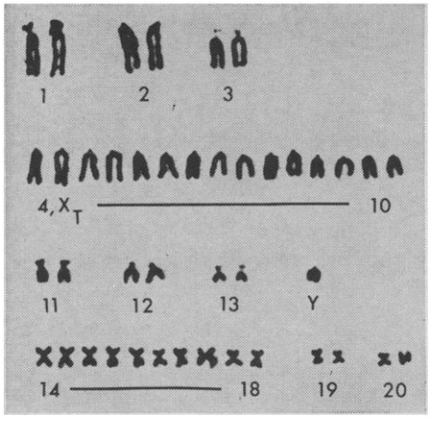

Fig. 1. Karyotype of diploid cultured cell from a male rat showing 42 chromosomes, X_T and small positively heteropyknotic Y. Pairs 3 and 13 are satellited.

as monolayers on glass or in suspension cultures, they more commonly disappear. When cells in serial culture lose such specialized functions they have not been observed to regain them. Evidence presented in this report shows that connective tissue cells may regain the differentiated function of collagen synthesis that has been lost during many serial passages as monolayers on glass.

We grew the cells used in these experiments from aorta of the rat by explantation into plasma clots, transferred them to prescription bottles, and maintained them for several months in serial culture as monolayers on glass. The nutrient medium was F-10 (*5*), supplemented with 10 percent fetal calf serum and 5 percent fetal human serum.

The culture medium was changed two times a week, and trypsinization and subdivision were carried out when growth became confluent. Cell doubling time was about 40 hours. The cells retained the normal diploid complement of 42 chromosomes (Fig. 1) for about 4 months, as long as they were observed (*6*). Analysis of the medium and cells during this time revealed no accumulation of collagen, as measured by the content of hydroxyproline, even in cultures allowed to remain confluent for several days.

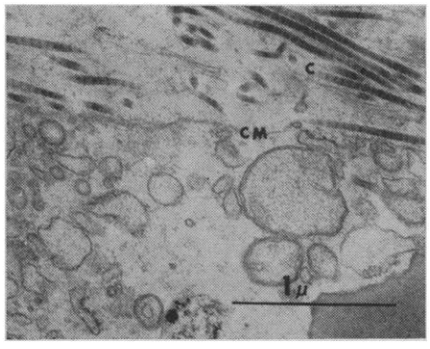

Fig. 2. Electron micrograph showing collagen bundles (*C*) near the cell membrane (*CM*).

Cells maintained in this way through 15 or more transfers were placed in diffusion chambers (*7*). The chambers were constructed by cementing a Millipore filter (pore diameter 0.22 μ) on one side of a Plexiglas disk 2.5 cm in diameter and 0.3 cm deep. The chamber was sterilized with ultraviolet light and 0.25 ml of growth medium that contained 5×10^5 cells was placed in the chamber. It was then closed by cementing another filter membrane to the other side. Two chambers were inserted into the peritoneal cavity of each rat. After 3 weeks a thin fibrous capsule surrounded the chamber. Within the chamber a layer of tissue six to ten cells thick was found adjacent to the filter membrane. The elongated cells forming the tissue within the chamber were separated from one another by an abundant extracellular material, part of which showed an affinity for the aniline blue of the Mallory trichrome stain and for light green of the Gomori trichrome stain. Both stains are used to show collagen. Electron micrographs reveal numerous fibers with a major periodicity of 640 Å, which is a characteristic of collagen (Fig. 2). Chambers that did not contain cells originally had only fluid that resembled plasma.

The thin fibrous capsules were removed from outside the chambers, and filter membranes and layers of tissue inside the diffusion chambers were hydrolyzed and analyzed for hydroxyproline, a component of collagen (Table 1) (*8*). The small amount of hydroxyproline present on the filter membranes from blank chambers probably was a result of contamination from the fibrous capsules.

