short and long columns of a Spinco automatic amino acid analyzer. Norleucine was used as an internal standard.

The amino acid composition of the *opaque-2* and normal endosperms is compared in Table 1. Both types of endosperms contain 8.69 percent crude protein ($N \times 6.25$) on a fat and moisture-free basis (micro-Kjeldahl). Thus, the amino acid contents are directly comparable.

The *opaque-2* endosperm contains 69 percent more lysine than the normal endosperm. The former contains less glutamic acid, alanine, methionine, leucine, and tyrosine, and more lysine, histidine, arginine, aspartic acid, glycine, and cystine than the latter. The same relationship is found when the amino acid compositions of glutelin and zein are compared (*3*).

Copper fractionation (*2*) of duplicate 0.5-g portions of ground, defatted *opaque-2* endosperm, and of a single 0.5-g portion of normal endosperm from the same ear of corn used above,

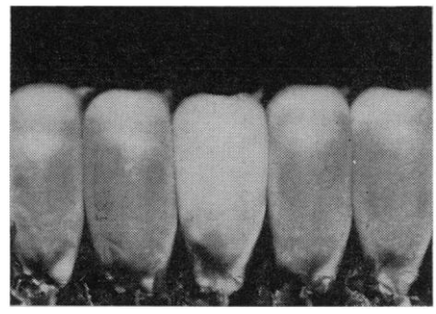

Fig. 1. A row of kernels on an ear of maize showing *opaque-2* mutant (center) and normal kernels.

gave the following distribution based on soluble nitrogen. *Opaque-2*: 35 percent acid-soluble, 26 percent alcohol-soluble (zein), and 39 percent alkali-soluble (glutelin); normal: 34 percent acid-soluble, 37 percent alcohol-soluble (zein), and 29 percent alkali-soluble (glutelin). This confirms the reduction in the zein to glutelin ratio observed previously.

Preliminary data on the basic amino acid and amide ammonia content of the above separated soluble copper fractions show that along with the reduction in the zein to glutelin ratio, important changes occur in the amino acid patterns of the acid-soluble and alcohol-soluble (zein) fractions. We have calculated the ratios of the three basic amino acids and amide ammonia

Table 1. Amino acids in normal and opaque endosperms from the same ear of corn (expressed as grams per 100 g of protein).

Amino acid	Endosperm	
	Opaque	Normal
Lysine	3.39	2.00
Tryptophan*		
Histidine	3.35	2.82
Amide ammonia	3.41	3.28
Arginine	5.10	3.76
Aspartic acid	8.45	6.17
Glutamic acid	19.13	21.30
Threonine	3.91	3.48
Serine	4.99	5.17
Proline	9.36	9.67
Glycine	4.02	3.24
Alanine	6.99	8.13
Valine	4.98	4.68
Cystine	2.35	1.79
Methionine	2.00	2.83
Isoleucine	3.91	3.82
Leucine	11.63	14.29
Tyrosine	4.71	5.26
Phenylalanine	4.96	5.29

* Peptide-bound tryptophan presents a special problem because it is destroyed by hydrolyzing agents (*8*).