Selected Enzyme Activities and Isoenzyme Patterns of Virus-Infected Cell Cultures[1] (35649)

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Morphologic changes in mammalian cell cultures infected with viruses are generally not observed until late in infection when virus particles are released from the cells. Alterations in host cell metabolism, however, may occur in infected cells prior to changes in morphology.

Viruses are taken up by animal cells through pinocytosis, which requires activity of cell membranes and expenditure of energy. Therefore, important biochemical changes may begin with adsorption and penetration of the virus. Novikoff (1) demonstrated a concentration of lysosomes around pinocytic vesicles. Kovacs *et al.* (2) studied early changes in activities of lysosomal enzymes from cells infected with poliovirus. It has been suggested that lysosomal enzymes may contribute to the uncoating of viruses (3, 4). Early changes in nucleic acid metabolism and protein synthesis of virus-infected cells may be reflected by changes in glucose metabolism, but only limited work has been carried out in this area (5–8).

It is possible that new or altered forms of enzymes may be produced in virus-infected cells due to altered metabolism. Rogers and Moore (9) demonstrated that Shope papilloma virus induced production of an arginase in rabbit squamous epithelium. There have been many publications on multiple forms of enzymes of cells in culture (10) but there are no reports of isoenzyme patterns in mammalian cells infected with animal viruses.

The present investigation was initiated to study changes in enzyme activity and isoenzymes in virus-infected cells prior to the appearance of morphologic changes. Specific activities and isoenzyme patterns were determined on cell-free extracts of uninfected and virus-infected cells. Enzymes of carbohydrate metabolism, fructose-1,6-diphosphate aldolase (aldolase), glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH); and hydrolytic enzymes, arginase, leucine aminopeptidase (LPH); and acid (Acid P) and alkaline (Alk P) phosphatase were tested.

Materials and Methods. Cell cultures and virus. An established pig kidney line, PK-15, primary swine kidney (SK), and primary bovine embryonic kidney (BEK) cell cultures were grown as stationary cultures at 37°. Confluent monolayers were formed in 5–6 days and were used at that time. Growth medium for SK and PK-15 cells consisted of Hanks' lactalbumin hydrolysate (HLA) supplemented with 10 to 20% calf serum. HLA with 5% calf serum was used as the maintenance medium. Modified lactalbumin tris(hydroxymethyl)aminomethane (Tris) buffer medium (11) with 20% calf serum was used as growth medium for BEK cultures. Maintenance medium was of the same composition but without serum. Phenol red was not used in any of the media.

Beran's swine enterovirus (12) was used to infect SK and PK-15 cells. Pseudorabies (Pr) virus was used to infect BEK and PK-15 cells. These viruses belong to different groups and differ in their ability to infect cells and cause different types of cytopathic effects (CPE) (12, 13). Virus pools were prepared by inoculating cells with one hundred 50% cell culture infective doses ($CCID_{50}$) and incubating at 37°. When

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CPE was 50 to 75% complete the cultures were harvested by shaking with powdered Pyrex glass and centrifuging at 600*g* for 15 min at 4°. Virus titers were determined on the cell-free supernatant fluids by test tube cell cultures and calculated by the Reed and Muench method (14).

Virus inoculation and controls. In each experiment, some containers were not inoculated with virus and were used as preinoculation controls.

Growth media was drained from the remaining monolayers and the cells were washed with Hanks' balanced salt solution (HBSS). Virus, contained in a small volume of inoculum, was added to half of the containers. The virus-to-cell ratio was 5 $CCID_{50}$/cell. The other half of the containers served as noninfected controls and were prepared by adding an equal volume of medium from uninfected cultures handled in the same manner as the virus pool. During the adsorption period, the containers were frequently tilted to insure even distribution of the virus. At the end of the 1-hr adsorption period, the inoculum was removed and maintenance medium was added to give the proper volume of fluid in each container. The cultures were incubated at 37° until harvested.

Harvesting and preparation of samples. Various methods of harvesting and preparing the samples were tested and results of the assays were compared. The following procedures produced the most satisfactory results.

