COMPARATIVE STUDIES OF THE ISOZYMES OF LACTIC DEHYDROGENASE IN RABBIT AND MAN

Observations during Development and in Tissue Culture*

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Isozymes, multiple molecular forms of an enzyme exhibiting similar substrate specificities (1), have provided a new and sensitive technique for the identification of various mammalian tissues, some of which contain a characteristic distribution of total lactic dehydrogenase (LDH) activity among the 5 isozymes (2–8). Sequential alterations in the pattern of LDH isozymes of several chick tissues in culture and during embryological development have been described (9). The present report extends these previous observations to 2 other species, rabbit and man. In the 3 species examined the regularity of the sequential isozymic alterations observed during embryological development suggests the operation of similar underlying mechanisms. In tissue culture the alterations in the pattern of lactic dehydrogenase isozymes are alike in the 3 species examined. Each species, however, retains a characteristic isozyme pattern. The specificity of the isozyme pattern of chick, rabbit, and human cells grown in tissue culture may provide a convenient method for distinguishing between cells derived from different species.

Materials and Methods

Liver, skeletal muscle, and heart were dissected from rabbit and human fetuses, and the LDH isozyme pattern of various rabbit tissues was examined at regular intervals after fertilization. Two human fetuses were obtained, one by cesarean section, the other by curettage, at approximately 10 weeks of gestation; and the LDH isozyme pattern of various tissues was compared with that of adult human tissues obtained at autopsy. The tissues, after disruption by ultrasonic oscillation in phosphate buffer pH 7.0, 0.01 M, were centrifuged at 15,000 *G* for 2 hours. The supernatant was subjected to vertical starch gel electrophoresis according to the method of Smithies (10). A phosphate–citric acid buffer, pH 7.0, 0.01 M, was employed in the gel; the molarity of the solutions in the proximal and distal vessels was 0.2. A potential gradient of 6 v per cm was employed for 12 hours at room temperature. The gel was then cooled at 4°C and stained for LDH activity by incubation for 1 hour at 37°C in the dark. Sodium lactate was employed as substrate, DPN as coenzyme, phenazine methosulfate as electron transporter, and the dye nitro blue tetrazolium was used. On reduction to the forma-

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zan this dye produces a brilliant purple color (4, 11). Prior to application on the gel most of the samples were assayed spectrophotometrically for LDH activity (12) and suitably diluted so that the samples had similar total LDH activities.

Trypsinized, filtered cell suspensions or cells derived from explants of various rabbit, chick, and human tissues were grown in culture bottles in reinforced Eagle's medium with 10 per cent fetal calf serum (13). When a monolayer of cells was seen microscopically to cover the bottom of the bottles, subculturing was performed by freeing and removing the cells with 0.25 per cent trypsin solution followed by centrifugation, washing, and resuspension in culture medium. An alternative subculturing procedure was also employed in which 0.25 per cent trypsin or, in the case of rabbit cultures, a mixture of 0.25 per cent trypsin and 0.05 per cent sodium versenate was added, and after 1 minute the solution was discarded, the bottle left at room temperature, and after 15 minutes fresh medium was supplied. The cells were then removed from the glass by pipetting or with a rubber policeman (14).

When approximately 10 to 15 million cells were obtained, harvesting with a rubber policeman or with trypsin was carried out. The cells were then washed three times in phosphate-buffered saline and stored at 4°C until used. The LDH isozyme pattern was unaltered by storage for several weeks.

