rare opportunity to compare some of these hypotheses to the actual fossil record. The important morphological features of this fossil reproductive axis—(i) numerous helically arranged carpels on an elongated axis, (ii) multiseeded carpels, (iii) seeds borne near the adaxial side of the carpels, (iv) an adaxial suture extending the length of the carpel, (v) conduplicate carpel, and (vi) extension of the carpel walls folding out from the adaxial suture to form an adaxial crest—have been considered primitive by comparative angiosperm morphologists (*12*). The existence of these features in an angiosperm at the beginning of the Upper Cretaceous confirms their antiquity and further supports the consideration of these features as primitive. The presence of other angiosperm reproductive remains from the Cenomanian and earlier sediments (*14*), however, suggests that angiosperm reproductive structures had already diversified morphologically by the early Upper Cretaceous. Most of these other remains have not yet been subjected to investigations aimed at elucidating their detailed morphology. This report establishes that many characters considered primitive in the angiosperm flower by comparative morphologists were actually present during the early history of the angiosperms.

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References and Notes

1. G. J. Brenner, *Md. Dep. Geol. Mines Water Resour. Bull. 27* (1963); J. A. Doyle, *J. Arnold Arbor. Harv. Univ.* **50**, 1 (1969); M. Muller, *Biol. Rev.* **45**, 417 (1970).
2. J. A. Doyle and L. F. Hickey, in *Origin and Early Evolution of Angiosperms*, C. B. Beck, Ed. (Columbia Univ. Press, New York, in press).
3. C. T. Siemers, thesis, Indiana University (1971).
4. J. A. Doyle, personal communication.
5. L. Lesquereux, *Rep. U.S. Geol. Surv. 6* (1874); *U.S. Geol. Surv. Monogr. 17* (1892).
6. ——— *Rep. U.S. Geol. Surv. 8* (1883).
7. J. S. Newberry, *U.S. Geol. Surv. Monogr. 26* (1895).
8. A detailed report on the reproductive axes and leaves will be presented elsewhere.
9. A. Cronquist, *The Evolution and Classification of Flowering Plants* (Houghton Mifflin, Boston, 1968), pp. 1–396.
10. A. Takhtajan, *Flowering Plants, Origin and Dispersal* (Oliver & Boyd, Edinburgh, 1969), pp. 1–310.
11. C. R. Metcalfe and L. Chalk, *Anatomy of the Dicotyledons* (Oxford Univ. Press, London, 1968), pp. 1–396.
12. I. W. Bailey and B. G. L. Swamy, *Am. J. Bot.* **38**, 373 (1951); A. J. Eames, *Morphology of the Angiosperms* (McGraw-Hill, New York, 1961).
13. G. L. Stebbins, *Flowering Plants, Evolution Above the Species Level* (Harvard Univ. Press, Cambridge, Mass., 1974).
14. V. A. Samylian, *Bot. Zh.* **45**, 335 (1960); V. Krassilov, *Lethaia* **6**, 163 (1973).
15. Supported by NSF grants GB 12803 and BMS 75-02268. We thank M. Walker for his help in the field and D. J. DesMarais and J. Hayes, Geochemical Laboratory, Indiana University, for the carbon isotopic analysis.
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Glycerol Phosphate Shuttle in Virus-Transformed Cells in Culture

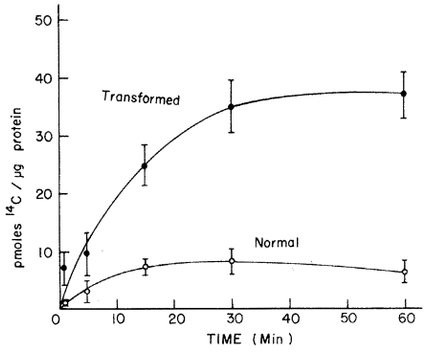
Abstract. *The glycerol phosphate shuttle is shown not only to be present and functional in virus-transformed cells, but its level is higher than in normal cells in culture. The increased aerobic glycolysis that has been demonstrated for these cells after transformation, therefore, is not due to an impairment of hydrogen transfer pathways.*

