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Changes in Lactate Dehydrogenase Enzyme Pattern in Chinese Hamster Cells Infected and Transformed with Simian Virus 40[1]

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SUMMARY

The lactate dehydrogenase (LDH) isozyme patterns of consecutive passages of Chinese hamster embryo cultures were monitored. At early passages the population displayed two LDH bands, M_4 and M_3H; however, at higher passages the cultures exhibited M_2H_2, M_3H, and M_4. When primary cultures of Chinese hamster embryo cells were infected with simian virus 40 (SV40), no change in the LDH pattern was observed; however, the total activity of LDH increased.

Twenty-three of 25 transformed colonies isolated from SV40-infected primary cells by their ability to grow in methyl cellulose produced only M_4 or M_4-M_3H isozymes bands. Four of the SV40-transformed clones that produced only the M_4 isozyme were tested for LDH activity and found to have activities 2.5 to 3 times greater than the control cells.

Chinese hamster kidney epithelial cells transformed with SV40 virus had a decrease in the H subunit production, from 57 to 31%, compared with normal kidney epithelial cells. This decrease in H subunit production led to an increase in the cathode-migrating isozymes. Therefore, a shift to the cathode-migrating isozyme was observed in SV40-transformed cells. This change in LDH pattern might represent a reversion to the enzyme pattern present in fetal cells.

INTRODUCTION

Lactate dehydrogenase is a tetrameric enzyme composed of 2 subunits, the M (muscle) and H (heart) subunits. There are separate genes controlling production of each subunit, and these 2 subunits can combine to give a total of 5 different tetrameric isozymes: M_4, M_3H, M_2H_2, MH_3, and H_4 (4, 10).

Interest in the LDH[3] isozyme patterns of neoplasms stemmed from the observation that their patterns often differed from those found in normal tissues. Most of the solid tumors studied, such as carcinoma of the colon (6), carcinoma of the lung (1), and malignant tumors of the brain (5), showed increased activity in those isozymes with a greater M subunit composition; however, there were a few solid tumors such as basal cell carcinoma (2) and several germinal tumors (21) that showed an increase in H subunit activity. It was hoped that these pattern changes could be used as an early diagnostic procedure for evaluating the malignancy of tumors, and encouraging in this regard was the work of Gerhardt *et al.* (5) on brain tumors, which showed that an increase in the M/H subunit ratio occurred in some tumors before histological signs of cancer developed. Prasad *et al.* (13) have shown that tumors induced in hamsters by both SV40 viruses and 7,12-dimethylbenzanthracene exhibit an increase in the activity of those isozymes with a large M subunit composition.

However, *in vivo* studies have been hampered by the fact that it has not been possible to observe the changes occurring in the ratio of M and H subunit production during the early time period following the transforming event in either spontaneous human tumors or those induced in laboratory animals by carcinogenic agents. Neither is it possible to conclude that the heterogeneous population of cells in the normal tissue could adequately serve as a control for a cancer that probably arose from only 1 cell in the population. In order to alleviate these shortcomings, we have used an *in vitro* system to study the changes occurring in LDH isozymes. These studies compare the LDH patterns of Chinese hamster cells cultivated *in vitro*, following infection and transformation with SV40 virus.

MATERIALS AND METHODS

Whole embryos and embryo kidneys from pregnant Chinese hamsters of 15 days gestation were used to initiate cell cultures. The methods have been published previously (8). The culture initiated from whole embryos will be designated ChH and those from kidney as ChH kidney epithelium. The ChH kidney epithelium was isolated by placing a cloning ring around cells characterized as epithelial in morphology and trypsinizing these cells to establish secondary cultures.

The growth medium used throughout these studies was minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2 times the con-

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[3] The abbreviations used are: LDH, lactate dehydrogenase; SV40, simian virus 40; PBS, phosphate-buffered saline [NaCl (8.0 g/liter), KCl (0.2 g/liter), Na_2HPO_4 (1.15 g/liter), KH_2PO_4 (0.2 g/liter), $MgCl_2 \cdot 7H_2O$ (1.5 g/liter, $CaCl_2 \cdot 2H_2O$ (0.10 g/liter)].

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centration of amino acids and vitamins. The medium also contained 5% fetal calf serum and 100 units of penicillin and 100 μg of streptomycin per ml.

The strain of SV40 used for these studies was RH 911 grown in CV-1 cells (8). The titer of the virus was determined by plaque assay (8) on CV-1 cells.

