The literature provides no answer to the question of what the function of the coralloid root may be, apart from its serving as a place of habitation for the alga. One may suppose the function to be storage of food and water, since it is highly charged with both. The significance, if any, of the relation between alga and plant remains a matter for conjecture.

Tissues inhabited by organisms in certain species of plants (for example, in root nodules of legumes) consist of polyploid cells (*8*). The existence of somatic reduction as a normal ontogenetic process, participating in development of a functional tissue in a plant, may be unique, however.

A significant aspect of somatic reduction is the ability of cells having reduced numbers of chromosomes to organize metabolic nuclei and function normally.

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Hexokinase Isozymes in Human Erythrocytes

In 1966 Eaton, Brewer, and Tashian (*1*) reporting from this laboratory described seven isozymes of hexokinase in hemolyzates from adult human bloods. Subsequently, Holmes, Malone, Winegrad, and Oski (*2*) published on this subject, including studies of an association between a particular type of hexokinase isozyme and fetal hemoglobin. The method used by Holmes *et al.* as adapted from Katzen and Schimke (*3*) was considerably different from that which we had used in our studies, and resulted in a demonstration of two bands of activity in hemolyzates of adult humans, rather than seven. In commenting upon these differences in isozyme patterns in the two studies, Holmes *et al.* suggested that in our system additional, presumably artifactual, bands were being produced in the course of preparing the hemolyzates or during electrophoresis. In the absence of any data, this does not seem to be any more likely, and is perhaps less likely, than the possibility that the method employed by Holmes *et al.* is failing to resolve all of the isozymes actually present. We have studied this problem by reinvestigating the conditions of assay in both systems.

We have used our method (*1*) as published except for two modifications, which, although they do not affect the number of isozymes, do increase the strength of the bands and make resolution sharper. These modifications are an increase in the concentration of adenosine triphosphate (ATP) in the developing solution from $2.4 \times 10^{-4}M$ to $2.4 \times 10^{-3}M$, and an increase in the concentration of magnesium chloride from $10^{-3}M$ to $10^{-2}M$.

Our initial comparative studies were carried out with the method of Holmes *et al.* (*2*). Subsequently, two modifications were introduced. The amount of nicotinamide-adenine dinucleotide phosphate (NADP), an expensive reagent, used in the method of Holmes *et al.* is in excess by at least tenfold (over $30 per 100 ml of staining solution). NADP concentration of 5×10^{-4} *M* rather than $5 \times 10^{-3}M$ was employed without affecting results. (We use $1.2 \times 10^{-4}M$ in our method.) One other modification in the method of Holmes *et al.* was adopted to avoid a partial encroachment of hemoglobin bands on the hexokinase patterns, namely, the gel buffer was *p*H 8.2, rather than 8.6. These modifications were compared with the original method in detail, and we were certain that the basic results and conclusions from the comparative study were unaffected by the modifications.

Holmes *et al.* list four differences between their technique and the one we have used as possible causes of the difference in results. Referring to our system they say, "Major differences include a preliminary extraction of the hemolyzate with toluene, the absence of 2-mercaptoethanol from the hemolyzate and gel buffers, the use of tris-boric acid gel buffer, and the use of an electric field of 8 volt/cm."

We have been able to demonstrate that, with both systems, extraction of the hemolyzate with toluene does not affect the number of isozymes. Such extraction does increase the sharpness of the bands, probably because it rids the hemolyzate of lipids and stromal components. We have also shown that the presence or absence of 2-mercaptoethanol does not affect the number of bands in either system. Also, it does not appear that the use of tris-boric acid gel buffer rather than the barbital-EDTA (ethylene diaminetetraacetic acid) buffer used by Holmes is crucial, since we have been able to resolve five or six bands of activity while using the barbital-EDTA buffer of Holmes *et al.* (see below). The barbital-EDTA buffer (normally used in hemoglobin electrophoresis) is considerably poorer, however, in providing band separation, and hence, band resolution. Concerning the use of an electric field of 8 volt/cm rather than the 6 volt/cm recommended by Holmes *et al.*, it is true that the lower voltage does not resolve seven isozymes unless the electrophoresis is allowed to run for an inordinately long period, in which case, resolution is poor. A voltage of 8 volt/cm is not unusual for starch-gel electrophoresis, particularly when the electrophoresis is carried out in a cold room, and we are aware of only one possible harmful effect of greater voltage, that is, heating of the gel. Carrying out the electrophoresis at 4°C eliminates this as a problem, and we are certain that voltage differences are playing no role in *producing* extra bands, although it does affect the resolution of bands by affecting the amount of migration.

The most important difference between the two methods appears to be one not mentioned by Holmes *et al.*, and involves the type of tetrazolium dye used for the histochemical identification of the enzyme. We have used MTT-tetrazolium, whereas Holmes *et al.* used nitro blue tetrazolium. In comparative studies of both methods, the MTT dye consistently resolved more bands of activity than the nitro blue dye. In hemolyzates from human adult blood, six isozymes of the red cells were uniformly and reproducibly detected with, basically, the technique of Holmes *et al.*, but with the use of MTT-tetrazolium as the staining dye and a slightly higher voltage.

