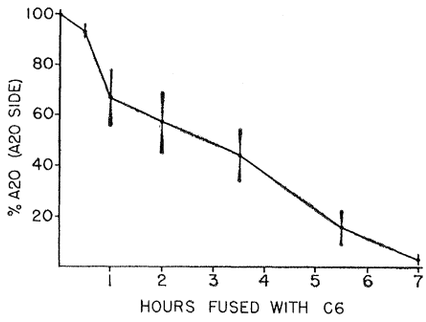

Fig. 2. The percentages of A20-type plaques recovered from the A20 side of a fused plasmodium when equal-sized plasmodia of C6 and A20 were fused. The vertical line equals 2 times the standard error.

mixer until no macroscopic pieces remained. Examination of the mixture with a phase-contrast microscope revealed that, although most of the plasmodia had simply lysed, some microplasmodia containing one or more nuclei remained. The mixtures were plated on agar containing glucose, peptone, and yeast extract with bacteria. Adjacent pieces of fused plasmodia were placed on nonnutrient agar, as usual, for fruiting. After 48 hours the plates bearing the microplasmodia were examined with a dissecting microscope and the smallest plasmodia present were cut out and subcultured to nonnutrient agar spread with bacteria. After these had fruited their spores were plated as usual and scored as C6-type or A20-type. About 20 percent of the microplasmodia yielded exclusively A20-type plaques, whereas less than 2 percent of the plaques from the controls were the A20-type. Microplasmodia isolated from C6 plasmodia in a similar manner gave rise to C6-type plaques only. The A20 characteristic therefore persists in the vegetative plasmodia after being mixed with C6 nuclei and cytoplasm. It disappears during fruiting. Microplasmodia isolated from C6 remained normal; no factor could be eliminated from them.

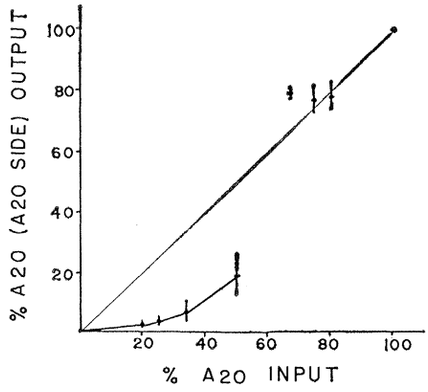

Fig. 3. The percentages of A20-type plaques recovered from the A20 side of a fused plasmodium when the plasmodia remained fused for 4 hours. Various ratios of C6 and A20 plasmodia were fused. The results expected on the basis of a simple mixing are represented by the fine line.

The disappearance of the characteristic of delayed plasmodium formation might be due to nuclear selection. Since the characteristic can be recovered from microplasmodia, the selection cannot take place in the vegetative plasmodium. Furthermore, when C6 and A20 plasmodia were fused on nonnutrient agar, where little growth would occur, and were left together until the fused plasmodium fruited, few A20 plaques were recovered. It is possible, however, that nuclear selection might occur during the formation of fruiting bodies. Cadman (*5*) reported that many nuclei in the developing fruit became pycnotic and did not undergo meiosis or become incorporated into spores. The possibility that C6 nuclei might be preferentially incorporated into spores was tested by means of radioactive tracers. Plasmodia were labeled by growing them on GPY/5 plates spread with bacteria, 250 μc of H^3-thymidine having been added to the plates as a narrow band near the inoculum. Twenty-four hours after the plasmodia had crossed the labeled band, they were fused with unlabeled plasmodia. Twelve hours later the fused plasmodia were transferred to nonnutrient agar for fruiting. Amoebas that germinated from the resulting spores were fixed with buffered osmium, air-dried on clean slides, and washed extensively with distilled water. Autoradiography was done according to the technique described by Prescott (*6*) with Kodak NTB-2 liquid emulsion. After 4 weeks of exposure, amoebas were examined by phase-contrast microscopy and scored as labeled or unlabeled. Comparisons were made between the percentages of labeled amoebas recovered from fused labeled A20 plus unlabeled A20 plasmodia and labeled A20 plus unlabeled C6 plasmodia, and from fused labeled C6 plus unlabeled C6 plasmodia and labeled C6 plus unlabeled A20 plasmodia. No significant differences were found. In every case 40 to 60 percent of the amoebas were labeled. Silver grains were seen above pieces of capillitium, indicating that there had been breakdown of nuclear material. The results obtained by autoradiography suggest that the phenomenon of the disappearance of the two characteristics, delayed plasmodium formation and resistance to cycloheximides, cannot be explained by nuclear selection.