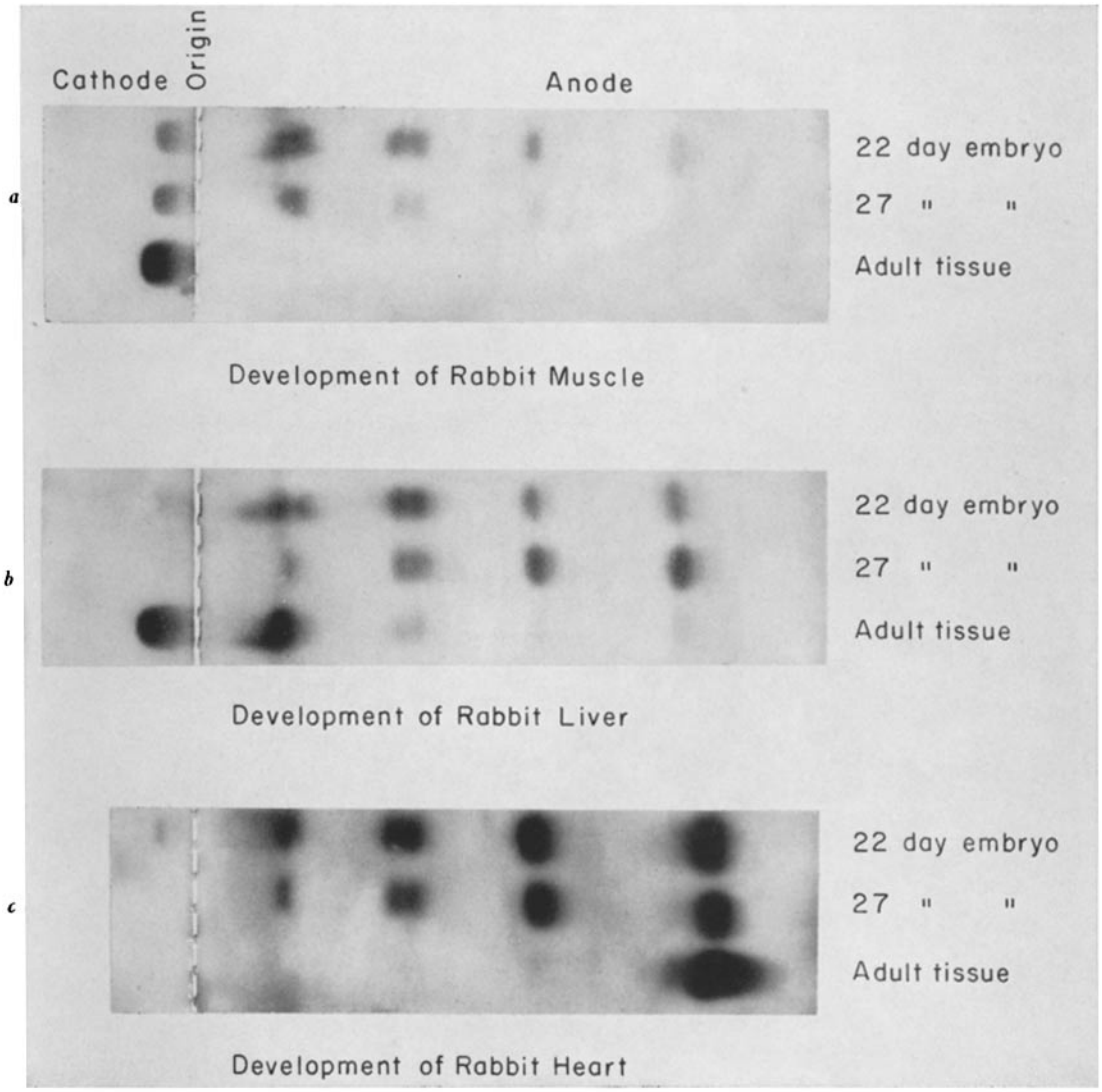
The chromosome complement of rabbit and human cells grown in long term culture in the above mentioned medium was examined by the method of Harnden (15) as modified by Philip *et al.* (16).

RESULTS

A. Sequential Alterations of Isozymes during Development.—

1. In rabbit muscle and liver, isozymic alterations during development are characterized by a loss of the most rapidly migrating anodal bands and increased intensity of staining of the cathodal band, whereas in heart the opposite sequence of events occurs. Fig. 1 *a* shows that in rabbit muscle 22 days after fertilization, there are observed 4 main anodal isozyme bands, the second slowest migrating of which invariably appears as a double band. At 27 days the most rapidly migrating band has almost vanished, and in adult skeletal muscle only a cathodal band is observed. Fig. 1 *b* illustrates that in developing rabbit liver two processes are observed. From 22 to 27 days after fertilization there is a tendency toward increasing activity of the most rapidly migrating anodal bands, whereas in the adult most LDH activity is found in the cathodal band and the slowest migrating anodal band. Fig. 1 *c* illustrates that in the rabbit heart 22 days after fertilization a faint cathodal band and 4 main anodal bands, the second slowest of which is apparently split, are observed. Most LDH activity appears in the 3 rapidly migrating anodal bands. The 27 day specimen shows that at the concentrations employed the cathodal band disappears and a greater part of the total LDH activity is observed in the most rapidly migrating anodal bands. In the adult rabbit heart almost the entire LDH activity is found in the most rapidly migrating anodal band.