Our results indicate quite clearly that at least one differentiated function, the production of collagen, can be regained by cultured cells following its disappearance. The genetic capacity of the cells to produce collagen is therefore not irreversibly lost during the period of growth as monolayers on glass. Because the ability to produce collagen is retained through 15 or more transfers in culture, by which time homogeneity of the cell population might be expected, it is probable that this capacity is a property of all the cultured cells rather than of only some.

Table 1. Hydroxyproline in diffusion chambers.

Empty chamber (μg hydroxyproline per chamber)	5×10^5 cells introduced (μg hydroxyproline per chamber)
18.6	176.0
18.0	135.0
6.4	129.0
34.0	124.0
11.6	138.0
16.0	156.0

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References and Notes

1. C. Champy, *Bibliog. Anat.* **23**, 184 (1913).
2. C. Grobstein, in *The Cell*, J. Brachet and A. Mirsky, Eds. (Academic Press, New York, 1959), vol. 1, pp. 479–480.
3. B. Goldberg, H. Green, G. Todaro, *Exptl. Cell. Res.* **31**, 444 (1963).
4. C. Morris, *Ann. N.Y. Acad. Sci.* **86**, 878 (1960); H. Green and D. Hamerman, *Nature* **201**, 710 (1964).
5. R. Ham, *Exptl. Cell. Res.* **29**, 515 (1963).
6. D. Hungerford and P. Nowell, *J. Morphol.* **113**, 275 (1963).
7. R. Curran and E. Rowsell, *J. Pathol. Bacteriol.* **76**, 561 (1958).
8. D. Prockop and S. Udenfriend, *Anal. Biochem.* **1**, 228 (1960).
9. This work was supported in part by a grant from the U.S. Public Health Service.

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C-Phycocyanin: Minimum Molecular Weight

Abstract. *Sedimentation and immunodiffusion experiments indicate that the molecular weight for the minimum molecular unit of C-phycocyanin is 30,000. This result agrees with an analysis of available data on amino acid content for C-phycocyanins from several different algae.*

The molecular weight and other properties of phycocyanin, a biliprotein from blue-green and red algae have been studied (*1*). Svedberg *et al.* (*2*, *3*) investigated the molecular weights of C-phycocyanin and other phycocyanins as a function of *p*H by sedimentation velocity and sedimentation equilibrium studies. Svedberg and Katsurai (*2*) estimated the molecular weight for C-phycocyanin as 208,000 and for the

dissociated molecules at 104,000. Hattori and Fujita (*4*) more recently made a slightly higher estimate of the molecular weights for C-phycocyanin.

In studies on fully deuterated phycocyanin (*5–7*) we have investigated the physical and chemical properties of several C-phycocyanins and found molecular weights (*8*) that agree substantially or in part with those of Svedberg and Katsurai (*2*). However, in purified preparations at *p*H 5.0 and *p*H 9.0, and in crude extracts, we have detected lower molecular weight components. Larger amounts of the lower molecular weight components were detected in the presence of urea and sodium dodecyl sulfate.

When phycocyanin solutions in 0.1*M*, 1*M*, and 2*M* urea and 0.1 ionic strength phosphate buffer at *p*H 7.0 are subjected to ultracentrifugation, the relative amounts of the resulting multiple peaks vary as a function of urea concentration, the slowest moving peak increasing with increasing urea concentration. This effect is almost totally reversed when the urea is dialyzed into buffer. We observed an analogous effect also in 0.01 percent, 0.1 percent, and 1 percent solutions of sodium dodecyl sulfate, in the 0.1 ionic strength phosphate buffer. The slowest moving peak is increased in relative concentration with increasing concentration of the dodecyl sulfate, so that at 1 percent a solution containing 2 percent protein has about 90 percent of 3.03*S* component. The same behavior is observed with sodium dodecyl sulfate solutions at *p*H 4.7. A 3.76*S* component is found in sedimentation patterns of phycocyanin in acetate buffer at *p*H 5.0, 0.1 ionic strength, and also at *p*H 9.0 The slowest sedimenting component is thus found in the *p*H range 4.7 to 9.0, and under the proper conditions it is the major component. This *p*H region is the region of greatest stability for this protein.