Our principal conclusion then is that the method of Holmes *et al.* does not resolve all of the hexokinase isozyme

bands actually present in human-blood hemolyzates. Apparently, there is internal evidence for this conclusion in the data given by Holmes *et al.* in their own paper. In 4 of 21 hemolyzates of adult human bloods, only one band, rather than two, was observed. Holmes *et al.* say nothing about the reproducibility of the absence of this band, and since, in our work, we have seen no genetic variation in blood from several hundred individuals, we must conclude that the method of Holmes *et al.* is failing to resolve, about 20 percent of the time, one of the two isozyme bands they themselves have described in the other 17 individuals. Given this information, it seems unlikely that the method of Holmes *et al.* would resolve the additional isozyme bands that we have described.

In our studies of six cord-blood samples we have not been able to confirm the existence of a unique band of hexokinase in fetal red cells with either technique. The bands from fetal cells are always more intense than those from the adult; and, in addition, the slower migrating hexokinase band of the red cell of the newborn tends to be somewhat wider. Possibly, this area of activity is resolved into two bands in the laboratory of Holmes *et al.*, but not in our laboratory. We are concerned, however, that the greater migration of adult hemoglobin than fetal hemoglobin, and the consequent greater encroachment of adult hemoglobin on the hexokinase patterns at *p*H 8.6 are also confounding the interpretation of the patterns in the method of Holmes *et al.*

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4 December 1967

Although it is impossible to exclude the existence of additional molecular forms of hexokinase activity in the soluble or particulate fractions of human red cells other than the three described in our initial studies (*1*), we are forced to conclude that the significance of the seven bands of hexokinase activity observed by Brewer and his coworkers remains to be established. The factors that lead us to this conclusion are as follows: (i) We are unable to reproduce the observations of Eaton *et al.* (*2*) using the conditions described in their report. (ii) In our laboratory the substitution of MTT-tetrazolium for nitro blue tetrazolium in the identification reaction does not reveal additional bands of hexokinase activity. (iii) There is an extensive body of information that substantiates the presence of a family of low K_m hexokinases (types I, II, and III) in the tissues of multiple mammalian species (*3*). This family of low K_m hexokinases has recently been demonstrated in rat erythrocytes (*3*). There is a significant potential for the formation of active fragments of Types I, II, and III (*3*). The comments by Brewer and Knutsen ignore the discrepancies between their own observations and this large body of information concerning hexokinase isoenzymes in multiple mammalian species and tissues. The possibility that some of the bands of hexokinase activity that they have observed are fragments of Types I, II, and III is not excluded. (iv) In the report of Eaton *et al.* (*2*) the pattern of hexokinase isoenzymes observed in leukocyte sonicates is not dissimilar from that reported in multiple mammalian tissues. Therefore it is difficult for us to concede that the system of Dr. Brewer and his co-workers permits greater resolution of hexokinase isoenzymes, for in their own experience the presence of seven bands of hexokinase activity appears to be unique to the red cell. (v) The existence of Types I and II hexokinase has been confirmed by their isolation from hemolyzates by means of column chromatography on DEAE-cellulose (*4*).

With regard to other points raised by Brewer and Knutsen, in our initial studies we purposely used a system which permitted the direct comparison of our observations on hexokinase activity in human erythrocytes with the rapidly developing body of information concerning hexokinase isoenzymes in other mammalian tissues. We have subsequently observed that the NADP in the identification reaction mixture can be reduced to $5 \times 10^{-4}M$ without changing the results. In addition, we have observed that reducing the 2-mercaptoethanol in the gel and electrode buffers to $5 \times 10^{-4}M$ greatly reduces the rate at which background staining develops and permits incubation for prolonged periods (up to 2.5 hours) in the staining solution at 37°C. The quality of the resultant gels is greatly improved. With this technique, a very faint band of Type II hexokinase can be demonstrated in the red cells of approximately 40 percent of hematologically normal adults; however, the staining intensity never approximates that of Types I and III in the adult nor that of Type II in the newborn. Type II is therefore probably not totally absent from the red cells of normal adults but is present in greatly reduced quantities. Our observations on the relative quantities of Type II hexokinase in adult and newborn red cells are supported by the work of Oski and Rose (*4*), who observed that 60 percent of the hexokinase activity recovered on DEAE-cellulose chromatography of hemolyzates of normal adult cells is eluted as Type I hexokinase, and no peak corresponding to Type II hexokinase could be recovered. The Type I hexokinase recovered from the DEAE-cellulose column gave a single band with the mobility of Type I erythrocyte hexokinase when run in our starch-gel system. With hemolyzates from newborn infants, 70 to 80 percent of the hexokinase activity had the chromatographic properties of Type II rat liver hexokinase and the electrophoretic mobility of Type II erythrocyte hexokinase. Moreover, the decrease in the quantity of red cell Type II hexokinase as the human ages should be viewed in the light of recent reports that the quantities of Type II hexokinase also decrease in rat liver and adipose tissue as the animal grows older (*3*).

Finally, there is additional information which suggests that the proportions of the three hexokinase isoenzymes in human erythrocytes may also be subject to genetic control, for we have observed increased quantities of Type II hexokinase in the erythrocytes of adults homozygous for hemoglobins C or S, as well as in the erythrocytes of adults with the trait for the hereditary persistence of fetal hemoglobin.

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21 December 1967