The observed phenomenon could be explained either by assuming that some nonchromosomal factor responsible for the two characteristics of strain A20 is selected against during the fruiting process, or by assuming that such a factor derived from the C6 parent is somehow incorporated into the A20 nuclei during the fruiting process.

NORMAN S. KERR
Department of Zoology, University of Minnesota, Minneapolis 55455

References and Notes

1. N. S. Kerr and M. Sussman, *J. Gen. Microbiol.* **19**, 173 (1958).
2. ———, *J. Protozool.* **6** (suppl.), 16 (1959).
3. C. J. Alexopoulos, *Botan. Rev.* **29**, 1 (1963).
4. N. Kamiya, *Protoplasmatologia* **8**, pt. 3a (1959).
5. E. J. Cadman, *Trans. Roy. Soc. Edinburgh* **57**, 93 (1931).
6. D. M. Prescott, in *Methods in Cell Physiology*, D. M. Prescott, Ed. (Academic Press, New York, 1964), vol. 1, p. 365.
7. This investigation was supported by PHS research grant AI 05521 from the National Institute of Allergy and Infectious Diseases.

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Erythropoiesis in the Chick Embryo: The Role of Endoderm

Abstract. *The endoderm can be separated from the ectomesoderm of the area vasculosa of the early chick embryo. The development of the separated germ layers in organ cultures shows that the endoderm is necessary for the normal formation of blood islands by mesodermal cells. In the absence of endoderm the mesodermal cells undergo erythropoiesis in dispersed groups of cells but do not form endothelium.*

An early developmental event leading to the initiation of erythrocyte formation in the chick embryo is the condensation of newly formed mesoderm of the area vasculosa, which results in the appearance of blood islands. Thus, at the head process stage, blood islands composed of a few thousand cells are observed in the splanchnic mesoderm (*1*). At about the seven-somite stage all the central cells of the blood islands begin to make hemoglobin and produce erythrocytes (*2*); the peripheral cells of the blood islands flatten and form the endothelium of the extraembryonic blood vessels (*3*). Organogenesis elsewhere in the embryo often involves similar compact groups of

cells, and this aggregated condition may often be necessary for normal organogenesis to occur (*4*).

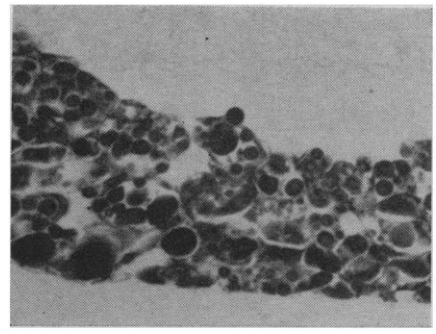
I have investigated the tissue interactions, primarily between endoderm and mesoderm, which occur during the establishment and differentiation of the blood island. Small explants (approximately 0.5 mm^2), from the area vasculosa near the pellucida-opaca junction surrounding the posterior two-thirds of the primitive streak, were cultured on Spratt's whole egg-agar (*5*) medium in watch glasses for 24 to 72 hours. These conditions allowed normal erythropoiesis and hemoglobin formation in all of the triploblastic control explants taken from donors at the various stages of development (see Table 1). Triploblastic fragments were separated into two layers, ectomesoderm and endoderm, by dissection at room temperature in calcium- and magnesium-free Tyrode's solution, after which the separate layers were cultured in watch glasses. Hemoglobin was detected by inspection of living cultures and by staining the explants by means of the sensitive dimethylbenzidine procedure (*6*). Over one-third of the cultures in each group were fixed in Bouin's solution, sectioned at 5 μ, and stained with hematoxylin and eosin.