The SV40 intranuclear tumor (T) antigen was assayed by immunofluorescence as previously described (8).

LDH Determinations. Confluent flasks of cells were trypsinized, washed, and counted with a hemocytometer. The cells were then pelleted by centrifugation at 1000 rpm for 10 min and washed once with 5 ml of sterile PBS. The cells were diluted in sterile deionized water to a concentration of 1×10^7 cells/ml and were sonically disrupted 3 times for 10 sec each at an intensity of 30 on a Biosonik III sonic oscillator (Bronwill, Rochester, N. Y.). The supernatant was then analyzed immediately for LDH activity and isozyme patterns. Samples of the supernatant were applied to agarose-coated (0.5%) microscope slides. The samples were subjected to electrophoresis for 30 min at 150 V in a barbital buffer with an ionic strength of 0.075 at a pH of 8.6.

The gels were stained with a solution containing 1.0 part 0.1 M phosphate buffer, pH 8.0; 0.2 part 0.5 M L-lactate; 0.14 part NAD at a concentration of 4 mg/ml; 0.5 part of methylthiazolyldiphenyl tetrazolium (2 mg/ml); 0.05 part phenazine methosulfate (2 mg/ml); and 0.02 part 1% potassium cyanide (all from Sigma Chemical Co., St. Louis, Mo.). The staining procedure was carried out at 37° for 20 min (16). The isozyme band migrating the farthest toward the anode was H_4 and that migrating the farthest toward the cathode was M_4.

Isozyme patterns were scanned with a Quick Quant and a Quick Scan densitometer (Helena Laboratories, Beaumont, Texas) at a wavelength of 520 nm, and a permanent record of the gel obtained. The percentage of M and H subunits was calculated from the relative intensities of each band recorded by the densitometer. Latner and Turner (7) have demonstrated that the intensity of the staining is directly proportional to the activity present in each band, provided that the isozyme concentration does not exceed 400 IU/liter. In the studies reported, we kept within this range so that comparisons between cells could be made.

Total LDH activities of samples were determined by using the LDH diagnostic kit supplied by the Boehringer Mannheim Corp. (New York, N. Y.). The assay is based upon the modified procedure of Wacker *et al.* (18) who used L-lactate and NAD as substrates for LDH and monitored the rate of production of NADH by the increase in absorbance at 340 nm. One International Enzyme Unit is defined as the activity of enzyme which converts 1 μmole of substrate in 1 min at standard conditions. Protein concentration was assayed by the procedure of Lowry *et al.* (9).

A microtechnique was utilized to obtain patterns on colonies containing approximately 1000 cells. This technique required removal of the medium, followed by a wash with PBS. Each colony was then surrounded with a small metal cloning ring dipped in sterile stopcock grease. The cells from each colony were scraped up in 50 μl of sterile H_2O delivered from a siliconized micropipet drawn to a fine point. The cell suspensions were placed in separate plastic microfuge tubes and the cells were disrupted by freeze-thawing 3 times. The tubes were spun for 5 min in a Beckman 152 microfuge to pellet the cellular debris. Electrophoresis, staining, and recording of results were carried out as described above.

Transplantation. Three-month-old Chinese hamsters were irradiated with 400 rads; then each hamster was given an

Table 1
LDH isozyme patterns of Chinese hamster embryo cells

Passage	M_4	M_3H	M_2H_2	MH_3	H_4
Primary	+	+			
1	+	+			
2	+	+			
3	+	+	+		
4	+	+	+		
5	+	+	+		
6	+	+	+		
7	+	+	+		
8	+	+	+		
9	+	+	+	+	

Table 2
Isozyme patterns of consecutive passages of Chinese hamster SV40-infected cells

Days postinfection	Passage[a]	% T antigen	M_4	M_3H	M_2H_2	M_2H	H_4
0	Primaries	0	+	+			
2	1	82	+	+			
5	2	90	+	+			
7	3	50	+	+			
11	4	69	+	+			
14	5	ND[b]	+	+			
20	6	76	ND				
30	8	ND	+	+			
39	9	100	+				
45	10	100	+				
51	11	100	+				

[a] Refers to the consecutive subcultivation of these cells.
[b] ND, not done.

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i.m. injection in the thigh with 0.2 ml of a known number of SV40-transformed cells. Injections were done on both sides of the animal.

The animals were palpated at the site of injection every 4 days, and when masses of 0.5 cm in diameter were palpated, the animals were sacrificed. Animals that did not develop masses at the site of injection were sacrificed after 2 months. At autopsy all animals were examined visually for evidence of metastases.