The containers were divided into groups and harvested at selected time intervals. The maintenance media were decanted, the cells were washed 3 times with HBSS and removed from the glass surfaces by scraping with a rubber policeman. The cell suspensions were transferred to centrifuge tubes, washed with HBSS, and centrifuged at 600*g* for 15 min at 4°. The supernatant fluid was discarded and the packed cells were suspended in 5 ml of 0.1 *M* Tris–HCl buffer (pH 7.4) with 0.25 *M* sucrose. The cell suspensions were quick-frozen in a bath of dry ice and alcohol and stored at —60° until all samples had been harvested. The samples were then thawed and kept at 4° during subsequent manipulations. Samples were sonified with a Branson sonifier with a microtip attachment until microscopic examination revealed that the cell structures had been disrupted. The extracts were then centrifuged at 800*g* for 20 min, the sediment was discarded and the supernatant was centrifuged at 98,000*g* for 10 min. The final supernatant fluid was used for the enzyme assays.

In each experiment, a few containers were not harvested but were kept to serve as a check on the morphology of the cells and cytopathogenicity of the virus.

Quantitative assays. Total protein was determined by the method of Lowry *et al.* (15). LDH, MDH, G6PDH, and arginase were asayed by procedures outlined in "Worthington Enzymes."[2] ICDH; Alk P and Acid P; LPH; and aldolase were determined according to the methods described in "Sigma Technical Bulletins" nos. 175, 104, 250, and 750, respectively.

Isoenzymes. In preliminary experiments cell-free extracts were subjected to starch gel electrophoresis and polyacrylamide disc electrophoresis. Starch gel electrophoresis yielded the most satisfactory results and was used throughout this study. The method of Marsh and Joliff (16) was used. For enzymes requiring divalent cations for activity the gel buffer was prepared without EDTA, but with the required concentrations of the divalent cations. One-tenth-ml samples of the cell-free extracts were mixed with starch gel and inserted into slots cut in the gels.

Following electrophoresis, the gels were incubated at 37° with appropriate substrates and stains until bands were visible. These solutions were prepared essentially according to the methods listed by Shaw and Koen (17). Bovine serum samples were used as controls.

Results. Specific enzyme activities. Samples from control cultures harvested at various time intervals showed some variation in specific enzyme activity. Usually changes from one time of sampling to the next were not very great. Table I shows the specific enzyme activities of uninfected PK-15 cells

[2] Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE I. Specific Activity of Enzymes from Uninfected and Pseudorabies-Infected PK-15 Cell Cultures.[a]