Primary attention was focused on two different stages of development: the stage before the formation of blood islands (definitive primitive streak) and the stage at which the formation of blood islands was in progress (head fold). Separation of triploblastic fragments from embryos with a definitive primitive streak into two layers could result in a complete separation of endoderm from ectomesoderm (Figs. 1 and 2). The isolated endoderm lived several days in culture, but it failed to display any sign of erythrocyte formation, except in the two out of 47 explants in which some mesodermal cells remained attached to the endoderm after dissection. The adherence of mesodermal cells to the endoderm during dissection was much more pronounced in fragments from the head-fold stage; this resulted in development of a substantial number of small but otherwise normal blood islands in endodermal explants (Fig. 3). Hemoglobin is easily detectable in these explants within 12 to 16 hours after explantation.

In contrast to the behavior of isolated endoderm, explants which were from donor embryos of both stages and which contained ectomesoderm displayed a very different kind of erythropoiesis. Erythrocyte formation was reduced in frequency compared to triploblastic control cultures, and hemoglobin synthesis seemed somewhat delayed, becoming obvious by 24 to 30 hours after explantation. This delay may be apparent only because of the difficulty of detecting the small number of erythrocytes arising in the explants. These erythrocytes did not seem to deviate from normal morphology or stainability for hemoglobin. Typical blood islands did not form, however, nor could endothelium be detected. Erythropoiesis either took place in small clusters of cells which were in contact with other mesodermal cells

Table 1. The differentiation of separated germ layers in organ culture. Abbreviations: DPS, definitive primitive streak; HF, head fold; HB, hemoglobin. "With blood islands" means cultures showing compact groups of erythrocytes with typical morphology as observed in living and stained explants.

Stage of donor embryo	Number of explants		
	Total	With HB	With blood islands
Endoderm alone			
DPS	47	2	2
HF	27	18	18
Ectomesoderm alone			
DPS	49	32	0
HF	24	16	2
Recombined endoderm plus ectomesoderm			
DPS	12	12	12
HF	6	6	6



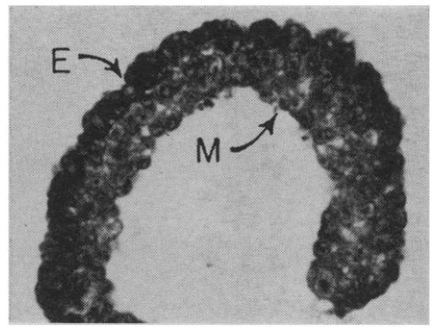
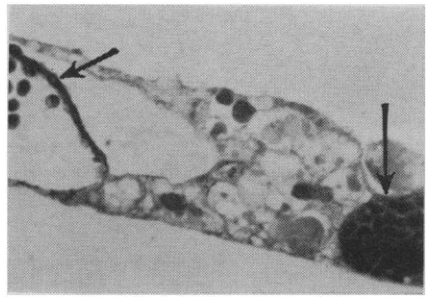


Fig. 1 (left). Endodermal layer resulting from separation of triploblastic fragments of the area vasculosa at the definitive primitive-streak stage (× 288). Fig. 2 (right). Ectoderm (*E*) and mesoderm (*M*) in ectomesodermal layer from which endoderm has been removed by dissection of area vasculosa at the definitive primitive-streak stage (× 208).



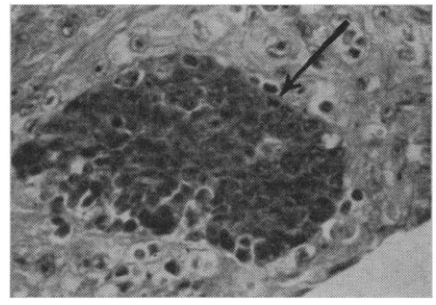


Fig. 3 (left). An endodermal fragment of an embryo at the head-fold stage, cultured for 40 hours after separation from the ectomesoderm. Some mesodermal cells were retained by the endoderm during dissection. The arrows point to developing blood islands (× 288). Fig. 4 (right). A fragment of ectomesoderm of an embryo at the definitive primitive-streak stage cultured for 40 hours in the absence of endoderm. The arrow points to a site of erythropoiesis. Endothelium is absent (× 288).