2. Tissues from 2 human fetuses were compared with those from the adult (Fig. 2). At the activities employed, embryo skin, liver, and lung (not shown in the figure) were characterized by the appearance of the majority of the total



FIGS. 1 *a* to 1 *c*. Photograph of starch gels stained for LDH activity.

FIG. 1 *a*. Rabbit muscle during development. Each specimen contained 8,000 units/ml of activity.

FIG. 1 *b*. Rabbit liver during development (8,000 units/ml).

FIG. 1 *c*. Rabbit heart during development (11,000 units/ml). Note that sequential alterations are the reverse of Figs. *a* and *b*.

LDH activity in the anodal region, whereas very little activity was observed in the cathodal region. In adult skin, liver, and lung the cathodal band was the most heavily stained. In embryo heart the greatest activity was observed in the 2 slowest migrating anodal bands and the cathodal band, whereas in adult heart the 2 fastest anodal bands were the most prominent. In embryo kidney no cathodal band was observed; in the adult a very faint cathodal band was seen at the activities employed.

B. Isozyme Patterns in Tissue Culture.—

In human and rabbit tissues in culture certain characteristic changes occur of which the most prominent are a gradual decrease in intensity of the most rapidly migrating anodal bands and increased intensity of cathodal bands. The presence of a cathodal band is more dependent upon the total LDH activity of

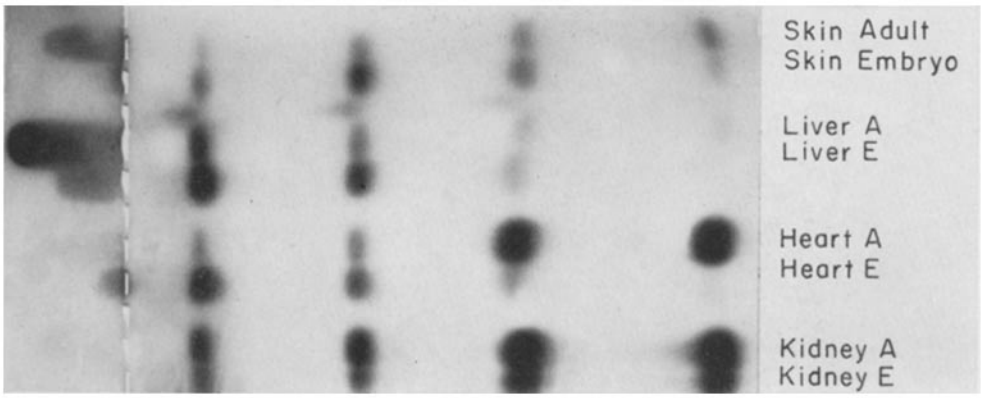


FIG. 2. Photograph of a starch gel contrasting the LDH isozyme pattern in fetal and adult human tissues. Each skin sample contained 4,000 units/ml; each liver sample contained 7,000 units/ml; each heart sample 10,000 units/ml and each kidney sample 6,000 units/ml.

the sample applied to the gel than are the anodal bands (17). Although these general changes in the distribution of total LDH activity among the isozymes occur in cells grown in culture regardless of whether they are derived from chick, rabbit, or man the relative mobility of the isozymes of cells grown in culture is characteristic of the particular species from which the cells have been obtained.

Rabbit skin, muscle, and heart from 21-day-old embryos and liver from 15-day-old embryos were grown in culture. Rabbit skin, muscle, and liver were characterized by 4 migrating anodal bands; the second slowest migrating band invariably appeared split (Figs. 3 to 5). Samples containing 6,000 to 7,000 LDH units/ml revealed no cathodal bands, whereas at 12,000 and 18,000 units a prominent cathodal band was observed (Fig. 3). In rabbit muscle in culture 2 slowly migrating anodal bands were seen which were not observed in embryonic or adult rabbit muscle (Fig. 4). In rabbit heart in culture a