The diffusion coefficients of the several aggregates present at *p*H 7.0 were determined by the agar diffusion method of Allison and Humphrey (*9*). The rabbit antibody preparations required for this method have been characterized previously (*6*). The diffusion coefficient determined for the major precipitin line was 4.2×10^{-7} cm²/sec, a value in good agreement with the 4.05×10^{-7} reported by Tiselius and Gross (*10*) for the major component of a phycocyanin and the value of 4.3×10^{-7} reported by Hattori and Fujita (*4*).

Table 1. Number of amino acid residues found in C-phycocyanins, based on one cystine residue per integral unit rounded off to the nearest residue. Values in parentheses are based on one histidine residue per integral unit. The names of organisms from which the phycocyanins were obtained are abbreviated in the table as follows: P.C., *Plectonema calothricoides;* P.L., *Phormidium luridum;* N.M., *Nostoc muscorum;* S.L., *Synechococcus lividus;* P.T., *Porphyra tenera;* C.C., *Cyanidium caldarium.*

Amino acid	Number of residues in C-phycocyanins from:					
	P.C.*	P.L.†	N.M.‡	S.L.†	P.T.§	C.C.‖
Lysine	10	13	10	11	4 (11)	7 (10)
Histidine	2	2	2	2	1 (1)	1 (1)
Arginine	14	17	16	19	7 (19)	12 (17)
Aspartic acid	28	29	31	28	14 (36)	24 (33)
Threonine	14	17	17	14	7 (18)	10 (14)
Serine	16	20	19	10	12 (32)	9 (13)
Glutamic acid	18	19	27	30	13 (38)	20 (28)
Proline	8	9	9	10	5 (10)	8 (11)
Glycine	20	24	23	10	11 (29)	16 (22)
Alanine	40	43	36	26	19 (52)	34 (47)
Cystine	1	1	1	1	1 (3)	1 (1)
Valine	16	20	14	17	9 (25)	13 (18)
Methionine	8	9	2	8	4 (11)	6 (8)
Isoleucine	14	18	13	16	7 (18)	12 (17)
Leucine	22	25	22	23	13 (36)	18 (25)
Tyrosine	10	14	11	16	6 (17)	8 (11)
Phenylalanine	8	11	7	10	3 (7)	5 (7)
	Calculated molecular weight¶					
	28,080	31,186	27,666	28,902	14,667 (38,807)	21,794 (30,209)

* See (*7*). † Based on work completed by K. O'Reilly and D. Berns at this laboratory. ‡ See (*11*). § See (*12*). ‖ Based on analysis performed by D. S. Berns at Argonne National Laboratory on sample supplied by L. Bogorad. ¶ Molecular weight calculated from summation of the residue weight contributions as listed in this table.

Precipitin lines representing faster diffusing species were also present. Diffusion coefficients of 7.45×10^{-7} and 1.32×10^{-6} were determined for these lines. By combining the diffusion coefficients of 7.45×10^{-7} with the sedimentation coefficients of 7.0×10^{-13} sec^{-1} for the slowest sedimenting species present at *p*H 7.0, and using the Svedberg equation, we calculated a molecular weight of 95,000. A similar calculation with the 1.32×10^{-6} diffusion coefficient and the 3.76×10^{-13} *S* value yields a molecular weight of 28,000. Therefore, it seems within reason to propose a minimum molecular weight of 30,000.