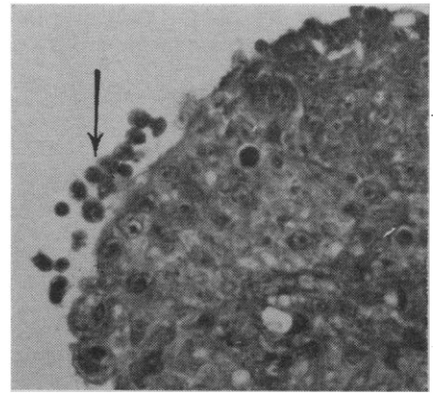


Fig. 5. A fragment of ectomesoderm from an embryo at the head-fold stage was dissected free of endoderm and cultured for 40 hours. An arrow points to a group of erythrocytes forming at the edge of the explant (× 288).

without an intervening endothelial barrier (Fig. 4), or, more commonly, erythrocyte formation took place on the edge of the explant and red cells were released to float on the fluid film surrounding the tissue (Fig. 5). Unless some endoderm was present, formation of a compact blood island and endothelium did not occur. Recombination at the time of explantation of the ectomesoderm with endoderm resulted in formation of typical blood islands and normal differentiation.

It is concluded that hemoglobin synthesis and blood island formation are dissociable events. While the mesoderm can carry out erythropoiesis in the absence of endoderm, the endoderm influences the frequency of erythrocyte formation and orients the mesoderm into blood islands in which endothelium formation can take place. Mere condensation into a blood island, however, is not a sufficient condition for erythropoiesis, for derangement of nucleic acid metabolism prior to the head-process stage allows formation of blood islands (*7*) without subsequent synthesis of hemoglobin.

FRED H. WILT
Department of Zoology, University of California, Berkeley

References and Notes

1. F. R. Sabin, *Carnegie Inst. Wash. Publ. No. 272* (contrib. embryol. No. 9), 213 (1920).
2. F. H. Wilt, *Proc. Natl. Acad. Sci. U.S.* **48**, 1582 (1962).
3. F. R. Lillie, *Development of the Chick* (Holt, New York, 1908), pp. 117–118.
4. C. Grobstein, in *The Cell*, J. Brachet and A. E. Mirsky, Eds. (Academic Press, New York, 1959), p. 437.
5. N. T. Spratt and H. Haas, *J. Exptl. Zool.* **144**, 139 (1960).
6. B. R. A. O'Brien, *Exptl. Cell. Res.* **21**, 226 (1960).
7. F. H. Wilt, *J. Mol. Biol.*, in press.
8. I was a PHS Special Fellow in residence at Stanford University during this investigation. I thank Dr. Clifford Grobstein and Dr. Norman Wessells and their technical staffs for their help. Portions of the research were supported by grants from the NSF to Dr. Grobstein and the author.

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Tricarboxylic Acid Cycle Mutants in Saccharomyces: Comparison of Independently Derived Mutants

Abstract. *A yeast mutant independently isolated as a glutamate auxotroph* (glt_{2-1}) *was similar to the* glt_{1-1} *mutant in exhibiting a blocked tricarboxylic acid cycle due to the lack of aconitate hydratase. The new mutant differed by exhibiting blocks in lysine and cytochrome biosynthesis which segregated together with the glutamate requirement.*

We recently described (*1*) some of the characteristics of a glutamate-requiring yeast mutant (glt_{1-1}) which lacked aconitate hydratase. A second glutamate auxotroph (glt_{2-1}) independently induced by ultraviolet irradiation (*2*) was nonallelic to glt_{1-1}. When the two mutant strains were mated, glutamate independent hybrids were always obtained. Tetrad analysis of the four-spored asci obtained from these diploid hybrids showed a high frequency of prototrophic recombinants and the independent assortment of glt_1 and glt_2 markers predicted for unlinked genes.