The mechanism of increased aerobic glycolysis of tumor cells, when it occurs, is as yet not understood. One of the more widely accepted explanations is that the enzymes necessary for generation of nicotinamide adenine dinucleotide (NAD^+) are lacking in malignant cells (*1*). Of the three reduced NAD (NADH) shuttles proposed for transport of electrons to the mitochondria (*2*), the glycerol phosphate (GP) shuttle is the most important. During the catabolism of one molecule of glucose, two molecules of NAD^+ are reduced to NADH when glyceraldehyde 3-phosphate (GAP) is converted to 1,3-diphosphoglycerate (1,3-DPGA). The cytoplasmic NADH is used, in turn, either to convert dihydroxyacetone phosphate (DHAP) to α-GP or to convert pyruvate to lactate. The affinity of glycerol phosphate dehydrogenase (GPDH) (E.C. 1.1.1.8) for NADH is greater than that of lactate dehydrogenase (LDH) (E.C. 1.1.1.27) (*3*). Thus, under aerobic conditions, little lactate is produced by normal cells in vivo. However, many tumor cells were reported to lack, or have drastically reduced levels of, cytoplasmic GPDH (*1, 2*). Boxer and Devlin proposed that LDH could now compete favorably for the cytosolic NADH with a resulting increase in lactic acid production (*2*). The NAD^+ thus produced would allow the continuous degradation of glucose.

The general applicability of this postulate has been questioned occasionally (*4*), although the postulate itself has gained general acceptability (*5*). More recently, in one kind of Ehrlich ascites tumor cells with a high rate of aerobic glycolysis, the shuttle was shown to be absent. However, in another strain with an equally high rate of glycolysis, the shuttle was fully operative (*6*). Ascites tumor cells do not have a normal counterpart in culture. A comparison between normal and malignant cells under comparable environmental conditions has not been reported and was therefore called for.

While normal chick embryo fibroblasts grown in tissue culture do produce an appreciable amount of lactic acid (*7, 8*), Rous sarcoma virus (RSV) –transformed cells have been shown to produce even more lactate (*8, 9*). The increase was demonstrated under steady-state conditions and was in excess of the changes due to growth rates (*8, 9*). An examination of the extent of the GP shuttle in these cells was undertaken to determine whether a decrease in shuttle activity accompanies the increased lactic acid production.

Primary cultures were prepared from 10-day-old chick embryos free of resistance-inducing factor (*10*). The single cells were plated in 100-mm culture dishes at 8×10^6 cells per plate in medium 199 supplemented with tryptose phosphate broth (2 percent) and chick and calf serums (1



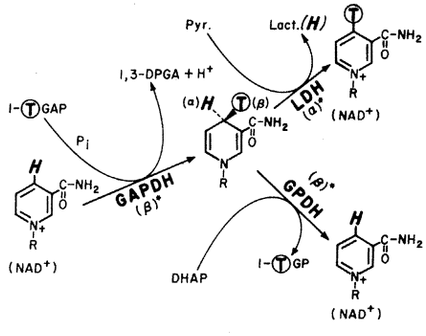


Fig. 1 (left). Glycerol 3-phosphate in normal and transformed cells. Secondary cultures were placed in the steady-state apparatus. After 1 hour of incubation in serum-free medium, medium containing uniformly labeled glucose (536 c/mole; 5.5 m*M*) was added. At intervals, the medium was removed, and the cells were washed rapidly and killed. After scraping and sonication, a portion was applied to filter paper for analysis by two-dimensional chromatography and autoradiography (*9, 13*). GP and DHAP traveled as a single spot under these conditions. The spot was eluted and the eluate was chromatographed on diethylaminoethyl-cellulose paper in a phenol, water, acetic acid (84 : 16 : 1) system for 72 hours; the two components separated under these conditions and the radioactivity was counted (*13*). Fig. 2 (right). Transfer of tritium from the 1-position of GAP to the β position of NADH; $(\alpha)^*$ and $(\beta)^*$ refer to the specificity of the dehydrogenases.

Table 1. LDH and GPDH activities (per milligram of protein) in normal and virus-transformed chick cells. One unit of activity is the absorption change of 0.1 per minute at 340 nm. The LDH was measured by the rate of NADH oxidation after the decrease in absorbance at 340 nm in the presence of pyruvate. GPDH was assayed after the rate of NADH oxidation in the presence of DHAP. The values are averages of three independent experiments.

Stage of cell	GPDH	LDH
Embryo before trypsin	0.013	1.20
Cells after trypsin	0.020	1.33
Primary cells after 24 hours	0.022	1.59
Normal secondary cells	0.014	2.81
Transformed secondary cells	0.012	5.50

percent each). The use of fungizone as a fungicide was eliminated entirely, as harmful side effects have been observed (*11*). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 35-mm tissue culture dishes. The concentration of glucose and calf serum was doubled (11 m*M* and 2 percent, respectively) at the time of secondary seeding.

For experiments with transformed cells, Schmidt-Ruppin strain of RSV, subgroup A, was added at a multiplicity of 0.2 infectious unit to half the cells from a single embryo at the time of primary seeding.