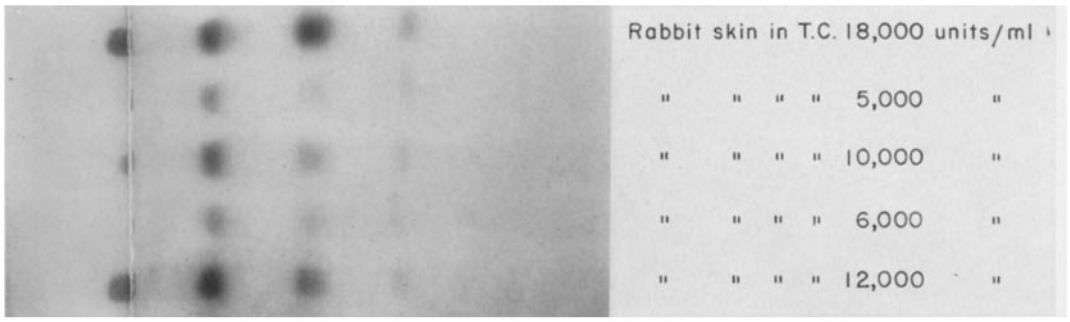


FIG. 3. Photograph of a starch gel of rabbit skin in tissue culture illustrating effect of concentration on LDH isozyme pattern.

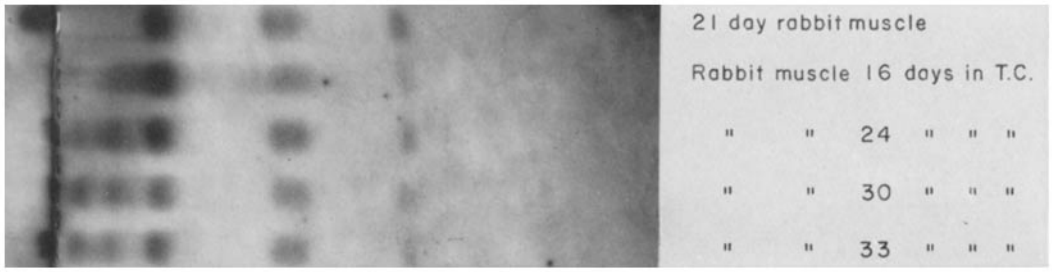


FIG. 4. Photograph of a starch gel illustrating LDH isozymes in rabbit muscle in tissue culture. Note the appearance of 2 slowly migrating anodal bands not seen in other tissues. Each sample contained 10,000 units /ml.

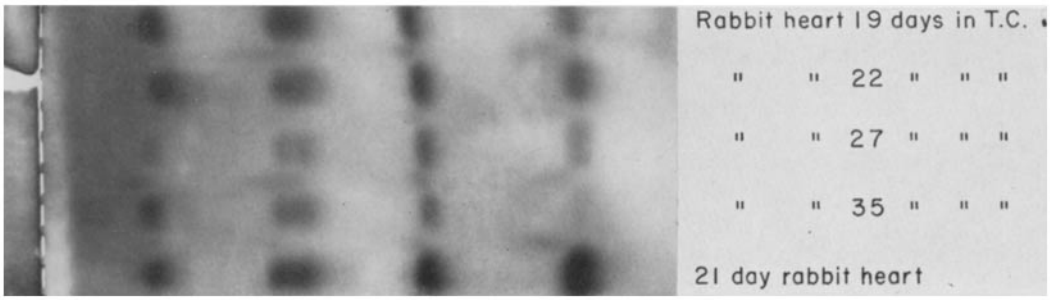


FIG. 5. Photograph of a starch gel illustrating LDH isozymes in tissue cultures of 15-day-old rabbit heart. Each sample contained 9000 units/ml. Note decreasing intensity of the 4th and 5th anodal bands with time.

fifth, the most rapidly migrating, anodal band is observed, not easily visualized in other rabbit tissues in culture at the activities used (Fig. 5), and this fifth band has a mobility similar to that of the most prominent band of 21-day-old embryo and adult rabbit heart.