Most of our physical studies have been made with extracts from *Plectonema calothricoides,* although work with C-phycocyanin from *Phormidium luridum* and *Synechococcus lividus* indicates analogous behavior. The amino-acid analyses of the C-phycocyanins from these and other algae have been made by the Moore-Stein method in this laboratory and similar analyses were made by Raftery and Ó hEocha (*11*) and by Kimmel and Smith (*12*). From available data, on the arbitrary basis of one cystine residue per minimum molecular unit, the approximate number of residues of each amino acid per arbitrary minimum unit may be calculated. This has been done for C-phycocyanin from six different algae (Table 1). The amino acid content of phycocyanin from *Plectonema calothricoides, Phormidium luridum,* and *Nostoc muscorum* is quite similar. The phycocyanin from the thermophilic *Synechococcus lividus,* although similar, does exhibit some interesting differences not necessarily associated with thermophilic proteins. The number of amino acid residues of phycocyanin from *Porphyra tenera* is in almost every case one-half that calculated for *Plectonema calothricoides, Phormidium luridum,* and *Nostoc muscorum.* In fact, if the arbitrary basis had been any one of ten other residues, the number of each amino residue on that basis would be quite similar to that of any one of the four phycocyanins already discussed. Cystine was used as the arbitrary basis so that one disulfide linkage per minimum unit would be included. In the case of phycocyanin from *Porphyra tenera,* however, the assignment of the arbitrary basis of one cystine per minimum unit is questionable, since this results in 0.4 histidine residues. It would probably be better to use the least abundant amino acid, histidine. If this is done, the number of residues and the molecular weight are those in parentheses in Table 1. These calculations are in better agreement with the proposed molecular weight. In the phycocyanins from *Plectonema calothricoides, Phormidium luridum, N.*

muscorum, and *S. lividus,* cystine is the least abundant amino acid; however, in the *Cyanidium caldarium* phycocyanin, histidine is the least abundant. The amino acid residues calculated on the cystine basis for *C. caldarium* show some real deviations; however, if histidine is used as the arbitrary basis, agreement with the other phycocyanins is quite good. The interesting deviation in the phycocyanins in both *Porphyra tenera* and *C. caldarium* is in the ratio of histidine residues to whole-cystine residues. Most striking is the fact that the molecular weight calculated from these analyses for a minimum molecular unit for five of the phycocyanins is between 28,000 and 31,000. The sixth (*P. tenera*) is in the 39,000 region. The molecular weight of the minimum unit observed in sedimentation studies is calculated to be in this same 30,000 molecular weight region.

The evidence presented seems to point to a minimum molecular weight unit of 30,000 for C-phycocyanin. There is good agreement between physical studies and analyses of amino acid content of the phycocyanins. It should be emphasized that in nearly all physical studies the smallest molecular weight component is not present in the largest amount. The conditions of preparation may be altered to cause a reversible formation of larger or smaller amounts of the smallest component.

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References and Notes

1. C. Ó hEocha, in *Physiology and Biochemistry of Algae,* R. A. Lewin, Ed. (Academic Press, New York, 1962), p. 421.
2. T. Svedberg and T. Katsurai, *J. Am. Chem. Soc.* **51**, 3573 (1929).
3. T. Svedberg and N. B. Lewis, *ibid.* **50**, 525 (1928); I.-B. Eriksson-Quensel, *Biochem. J.* **32**, 585 (1938).
4. A. Hattori and Y. Fujita, *J. Biochem. Tokyo* **46**, 633 (1959).
5. D. S. Berns, *Biochemistry* **2**, 1377 (1963).
6. ———, *J. Am. Chem. Soc.* **85**, 1676 (1963).
7. ———, H. L. Crespi, J. J. Katz, *ibid.*, p. 8.
8. E. Scott, unpublished observations.
9. A. C. Allison and J. H. Humphrey, *Immunology* **3**, 95 (1960).
10. A. Tiselius and D. Gross, *Kolloid Z.* **66**, 11 (1934).
11. M. A. Raftery and C. Ó hEocha, *Biochem. J.*, in press.
12. J. R. Kimmel and E. L. Smith, *Bull. Soc. Chim. Biol.* **40**, 2049 (1958).
13. Supported by NSF grants GB 422 and GB 2222. We thank Dr. Raftery and Dr. Ó hEocha for allowing us to see their results prior to publication.