A comparison of GPDH and LDH enzyme activities indicated that the GPDH was low, but comparable in normal and RSV-transformed cells (Table 1). The total enzyme activity fluctuated after the cells were grown in culture for 24 hours and was slightly lower for both normal and transformed secondary cultures. The activity of LDH, on the other hand, was increased after the cells were grown in culture and after transformation (Table 1) (*12*).

Since the measurement of enzyme activities in extracts does not give indications of the actual activity of the enzyme in the cells, the rate of ^{14}C flow from uniformly labeled [^{14}C]glucose into GP was compared in normal and virus-transformed cells. Forty-eight hours after secondary plating, the cells were placed in the steady-state apparatus and labeled with [^{14}C]glucose (uniformly labeled) for various periods of time (*9, 13*). The killed cells were analyzed for the distribution of label among various pools by two-dimensional paper chromatography and autoradiography (*9, 13*). The initial rate of ^{14}C flow from glucose into the pool of GP, as well as the final level of ^{14}C in GP, was three- to sixfold higher in transformed cultures than in normal cells (Fig. 1), with the magnitude of the increase being dependent on the degree of transformation. If one assumes that the flow of ^{14}C into GP is via DHAP, the most likely possibility, the initial rate of flow of ^{14}C into the pool of GP (Fig. 1) would indicate that the enzyme is not only active in transformed cells, but its activity is higher than in normal cells.

There are no indications that the mitochondrial GPDH is deficient in malignant cells (*2*). Nevertheless, while the above data indicate that GPDH is active in transformed cells, the rate of electron transfer via the GP shuttle cannot be determined by the measurement of the pool sizes. To gain further information about the extent of the shuttle, we took advantage of the stereospecificity of dehydrogenases with respect to the hydrogen atom transferred during NADH reduction and oxidation. GAP-dehydrogenase transfers the hydrogen on C-1 of GAP to the β position of NADH during the conversion of GAP to 1,3-DPGA (Fig. 2). Whether or not this hydrogen remains on the subsequently oxidized NAD^+ depends on which enzyme attacks the NADH molecule. While GPDH removes the β hydrogen, LDH removes the hydrogen from the α position. If the hydrogen on the C-1 position of GAP is labeled with tritium, the NAD^+ produced by the action of LDH may be distinguished from that produced by GPDH. Such labeling of the C-1 hydrogen of GAP is accomplished by allowing the cells to metabolize in tritiated water for 1 hour (*14*). In order to locate the metabolic products on the paper chromatogram, uniformly labeled [^{14}C]glucose was also added to the medium. During the aldolase and triose phosphate isomerase reactions, there is an exchange with the protons of the medium

Table 2. The ratio of ^3H to ^{14}C in NAD^+ after metabolism in tritiated water and [^{14}C]glucose. The specific activity of [^{14}C]glucose (uniformly labeled) was 70 mc/mmole. Normal and transformed cells were brought into a closed system containing 5 percent CO_2 in air at 38° to 39°C. The entire apparatus was placed in a large glove box with the pressure below atmospheric level for safe handling of the tritiated water. The procedure was then as described for Fig. 1. The isolated NAD^+ spot was cut out of the chromatograms and prepared by combustion for counting the ^3H and ^{14}C content by liquid scintillation. The error in determination of the ^3H to ^{14}C ratio for individual compounds on the paper chromatogram (based on triplicate samples of the same material) was less than 1 percent.

Triated water (mc/ml)	^3H to ^{14}C ratio	
	Normal	Transformed
200	0.086	0.082
600	0.250	0.277
600	0.298	0.265

Table 3. Comparison of [^3H]NAD^+ and [^{14}C]NAD^+ in normal and transformed cells. The procedure is described in the legend to Table 2. The specific radioactivity of the tritium was 600 mc/ml. The results represent an average of two analyses, and are given as 10^3 disintegrations per minute per milligram of protein.

Time (min)	Radioactivity			
	^3H		^{14}C	
	Normal	Transformed	Normal	Transformed
30	16.0	13.4	41.5	42.7
60	22.0	23.1	91.0	84.8

which results in labeling the C-1 position of GAP with tritium. The fact that other positions on the carbohydrate moiety of NAD^+ may also be labeled by other reactions does not affect the interpretation of the results, since the hydrogen transfer via the dehydrogenases is very specific (*15*). The NAD^+ was isolated from paper chromatograms and the ratio of ^3H to ^{14}C was determined (*14*). The ^{14}C radioactivity in the isolated NAD^+ was comparable in normal and transformed cells under the conditions chosen. If one assumes that the amount of ^{14}C labeling is proportional to the total amount of nucleotides present, then the ratio of ^3H to ^{14}C gives the specific tritium radioactivity in NAD^+. The results (Table 2) indicate that the ^3H to ^{14}C ratio in NAD^+ is the same in normal and transformed cells.