			After removal of inoculum (hr)					
		20-min adsorption	0	1	2	6	9	18
Aldolase	U[b]	6.0 ± 0.92	7.5 ± 0.94	5.5 ± 0.33	5.0 ± 0.46	4.8 ± 0.46	4.8 ± 0.54	5.3 ± 0.22
	I[c]	4.5 ± 0.31[d]	8.1 ± 1.16	11.6 ± 0.40[d]	4.3 ± 0.36	5.0 ± 0.32	6.2 ± 0.53	9.0 ± 0.26[d]
G6PDH	U	11.9 ± 0.95	7.8 ± 0.2	7.2 ± 0.46	8.7 ± 0.44	7.5 ± 0.36	4.3 ± 0.22	2.1 ± 0.17
	I	7.2 ± 0.17[d]	6.5 ± 0.7	11.4 ± 0.55	7.8 ± 0.8	6.8 ± 0.3	6.4 ± 0.26[d]	7.6 ± 0.21[d]
LDH	U	65 ± 5.5	114 ± 16.7	85 ± 4.8	92 ± 8.7	118 ± 13.2	82 ± 7.3	51 ± 3.5
	I	52 ± 6.4	115 ± 19.2	169 ± 5.9[d]	60 ± 7.1[d]	93 ± 10.8	71 ± 9.0	67 ± 3.1[d]
ICDH	U	11.6 ± 1.33	12.8 ± 1.53	9.3 ± 1.05	9.5 ± 0.5	9.0 ± 0.35	8.8 ± 0.47	6.5 ± 0.38
	I	8.7 ± 1.08[d]	12.4 ± 1.25	9.8 ± 0.86	7.7 ± 0.41	7.7 ± 0.28	13.0 ± 0.77[d]	9.7 ± 0.32[d]
MDH	U	72 ± 6.3	69 ± 3.7	76 ± 3.2	90 ± 4.4	92 ± 2.6	86 ± 9.3	76 ± 3.7
	I	68 ± 7.3	63 ± 4.6	76 ± 3.8	88 ± 5.3	101 ± 2.9	111 ± 4.9[d]	130 ± 4.2[d]
Acid P	U	1.81 ± 0.15	1.97 ± 0.16	1.74 ± 0.28	2.00 ± 0.17	1.86 ± 0.14	1.77 ± 0.4	1.44 ± 0.06
	I	2.18 ± 0.19	1.57 ± 0.2	1.65 ± 0.35	1.64 ± 0.13	1.77 ± 0.17	1.92 ± 0.13	1.29 ± 0.51
Alk P	U	3.00 ± 0.36	3.30 ± 0.22	3.2 ± 0.22	3.56 ± 0.46	3.9 ± 0.3	3.78 ± 0.19	3.09 ± 0.42
	I	3.54 ± 0.3	2.91 ± 0.36	3.7 ± 0.26	4.41 ± 0.21[d]	3.7 ± 0.36	3.48 ± 0.23	3.48 ± 0.33
Arginase	U	20.7 ± 1.8	25.2 ± 2.05	30.9 ± 3.4	39.6 ± 2.09	38.9 ± 2.7	25.5 ± 1.1	41.3 ± 2.58
	I	22.2 ± 2.2	20.0 ± 1.20[d]	24.5 ± 2.8[d]	52.5 ± 2.50[d]	38.8 ± 2.2	36.7 ± 1.3[d]	24.5 ± 2.10[d]
LPH	U	0.9 ± 0.22	1.0 ± 0.15	1.2 ± 0.14	1.9 ± 0.17	1.4 ± 0.01	1.2 ± 0.1	0.6 ± 0.02
	I	1.0 ± 0.26	1.1 ± 0.18	1.3 ± 0.17	2.6 ± 0.21[d]	1.3 ± 0.17	1.9 ± 0.15[d]	0.8 ± 0.15

[a] Each value is the mean ± standard error of three or four determinations.

[b] Extracts from uninfected cells.

[c] Extracts from virus-infected cells.

[d] Difference as compared with the control group is significant at $p < 0.05$.

and cells infected with Pr virus. Increases and decreases in specific activities of enzymes from infected cultures as compared to noninfected were calculated. Extracts of samples harvested after a 20-min adsorption period showed less activity of enzymes involved in carbohydrate metabolism and slightly greater activity of some hydrolytic enzymes. Samples harvested after the end of a 1-hr adsorption period, before the addition of maintenance media (0 hr), exhibited fewer differences. One hr after removal of the inoculum and addition of maintainence medium the glycolytic enzymes from extracts of virus-infected cultures showed a higher level of activity than noninfected cultures. During the time period of intracellular viral replication (2–9 hr) the activities were more nearly like the controls, although some hydrolytic enzymes still exhibited greater activity. There was a general increase in enzyme activity from 9 to 18 hr when viral particles were accumulating in the cells. In this experiment, CPE was evident in 24 hr.

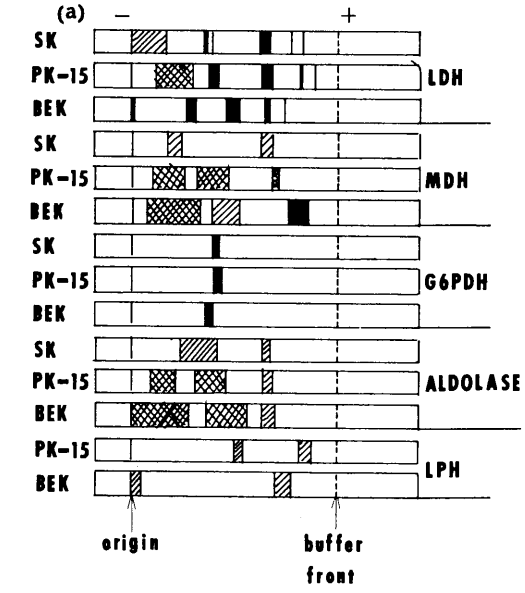
When BEK cell cultures were infected with Pr virus activities similar to those given in Table I were noted immediately after the adsorption period. Very few other differences were detected until 24 hr after infection when there was a general increase in enzyme activity as compared to controls. CPE was not observed until 48 hr after infection.