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Erythrocyte Glucose-6-Phosphate Dehydrogenase in Caucasians: New Inherited Variant

Abstract. *A new inherited variant of glucose-6-phosphate dehydrogenase having both a lowered enzyme activity and an altered electrophoretic mobility was discovered in two unrelated American families, one of Irish and the other of German ancestry. Family studies of the trait indicate that it is due to a sex-linked gene.*

Deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G-6-PD) activity is recognized as a heterogeneously expressed, sex-linked, enzyme defect which has been found in all major races of man in varying frequencies. The enzyme defect is manifest clinically by hemolytic anemia in apparently healthy individuals upon ingestion of the antimalarial drug primaquine, fava beans, or a large number of other hemolytic compounds. The general subject has been recently reviewed (*1*). In enzyme-deficient Negro males (13 percent of the American Negro male population), the erythrocytic G-6-PD activity is 8 to 15 percent of normal (*2*). Porter *et al.* (*3*) reported two electrophoretic forms (fast and slow) of erythrocytic G-6-PD in American Negroes, and they were designated as types A+ and B+ respectively (*4*); a plus (+) indicates normal enzyme activity. Studies by Kirkman and Hendrickson (*5*) demonstrated that these two phenotypes distinguishable by electrophoresis follow a sex-linked mode of inheritance. The G-6-PD in deficient Negro males usually migrates to the type A position and is designated A—. The B+ form of the enzyme is the common type found in Americans of European origin and in Mediterranean Caucasians (*3, 4*). Enzyme-deficient Mediterranean Caucasian males have 3 to 6 percent of normal enzyme activity (*2*), and their G-6-PD migrating to the B position is designated type B— (*4*). In addition to the above G-6-PD variants, other variants, all rare, have been reported in which electrophoretic and/or kinetic alterations are associated with the enzyme deficiency (*2, 6, 7*).

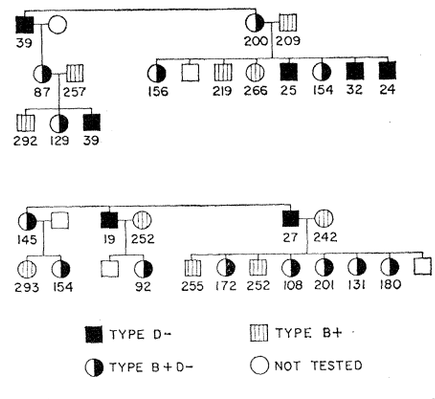

Fig. 1. Pedigrees showing segregation of the glucose-6-phosphate dehydrogenase variant, D— (*11*). The numbers beneath the tested individuals give G-6-PD activity in μmoles of reduced triphosphopyridine nucleotide (TPNH) formed per gram of hemoglobin per hour assayed at 22.5°C. The G-6-PD activity of the erythrocytes of normal males is 237 ± 36.

This report describes a new inherited variant of G-6-PD which is distinguished phenotypically by both a diminished enzyme activity and an altered electrophoretic mobility. In the course of a study of erythrocytic G-6-PD activity of 215 Caucasians (*8*), in which the enzyme was assayed by both a spectrophotometric method (*9*) and the methemoglobin reduction test (*10*), two individuals (a male and a female) with significantly lowered enzyme activity were found in two unrelated families, one of German and the other of Irish ancestry. In both families, there was a total of eight male members with the lowered enzyme activity. Their erythrocytic G-6-PD activity was 8 to 16 percent of normal (Fig. 1), an amount equivalent to that usually found in enzyme-deficient Negro males (8 to 15 percent of normal), and higher than that usually observed in enzyme-deficient Caucasian males (3 to 6 percent of normal) (*2*).

Electrophoretic studies were carried out on the erythrocytic G-6-PD of members of these two families. Under the electrophoretic conditions used (Fig. 2), all enzyme-deficient males had a single band of G-6-PD activity which migrated about 10 percent more slowly than the B form and 15 percent more slowly than the A form (Fig. 2). This slowly migrating band will be referred to here as D— (*13*). In eight of 25 male relatives tested, D— was the only band observed; all eight were enzyme deficient. In 15 of 25 female relatives tested, B+ and D— bands were present; the enzyme activity in these 15 females ranged from 35 to 80 percent of normal.