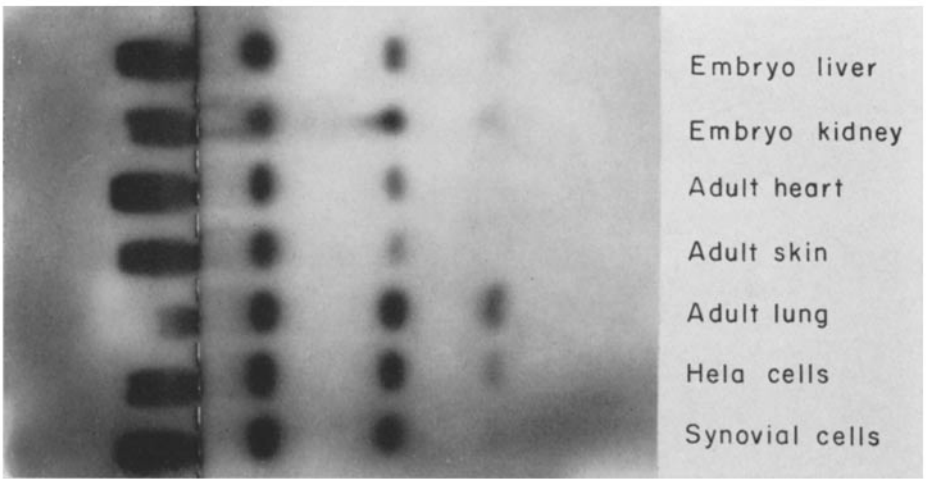


FIG. 6. Photograph of a starch gel illustrating the LDH isozyme pattern of various human tissues in culture. Each sample contained 10,000 units/ml. Embryo liver was in culture 82 days; embryo kidney, 58 days; heart, 91 days; skin, 112 days; lung, 180 days; Hela cells, several years; synovia, 120 days. Note that lung retains in culture its isozyme pattern more closely than the other tissues, most of which were grown in culture for shorter periods.

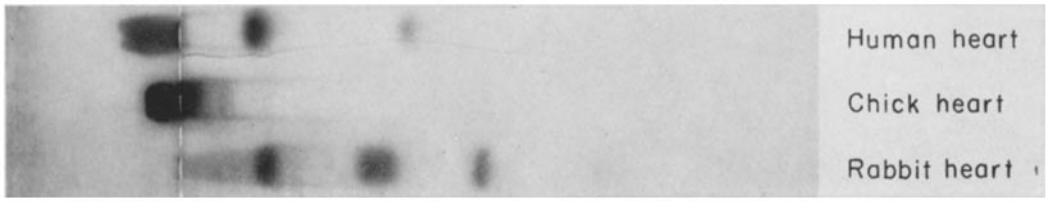


FIG. 7. Species specificity of isozymes in tissue culture. Photograph of a starch gel illustrating differences among the LDH isozyme patterns in heart from 3 species grown in culture. Each sample contained 10,000 units/ml.

Primary cultures of human skin, heart, liver, lung, kidney, and synovia were initiated and Hela cells (18), derived from a human cervical carcinoma, and Chang's conjunctival cells (19) were grown in tissue culture. The isozyme patterns obtained from all tissues consist principally of 4 anodal bands and 1 cathodal band (Fig. 6). In long term culture the most rapidly migrating anodal bands may disappear until, under the conditions of time and total enzyme ac-

tivity employed in these experiments, 2 or 3 anodal bands remain (Fig. 6). Fig. 6 also illustrates variations among certain human tissues in culture; lung retains prominent anodal bands, although it has been 180 days in culture, whereas its cathodal band stains less intensely than that of other tissues. The Hela and Chang cell lines, found to be aneuploid, revealed no alterations in their isozyme pattern when compared to other cell strains employed in this study which were demonstrated by karyotype analysis to be euploid. The fact that Hela cells grown in culture for more than 10 years retain the isozyme pattern characteristic of other human tissues in culture (Fig. 6) suggests that following the initial alterations in early subcultures the isozyme pattern is stable. Although a similar series of isozymic changes occur in cells from rabbit, human, and chick tissues grown in culture, each of these species maintains *in vitro* an isozyme pattern which distinguishes it from the other 2 species (Fig. 7).

DISCUSSION

These experiments indicate that rabbit and human cells under the *in vivo* conditions of embryonic development and also under the *in vitro* conditions of tissue culture exhibit characteristic alterations of their LDH isozyme pattern, already described in the chick (9). In the embryologic development of muscle and liver from rabbit and chick there is a gradual redistribution of total LDH activity from concentration in the anodal isozymes early in development toward concentration in the cathodal and less rapidly migrating anodal bands later in development. Rabbit and chick heart exhibit the reverse series of events. Comparison of LDH isozyme patterns in embryologic tissues prepared from human fetuses with adult tissues reveals that the same sequential alterations probably occur. The changes in the isozyme pattern of cells grown in tissue culture consist generally of a loss of one or more of the rapidly migrating anodal bands and an increased intensity of the cathodal bands and of the slower migrating anodal bands. Sequential alterations of LDH isozymes are, therefore, generally similar in the *in vivo* and *in vitro* environments. However, the sequential alterations of LDH heart isozymes in development are the reverse of those observed in tissue culture.