RESULTS

Since these studies were designed to monitor the shift in isozyme pattern of virally infected and transformed cells, it was necessary to follow the changes in isozyme patterns of normal Chinese hamster cells kept in tissue culture for varying periods of time. Table 1 shows the LDH isozyme pattern of different passages of these cells ranging from primary to senescent cells at passage 9. The primary cells contained only M_3H and M_4 but, by passage 3, the cells produced a 3rd band (M_2H_2). The band persisted until senescence of the cells (passage 9). The data shown in Table 1 were obtained from cells subcultivated once a week; similar results were also obtained from cells subcultivated twice a week.

Chinese hamster primary cultures were infected with SV40 at a multiplicity of 200 plaque-forming units per cell, and the changes in both total activity of LDH and the isozyme patterns were monitored. The total levels (347 to 387 milliunits/ml/10^7 cells) were constant through the 1st 6 hr, but thereafter there was an increase so that, between 12 and 72 hr, the values (617 to 572 milliunits/ml/10^7 cell) were double those seen during the 1st 6 hr.

When the LDH isozyme patterns were analyzed, no changes in the bands were observed within 5 days postinfection (Table 2). Both infected and uninfected cells were producing the M_3H and M_4 isozymes. However, at higher passages when control cells (Table 1) were producing M_2H_2 through M_4, the virally treated cells were producing only M_4 and decreasing amounts of M_3H. By passage 9, when control cells had reached senescence, the virally transformed cells were producing only the M_4 band. The correlation of the percentage of T-antigen-positive cells with the change in LDH pattern showed that, by passage 9, all of the Chinese hamster cells contain the SV40 genome and possess the property of infinite growth in culture. A similar pattern was observed for ChH cells that were infected at the 8th passage with SV40 virus. At 12 passages postinfection (100% T-positive), only the M_4 subunit was produced.

For further establishment that a shift in isozyme pattern followed SV40 transformation, cells from both the primary and passage 8 experiments were seeded into methylcellulose (14). Colonies isolated from the methylcellulose were seeded into Petri dishes and allowed to multiply to approximately 2000 cells. These colonies were assayed for LDH isozyme patterns by the microtechnique method. The total amount of time between seeding the cells into methylcellulose and assaying for LDH isozyme patterns was approximately 3 weeks. Not all colonies arising from transformed Chinese hamster primary cells exhibited the same isozyme pattern. Thirteen transformed colonies had only M_4, and 10 colonies were producing M_4 and M_3H. In 2 of the 25 transformed colonies, LDH isozymes M_4 through MH_3 were produced. This result could be explained on the basis that SV40 may have infected and transformed a cell in the primary culture that was producing more H subunits. Most of the transformed colonies were derived from fibroblasts, as deter-

Table 3

Comparison of cell protein with total LDH activity of SV40-transformed Chinese hamster clones

Cell type	Cell mass (mg protein/ 1 × 10^7 cells)	Total LDH activity (milliunits/ 1 × 10^7 cells)	Milliunits of activity (mg protein)
ChH-primary	2.36	347	147
ChH-SV40 Cl_1	1.50	618	412
ChH-SV40 Cl_4	1.72	804	467
ChH-SV40 Cl_7	1.20	489	408
ChH-SV40 Cl_9	1.18	474	401