There are three situations to be considered. (i) If there were no LDH activity, NAD^+ would never be labeled in position 4 of the pyridine ring from tritiated water. High levels of LDH are present (*12*), and LDH is obviously very active, as demonstrated by the level of lactate production in these cells (*8, 9*). (ii) If GPDH were not present, the tritium at position 4 of NAD^+ would approach the same specific activity as tritiated water at the steady state. We know from the above experiments that GPDH is present and active in both cells. However, we do not know the extent of its activity in the two cell types. (iii) If GPDH were more active in one cell type, this would cause the ratio of ^3H to ^{14}C to be less than in (ii) initially. When a steady state was reached, where no further increase in specific activity of tritiated NAD^+ occurred, that steady-state labeling would also approach the specific activity of tritiated water. It is therefore apparent that (ii) and (iii) may be distinguished by ^3H to ^{14}C ratios in NAD^+ if the 1-hour time point is prior to complete equilibrium.

It is not possible to carry these experiments with transformed cells for longer than an hour. In addition to a rapid decrease in [^{14}C]glucose (and hence not a steady-state situation), the monolayer is

detached from the dish because of handling in the tritium box. At 30 minutes, the [^{3}H]NAD$^+$ pool is comparable in normal and transformed cells, is not saturated, and is nearly one-half the value at 60 minutes (Table 3). Therefore GPDH is not only present (Table 1) and active (Fig. 1) in transformed cells, but the ratio of the activity of the enzymes of the two reactions that produce NAD$^+$, that is, LDH and GPDH, are comparable in normal and transformed cells. Since LDH level (Table 1) and activity (*8*) are higher in transformed cells than in normal cells, the GPDH activity must also be higher to give comparable ratios of the two enzyme activities.

Many postulates have been put forth regarding possible reasons for increased aerobic glycolysis of tumor cells, some of which are no longer accepted (*16*). Boxer and Devlin based theirs on measurement of enzyme activities alone, and their postulate is no longer tenable as a general explanation for increased aerobic glycolysis. There are, of course, changes in many enzyme levels after transformation, such as the rise in LDH. Whether these changes result from rapid growth and nutritional conditions of the culture or whether they are direct consequences of transformation needs further scrutiny.

We have proposed (*9*) that the initial increase in glycolysis that occurs after virus transformation is the result of altered glucose uptake in these cells. An increase in the rate of glucose transport after virus transformation has been demonstrated for chick cells in culture (*17*). The changes in glycolytic enzyme patterns and levels as well as in glycogen synthesis and pentose shunt are then seen as secondary events that may also be produced in normal cells if the level of glucose uptake is varied (*9*).

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References and Notes

1. S. Weinhouse, *Science* **124**, 267 (1956).
2. G. E. Boxer and T. M. Devlin, *ibid.* **134**, 1495 (1961).
3. T. E. Barman, *Enzyme Handbook* (Springer-Verlag, New York, 1969), vol. 1, p. 32.
4. C. E. Wenner, *Adv. Enzymol.* **29**, 321 (1967).
5. A. L. Lehninger, *Biochemistry* (Worth, New York, 1970), p. 409.
6. O. Dionisi, A. Cittadini, T. Gelmazzi, T. Galeotti, T. Terranova, *Biochim. Biophys. Acta* **216**, 71 (1970).
7. T. L. Steck, S. Kaufman, J. P. Bader, *Cancer Res.* **28**, 1611 (1968).
8. M. J. Bissell, C. Hatie, H. Rubin, *J. Natl. Cancer Inst.* **49**, 555 (1972).
9. M. J. Bissell, R. C. White, C. Hatie, J. A. Bassham, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2971 (1973).
10. A. Rein and H. Rubin, *Exp. Cell Res.* **49**, 666 (1968).
11. D. Dolberg and M. J. Bissell, *In Vitro* **10**, 26 (1974).
12. M. J. Bissell, H. Rubin, J. Hatie, *J. Exp. Cell Res.* **68**, 404 (1971).
13. J. A. Bassham, M. J. Bissell R. C. White, *Anal. Biochem.* **61**, 479 (1974).
14. W. A. Rambeck, M. J. Bissell, J. A. Bassham, *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 203 (1975).
15. N. O. Kaplan, in *The Enzymes*, P. D. Boyer, H. A. Lardy, K. Myrbäck, Eds. (Academic Press, New York, 1960), vol. 3, p. 115.
16. J. Paul, in *Cells and Tissues in Culture*, E. N. Willmer, Ed. (Academic Press, London, 1965), pp. 239–276.
17. M. J. Weber, *J. Biol. Chem.* **248**, 2978 (1973); S. Venuta and H. Rubin, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 653 (1973); F. R. Kletzien and J. F. Perdue, *J. Biol. Chem.* **249**, 3375 (1974).
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Demonstration of Kinetoplast DNA in Dyskinetoplastic Strains of *Trypanosoma equiperdum*