After infection of SK and PK-15 cells with Beran's virus quantitative assays were quite similar to those shown in Table I, although extracts from the infected cells were more nearly like the controls. CPE was evident at 24 hr.

Isoenzyme patterns. Arginase, ICDH, Acid or Alk P could not be detected in starch gels, although patterns were visible with the sera used as controls for the staining reactions. The isoenzyme patterns of extracts from uninfected cell cultures are illustrated in Fig. 1. The only differences detected in patterns from uninfected and infected cells were with MDH isoenzymes from PK-15 and BEK cells infected with Pr virus and harvested at 18 and 24 hr, just prior to the appearance of CPE (Fig. 2). The differences in electrophoretic pattern of extracts from infected cells involved a predominance of the anodal band with some deletion of the other bands that remained closer to the origin. In both cases, the amount of total protein and specific enzyme activity was quite similar in the paired samples.

Discussion. Changes in intracellular activity of an enzyme may be brought about through modification of existing enzyme activity. This would be governed by the availability of cofactors or the presence of activators or inhibitors which serve to increase or decrease enzyme activity. Also, there may be alterations in the concentrations of enzymes due to changes in the rate of synthesis, degradation of the enzymes, or to loss from the normal site of action. In these experiments, alterations in enzyme activitites were detected during the early time period after contact of the virus with the cells. These initial changes may be due to events associated with adsorption of virus, pinocytosis, digestion of pinocytic vesicles, and uncoating of virus. The increase in activity of hydrolytic enzymes is probably due to increased activity of bound lysosomal enzymes in the digestion of pinocytic vesicles. The decrease in activity of enzymes of carbohydrate metabolism at this early time period may be due to changes in membrane permeability or degradation of enzyme. During the time of virus replication, enzyme activities of extracts from infected cells were more nearly similar to those from uninfected cells. The experiments were designed so that the last sample would be harvested at a time when complete virus particles were present in the cells and prior to morphologic changes. Increased activity of hydrolytic enzymes at this time may be reflected later by morphologic changes. The general increase in activity of enzymes of carbohydrate metabolism during this late time period indicates that the infected cells did not preferentially metabolize glucose by a particular pathway.

Isoenzyme patterns were not obtained with all of the enzymes, although the reaction mixtures were satisfactory for staining. The concentrations of these enzymes were apparently too low to be detected by these procedures. The only difference detected between isoenzyme patterns of infected and uninfected cells was in Pr-infected cells har-