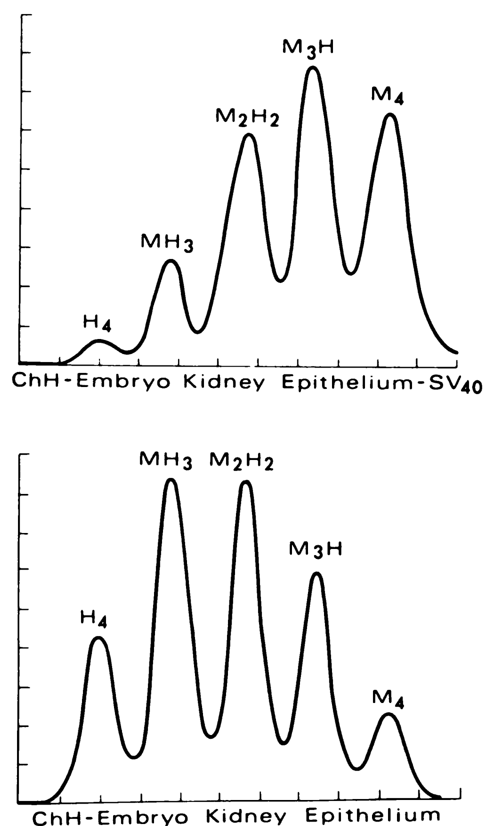

Chart 1. LDH isozyme patterns of ChH-embryo kidney epithelial cells and SV40-transformed ChH-embryo kidney epithelial cells. Colonies of normal ChH-embryo kidney epithelial cells were analyzed for LDH isozyme patterns by the microtechnique, and transformed cells were assayed by the procedure for LDH determinations on large quantities of cells. All gels were scanned with the Quick Scan densitometer at a wavelength of 520 nm. The cathode is to the *right* and the anode to the *left*. The relative intensity is plotted along the *ordinate*.

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mined by morphology, but a few colonies possibly originated from other cell types, such as kidney epithelium.

Total LDH activities were determined for 4 of the SV40-transformed clones that had been isolated in methylcellulose. All of the clones tested were producing only the M_4 band. The transformed cell values are 2.5 to 3 times greater than those of the control cells (147 milliunits/mg protein) (Table 3). These clones were analyzed for karyological changes, but no consistent change was observed, nor was there any correlation between LDH production and the presence of an extra chromosome.

In order that a more homogeneous population of cells might be examined, 8 colonies of normal kidney epithelial cells were analyzed for isozyme patterns, and similar results were obtained in all cases (Chart 1). Isozyme determinations were also performed on 3 lines of SV40-transformed kidney epithelial cells, and the lines exhibited a shift toward the more cathode-migrating isozyme, although all 5 of the isozymes were still expressed in the transformed cells (Chart 1). If calculations are made from the densitometer printout of the percentage of M and H subunits for the normal and transformed cells, there has been a decrease of 26% in the H subunit composition of transformed Chinese hamster embryo epithelial kidney cells (H subunits, 31.1%) compared with normal cells (H subunits, 56.9%). To confirm the fact that the epithelial cells were indeed transformed, they were tested for the presence of T-antigen and found to be 100% positive, and they also demonstrated infinite growth in tissue culture.

The tumorigenicity of various transformed clones was compared to determine whether there existed an association between the degree of M subunit production and the ability of these cells to produce tumors when injected into Chinese hamsters. Chart 2 shows the isozyme patterns of 4 clones of ChH-transformed cells. The results of transplantation of each of these cell lines into irradiated 3-month-old male Chinese hamsters are presented in Table 4. Only the fibroblastic ChH-S√40 1-cell line produced tumors. The tumors were poorly differentiated fibrosarcomas consisting of cells with a large nuclear-to-cytoplasmic ratio, a large number of nucleoli (approximately 7/cell), an increased mitotic rate, and numerous multinucleated giant cells.

LDH isozyme analyses of 3 of the 5 tumors exhibited patterns similar to those of the original cells. The appearance of a small amount of LDH_3 (M_2H_2) in the tumor, which is not present in the cultured cells, was explained by the presence of erythrocytes and leukocytes. Studies on various mammals have shown that these cell types have predominantly anode-migrating isozymes (17). LDH isozyme patterns of tumor cells placed in culture and then assayed showed only M_3H and M_4. The tumor cells were 100% T-antigen positive. All the cell lines tested for tumorigenicity were seeded into methylcellulose, and the efficiency of colony formation was determined. Only ChH-SV40 1 cells formed colonies in the methylcellulose (0.06% efficiency).