Abstract. *4,6-Diamidino-2-phenylindole (DAPI) forms a highly fluorescent complex with DNA which allows detection of mitochondrial DNA (K-DNA) in normal and dyskinetoplastic strains of* Trypanosoma equiperdum. *The K-DNA DAPI complexes in the dyskinetoplastic cells, cells lacking detectable K-DNA by other cytochemical methods, are not restricted to a single region of the organism as in the normal strain, but are seen as a row of particles. These observations support the hypothesis that the K-DNA is retained in dyskinetoplastic cells.*

Mitochondrial DNA of trypanosomatid flagellates is localized in an enlarged region of the mitochondrion, the kinetoplast, where electron microscopy shows that it is organized in a fibrous band. The kinetoplast is readily demonstrable by phase-contrast microscopy in living cells and by Giemsa or Feulgen staining and acridine-orange fluorescence microscopy in fixed cells. In dyskinetoplastic cells, these techniques do not show the kinetoplast; electron microscopy shows that the mitochondrion is still enlarged in the kinetoplast region but the fibrous band of kinetoplast DNA (K-DNA) is replaced by clumps of fibrous material; similar clumps occur at intervals along the length of the mitochondrion. Renger and Wolstenholme (*1*) reported that in both normal and spontaneously dyskinetoplastic strains of *Trypanosoma equiperdum* analytical ultracentrifugation shows the presence of a satellite DNA, buoyant density 1.692, which they identified as K-DNA. I demonstrate here by fluorescence microscopy the presence of K-DNA in living cells of normal and of dyskinetoplastic strains of *T. equiperdum*, using 4,6-diamidino-2-phenylindole (DAPI) as a fluorescent probe.

DAPI was first synthesized as a trypanocidal drug by Dann *et al.* (*2*) and has been shown to bind differentially to yeast mitochondrial and nuclear DNA forming

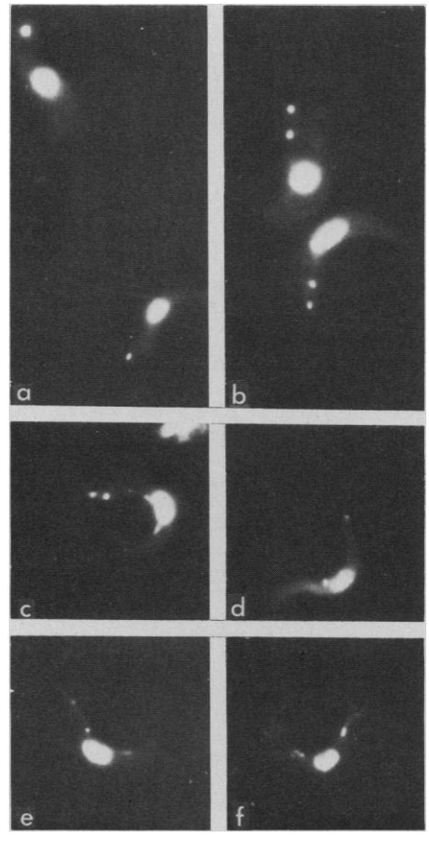

Fig. 1. (a) Photomicrograph of the kinetoplastic strain of *T. equiperdum*, treated with DAPI (0.1 μg/ml) at 37°C for 20 minutes. A single fluorescent nucleus and kinetoplast can be observed in both cells. (b) Photomicrograph of the kinetoplastic strain of *T. equiperdum*, treated with DAPI as in (a). The kinetoplasts in both cells have completed division and the nuclei have not. (c and d) Photomicrographs of the spontaneously dyskinetoplastic strain of *T. equiperdum*, treated with DAPI as above. Fluorescent bodies of varying size can be observed in the cells; the nuclei appear as in the normal cells. (e and f) Photomicrographs of the acriflavin induced dyskinetoplastic strain of *T. equiperdum*, treated with DAPI as above. Distribution of fluorescent bodies in the cells is similar to that seen in the spontaneously dyskinetoplastic cells (×3860).