Several practical applications of LDH isozymes in cells grown in tissue culture emerge from these studies. The LDH isozyme patterns of cells in culture from chick, rabbit, and man are readily distinguishable; isozymes may, therefore, provide a method for identifying cells from various species grown in culture. Comparison of LDH isozymes in erythrocytes from more than 20 vertebrate species has shown that although many species have characteristic patterns others tend to exhibit somewhat similar isozyme patterns (10). If some of the tissue-specific LDH isozyme patterns observed in some mammalian organs (1–8) had been preserved in culture it would have been possible to distinguish

within a species the organ from which cells in culture originated. However, various tissues from a single species tend to assume a similar LDH isozyme pattern after long term culture. Another application of isozymes in culture concerns efforts to grow a cell *in vitro* with closer physiologic resemblance to the cell in *vivo*. It is possible that by appropriate modification of the cultural conditions a greater similarity of the cell *in vitro* to that *in vivo* might be achieved.

Two alternative theories have been proposed to explain the emergence of a "common cell type" in tissue culture. The first, the selection theory, suggests that the *in vitro* environment favors the growth and development of a specific cell type. The second, the dedifferentiation theory, suggests that the relative simplicity of the culture medium fails to maintain the complex biochemical gradients and interactions existing *in vivo*; thereby specialization of the cell is lost. If selection of a non-parenchymal, interstitial cell type occurs *in vitro*, the sequential alterations of the isozyme pattern observed during culture might be compatible with the gradual decrease in number of parenchymal cells in early subcultures and the proliferation and overgrowth of a non-parenchymal cell type. Attempts to attain an organ-specific isozyme pattern by altering the *in vitro* environment could be realized only by selection of the parenchymal cell type. In favor of selection of a ubiquitous cell type under the conventional *in vitro* environment is the work of Sato *et al.*, who demonstrated that rat liver cells in culture lacked certain antigens present in liver cells *in vivo*, that these cultured cells derived from liver possessed antigens common to those from cultured cells derived from kidney, and that the antigenic properties of cells grown in culture were present in some of the cells in the inoculum from which the culture was initiated (20). Furthermore, it has been shown that under appropriate tissue culture conditions, specific properties of certain cells *in vivo* have been maintained *in vitro*. Liver cells growing on rat collagen retained in culture both the morphology and certain of the biochemical characteristics of human liver cells (21). Leukemic mast cells grown in culture maintained high intracellular levels of 5-hydroxytryptamine and histamine (22), and synthesis of pituitary hormone has also been demonstrated in tissue culture (23). Our attempts to modify the *in vitro* environment by omission of pyruvate or by addition of insulin or lactate to cultures of chick tissues were performed on cell strains in long term culture and produced no demonstrable effect on the isozyme pattern. These results could indicate that selection had already taken place and that these cells were therefore incapable of reverting to the isozyme pattern of the organ of origin.

To accommodate the selection theory to the maintenance of specialized biochemical properties in cultures it is necessary to postulate that in addition to the ubiquitous cell types there is proliferation of differentiated cell types. However, sequential alterations in the isozyme pattern may also be explained by the dedifferentiation hypothesis according to which cells in culture regardless of their origin would be expected to assume common biochemical properties.

This is compatible with the observation that various types of human cells with different *in vivo* enzymatic activities assume common enzymatic levels in culture (24). Conversely various human tissues, each exhibiting a similar pattern of 7 esterase bands, retained their esterase pattern during both short term and long term culture (25). It seems likely that the correct choice between the two theories must await studies in which clones of parenchymatous cells from different tissues are grown *in vitro*. Studies on LDH isozymes appear to provide a convenient and sensitive method for monitoring the biochemical changes which may occur during prolonged tissue culture.

SUMMARY

During development of rabbit tissues, characteristic sequential alterations in the LDH isozyme pattern occur, and consist for liver and muscle in loss of the most rapidly migrating anodal bands, and increased activity in the cathodal bands and slower migrating anodal bands. In heart the reverse changes were observed. Comparison of the isozyme patterns observed in various fetal and adult human tissues suggests that these same sequential alterations probably occur.

A species-specific isozyme pattern is obtained in long term culture of rabbit, chick, and human cells. The alterations in tissue culture are characterized by a gradual redistribution of total LDH activity in which there is decreased intensity of rapidly migrating anodal bands. These sequential alterations are independent of the organ of origin. The number of bands observed in the starch gel is partly dependent upon the total activities applied.

Isozymes may provide a convenient method for determining the species of origin of cell lines in common use and for investigating the effects of various alterations in the *in vitro* environment on cells grown in tissue culture.

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