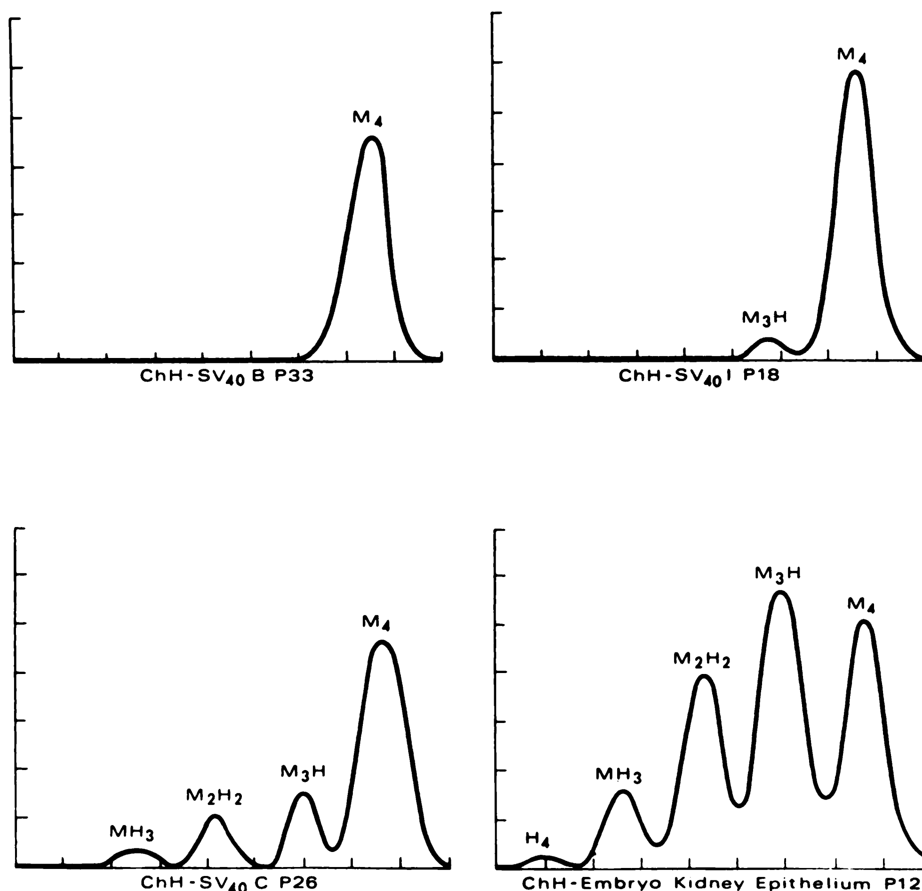

Chart 2. Densitometer tracings of the LDH isozyme patterns of SV40-transformed lines. Cells extracts containing 1×10^7 cells/ml of each cell line were assayed for LDH isozyme patterns by the technique outlined for LDH determinations on large quantities of cells. The anode is to the *left* and the cathode is to the *right*. The relative intensity is plotted along the *ordinate*.

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Table 4
Tumorigenicity of Chinese hamster SV40-transformed cells

Clone (passage)	LDH pattern	No. of cells injected	No. of animals	No. of tumors
ChH-SV40 B (P34)	M_4	2.8×10^7	8	0
		6.0×10^7	1	
		4.4×10^7	1	
ChH-SV40 I (P19)	M_4 M_3H	2.5×10^7	2	5
		2.0×10^7	4	
ChH-SV40 C (P27)	M_4 M_3H M_2H_2 MH_3	4.0×10^7	1	0
		2.0×10^7	4	
		2.5×10^7	1	
ChH embryo kidney-SV40 (P12)	M_4 M_3H M_2H_2 MH_3 H_4	4.0×10^7	10	0
		1.2×10^7	2	
ChH (control cells) (P3)	M_4 M_3 M_2H_2	1.4×10^7	3	0

DISCUSSION

The LDH isozyme pattern utilizing gel electrophoresis was monitored in normal ChH embryo cells, cells infected with SV40, and transformed cells. The utilization of a tissue culture system enabled us to follow these changes without contamination of cells not involved in the infection and transformation.

Normal Chinese hamster embryo cultures have an increase in the anode-migrating LDH isozymes as the length of time in culture increases. When these cells were infected with SV40 virus, an increase in the total LDH activity was seen at 12 hr postinfection; however, there was no change in the isozyme patterns. This increase in total activity may be related to the induction of a number of host enzymes systems following infection with SV40 (8). However, a shift in the LDH isozyme pattern toward isozymes with greater M subunit composition was observed in transformed cells. These transformed cells also had an increased LDH activity, compared with that of control cells, which may be due to the presence of predominantly the M_4 subunit. There was no correlation between the presence of the LDH M_4 subunit and the tumorigenicity of transformed cells.

An explanation for this shift in LDH isozyme patterns might be a reversion to a more fetal pattern of protein synthesis. Although extensive studies have not been performed on Chinese hamster embryonic tissues, another rodent, the mouse, has been examined, and these studies show that fetal mouse tissues have a preponderance of the M subunits (11). A series of studies has suggested that certain neoplastic tissues may produce fetal proteins. Weinhouse *et al.* (19) have shown that aldolase and glucose-ATP phosphotransferase in poorly differentiated hepatomas have a fetal isozyme pattern. Fetal α-globulin has also been demonstrated in many experimental and human hepatomas (15).