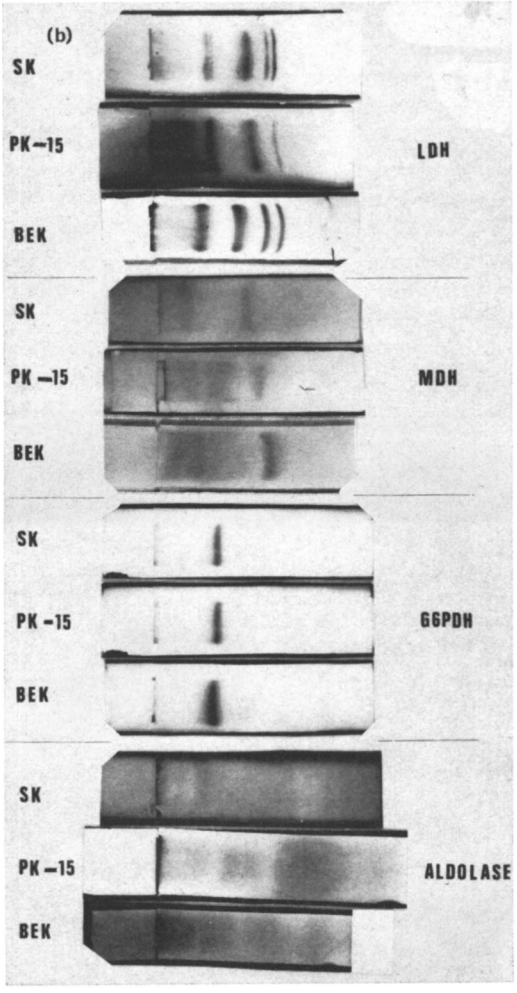


FIG. 1(a). Isoenzyme patterns of extracts from uninfected cell cultures; intensity of staining reaction: (■) intense; (▩) moderate; (▨) weak. (b) Photographs of starch gel patterns. LPH patterns did not show enough contrast to photograph.

vested a few hours before morphologic changes occurred. The anodal band of MDH was more prominent and some of the bands that remained closer to the orgin were deleted or showed a decrease in staining intensity. It has been demonstrated (18) that the MDH cytoplasmic isoenzyme is the anodal band and the mitochondrial isoenzyme remains near the origin. Substrate inhibition studies have lead to the suggestion that multiple forms of enzymes may reflect control mechanisms of cells. A predominance of the cytoplasmic isoenzyme would shift the equilibrium reaction toward the reduction of oxalacetate. Staples and Stahmann (19) have reported an absence of mitochondrial MDH isoenzymes in rust-infected bean leaves. They have interpreted this to indicate a destruction of leaf mitochondria due to infection by fungus. However, from these studies it is impossible to determine if there is actual destruction of mitochondria or if some other factor is responsible for the change in isoenzyme pattern.

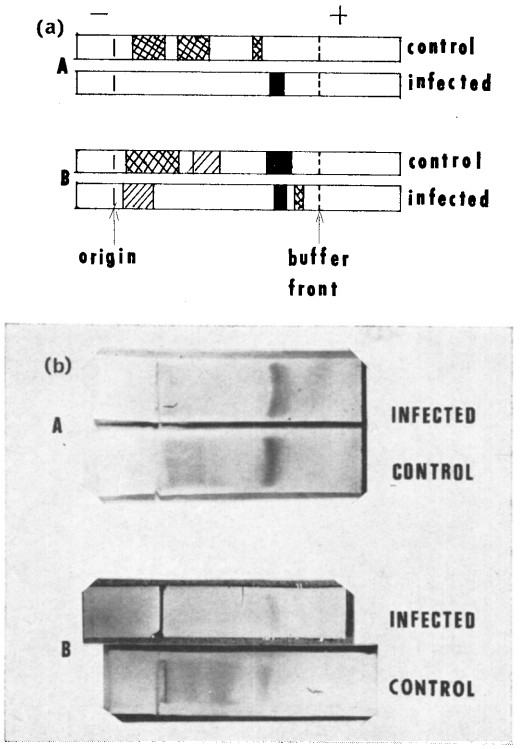


FIG. 2. MDH isoenzyme patterns; control and pseudorabies-infected cell cultures: (A) PK-15 cell cultures harvested 18 hr after inoculation with virus. (B) BEK cell cultures harvested 24 hr after inoculation with virus. (a). Diagramatic representation. Staining reaction is as indicated for Fig. 1. (b). Photographs of starch gel patterns.

Summary. Specific enzyme activities and isoenzyme patterns were determined in cell-free extracts from uninfected and virus-infected mammalian cultures. Samples were harvested at intervals prior to the appearance of morphologic changes in the cells. Two enzymes of the Embden-Meyerhof pathway, 2 of the tricarboxylic acid cycle, 1 in the hexose monophosphate shunt, and 4 hydrolytic enzymes were tested. Enzyme assays revealed differences in specific activities from infected cells as compared to controls. The main differences occurred during the early stages of contact between virus and cells and at a late time period when viral particles were accumulating in the cells. The malate dehydrogenase isoenzyme patterns from cultures infected with pseudorabies virus and harvested just prior to the appearance of morphologic changes had a different pattern than those of comparable uninfected cultures. The difference involved a predominance of the anodal band with some deletion of the bands that remained closer to the origin.

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