The glutamate requirement in both mutant strains is due to the lack of aconitate hydratase, and this lack leads to the failure of the mutants to make α-ketoglutarate (the immediate precursor of glutamate) by way of the tricarboxylic acid cycle. It thus appears that at least two unlinked genes take part in the biosynthesis of aconitate hydratase. The possibility that two polypeptides, one controlled by each gene, might participate in aconitate hydratase structure or activity was tested by assaying mixed, cell-free preparations derived from each mutant under a variety of preparative and assay conditions. Complementation in vitro was not observed but this may still be a matter of technical difficulty since the mutants complement each other in vivo. Aconitate hydratase activity per milligram of protein (*3*) in the complemented diploid (*362 ± 13 units*) was comparable to that in a wild-type diploid (*323 ± 77 units*).

In addition to the glutamate requirement based on a lack of aconitate hydratase, glt_2 mutant strains required lysine for growth and lacked cytochromes *a* and *b*. This complex phenotype segregated regularly as a unit; glutamate-requiring segregants required lysine and were nonrespiring with all substrates tested. Regular segregation of the inability to respire has been seen before in the "segregational petites" (*4, 5*). The current phenotype is similar to that of the ly_6 and ly_8 mutants in which a requirement for lysine and a block in cytochrome biosynthesis segregated as a unit and appeared in each case to be primarily due to an alteration in a single gene (*5*). We, therefore, examined the ly_6 and ly_8 mutants for a glutamate requirement, which had, up to this point, not been reported. Indeed they do require glutamate for growth. This requirement was also based on the lack of aconitate hydratase. These mutants are thus all of similar phenotype even though the glt_2 mutant was isolated as a glutamate auxotroph and the ly_6 and ly_8 mutants as lysine auxotrophs. By contrast no requirement for lysine is associated with the glt_1 marker and no requirement for glutamate with the ly_1, ly_2, ly_3, ly_4, ly_5, ly_7, ly_9, or ly_{10} markers.

Complementation tests on media lacking lysine indicate that the glt_2 lesion is probably heteroallelic to ly_8 and that ly_6 is nonallelic to ly_8 and glt_2.

Complementation tests were also performed against appropriate petite test strains. The results indicated that most glt_2 segregants were ρ^- (lack the cytoplasmic factor for cytochrome biosynthesis). From 5 to 10 percent of the strains, which contained 0.01 to 0.03 percent of ρ^+ cells, exhibited a very weak ρ^+ response.

One interesting problem raised by the isolation of these mutants is the interrelation between and control of glutamate, lysine, and cytochrome biosynthesis. α-Ketoglutarate is a common precursor in all three pathways. The block in the biosynthesis of aconitate hydratase does not appear to interfere with lysine and cytochrome biosynthesis in the glt_1 mutant but does appear to in the glt_2, ly_6, and ly_8 mutants. The mechanism underlying this difference is not yet known.

M. OGUR
A. ROSHANMANESH
S. OGUR
Department of Microbiology and Biological Research Laboratory, Southern Illinois University, Carbondale

References and Notes

1. M. Ogur, L. Coker, S. Ogur, *Biochem. Biophys. Res. Commun.* **14**, 193 (1964).
2. We thank Dr. R. Mortimer for strain X1251-1B. Genetic symbols are based on the Carbondale Yeast Genetics Conference, November 1961, summarized in the *Microbial Genet. Bull.*, suppl. to No. 19, January 1963.
3. E. Racker, *Biochim. Biophys. Acta* **4**, 211 (1950).
4. S. Y. Chen, B. Ephrussi, H. Hottinguer, *Heredity* **4**, 337 (1950).
5. F. Sherman, *Genetics* **48**, 375 (1963).
6. Supported in part by grant E98D from the American Cancer Society. A preliminary report was presented to the 6th International Congress of Biochemistry, New York, July 1964.

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