In regard to SV40 transformation of cells, certain malignant properties of the transformed cells have been suggested to be a reversion to a fetal pattern, such as increased agglutination with plant lectins (12, 20) and the presence of fetal antigens (3) on the surface of tumor cells. The change in LDH isozyme patterns may be related to the acquisition of a more fetal LDH pattern in neoplastic cells.

REFERENCES

1. Blumelova, J., Elleder, M., Pesek, M., and Vecerek, B. Isozymes of LDH in Human Lung Tissue and in Malignant Lung Tumors. Neoplasma, *16:* 645–650, 1968.
2. Bohn, L., and Rothenborg, H. W. LDH Patterns in Verrucal Vulgares, Basal Cell Carcinoma and Malignant Melanomas. J. Invest. Dermatol., *48:* 256–259, 1967.
3. Coggin, J. H., Ambrose, K. R., and Anderson, N. G. Fetal Antigen Capable of Inducing Transplantation Immunity against SV40 Hamster Tumor Cells. J. Immunol., *105:* 524–526, 1970.
4. Fine, I. H., Kaplan, N. O., and Kuftinic, D. Developmental Change in Mammalian Lactic Dehydrogenase. Biochemistry, *2:* 116–121, 1963.
5. Gerhardt, W., Clausen, E., Christensen, E., and Riiskede, J. LD Isozymes in the Diagnosis of Human Benign and Malignant Brain Tumors. J. Natl. Cancer Inst., *38:* 343–357, 1967.
6. Langrad, E. LDH Isozyme Patterns in the Tumor Bearing Colon. Intern. J. Cancer, *3:* 17–29, 1968.
7. Latner, A. L., and Turner, D. M. Quantitative Assay of LDH Isozymes by Reflectance Densitometry. Clin. Chim. Acta, *15:* 97–101, 1967.
8. Lehman, J. M., and Defendi, V. Changes in DNA Synthesis Regulation in Chinese Hamster Cells Infected with Simian Virus 40. J. Virol., *6:* 738–749, 1970.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with Folin Phenol Reagent. J. Biol. Chem., *193:* 265–275, 1951.
10. Markert, C. L. The Epigenetic Control of Specific Protein Synthesis in Differentiating Cells. *In:* M. Locke (ed.), Cytodifferentiation and Macromolecular Synthesis. pp. 65–84. New York: Academic Press, Inc., 1963.
11. Markert, C. L., and Urspung, W. The Ontogeny of Isozyme Patterns of Lactate Dehydrogenase in the Mouse. Develop. Biol., *5:* 353–381, 1962.
12. Moscona, A. A. Embryonic and Neoplastic Cell Surfaces: Availability of Receptors for Concanavalin A and Wheat Germ Agglutinin. Science, *171:* 905–906, 1971.
13. Prasad, R., Prasad, N., and Tevethia, S. Expression of Lactate and Malate Dehydrogenase in Tumors Induced by SV40 and 7,12-Dimethylbenzanthracene. Science. *178:* 70–71, 1972.

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14. Stoker, M. Abortive Transformation by Polyoma Virus. Nature, *218:* 234–238, 1968.
15. Uriel, J. Transitory Liver Antigens and Primary Hepatoma in Man and Rat. A. Pathol. Biol., *17:* 877–882, 1969.
16. Vander Helm, H. J. A Simplified Method of Demonstrating LDH Isozyme in Serum. Clin. Chim. Acta, *7:* 124–128, 1962.
17. Vessel, E. S., and Bearn, A. C. Isozymes of Lactic Dehydrogenase in Human Tissues. J. Clin. Invest., *40:* 580–591, 1961.
18. Wacker, W. E. C., Ulmer, D. D., and Vallee, B. L. Metalloenzymes and Myocardial Infarction. II. Malic and Lactic Dehydrogenase Activities and Zinc Concentrations in Serum. New Engl. J. Med., *255:* 449–456, 1956.
19. Weinhouse, S., Shatton, J. B., Criss, W. E., Farina, F. A., and Morris, H. P. Isozymes in Relation to Differentiation in Transplantable Rat Hepatomas. Gann Monograph, *13:* 1–17, 1972.
20. Weiser, M. M. Concanavalin A Agglutination of Intestinal Cells from the Human Fetus. Science, *177:* 535–536, 1972.
21. Zondag, H. A. Activity of LDH in Patients with Malignant Tumors of Testes and Ovary. *In:* R. Ruyssen and L. Vandendriessche (eds.). pp. 120–129. Enzymes in Clinical Chemistry, Amsterdam: Elsevier Press, Inc., 1965.

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