Isoenzyme Characterization of Animal Cell Cultures[1] (34238)

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Studies of polymorphic enzymes have shown that characteristic electrophoretic patterns are obtained from freshly isolated tissues of different animal species and from cells cultivated *in vitro* (1–11). Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD) in particular, have been widely investigated, but there appear to be no comprehensive comparative studies on cell cultures derived from a wide variety of animal species. In 1962 Vesell *et al.* (4) examined the LDH patterns of cell cultures derived from a few animal species and suggested that isoenzyme analysis may provide a method for identifying cells from various species when grown in tissue culture. Gartler (9), in 1967, pointed out that electrophoretic variants of polymorphic enzymes might also serve as useful genetic markers for the detection of intraspecific contamination of cell cultures.

The present study was initiated to: (i) survey animal cell cultures derived from a wide variety of species to determine whether isoenzyme analysis might be a reliable and practical method of species identification; (ii) investigate the reports by Gartler (9, 10) that many commonly used human cell lines, presumably of Caucasian origin, possess the electrophoretic A type variant of G6PD found only in approximately 30% of the Negro race; (iii) determine if intraspecies differences in LDH or G6PD isoenzymes might be detected in animal cell lines of other than human origin.

Material and Methods. Animal cell lines assayed. A total of 86 characterized animal cell lines certified by the Advisory Committee to the Animal Cell Culture Collection (12) were studied. The cells were tested directly from freshly thawed ampules. Each sample contained 4 to 8×10^6 viable cells. In a few experiments T-60 monolayer cultures initiated from frozen specimens were also assayed.

The species of origin of all cell lines used in this study had previously been verified (12) by immunological techniques or, in a few cases by chromosome analysis.

Preparation of extracts. The cells were washed three times with 0.9% NaCl solution containing 6.6×10^{-4}M ethylenediamino tetraacetate, and were suspended in the same solution to yield a concentration of approximately 1×10^7 cells/ml. The extract was prepared by freezing the cells in liquid nitrogen and thawing at room temperature. The freeze–thaw procedure was repeated three times. The crude extract was then centrifuged at 20,000*g* for 0.5 hr at 4°, assayed without purification, and when not used immediately, stored at —95°. In a few cases the cells were disrupted by ultrasonic vibration (fresh tissues) or by treatment with octyl alcohol. The mobility of the isoenzymes was the same regardless of the method employed for preparation of the extracts.

Electrophoresis. The vertical starch-gel technique described by Kirkman (13) was used. Extracts were assayed for G6PD and/or LDH activity by a modification of the methods of Glock and McLean (14) and Vesell (15) and were diluted to approximately equal activity.

Development of zymograms. After electrophoresis was completed, the gel was sliced in half horizontally and covered with a thinner gel consisting of 3.5 g of starch in 30 ml of 0.25 *M* Tris–HCl buffer at pH 7.5, to which the incubation mixture was added. This mix-

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ture contained 2 mg of phenazine methosulfate and 5 ml each of nitroblue tetrazolium (2 mg/ml), 0.025 *M* glucose-6-phosphate, 0.005 *M* nicotinamide adenine dinucleotide phosphate and 0.1 *M* $MgCl_2$. For LDH, 1.0 *M* sodium lactate and nicotinamide adenine dinucleotide (10 mg/ml) were substituted for the last three components. The gels were incubated at 37° for a period of time depending on the activity of the extracts (usually from 0.5 to 1 hr). Immediately thereafter the gels were washed with cold tap water and a photographic record was made. The gels were treated by immersion in a 5:5:1:1 mixture of methanol, water, acetic acid, and glycerol, and stored at room temperature.

Results. The results of a comparative electrophoretic analysis of G6PD and LDH isoenzymes in 86 animal cell lines representing 22 different taxonomic groups (species or genera) are summarized diagrammatically in Fig. 1. A high degree of heterogeneity in electrophoretic mobility was obtained. The differences permit the cells in almost all of the taxonomic groups to be readily distinguished from each other upon comparison of both the G6PD and LDH zymograms. The majority of the cell lines exhibited a single G6PD band. Multiple bands were obtained only in cells derived from cattle, the buffalo, horse, trout, and minnow. The number of LDH bands ranged from two to five in most of the species. Ten cell lines derived from the mouse exhibited a single band identical in mobility to the tissue LDH_5. Although it is not depicted in Fig. 1, five other mouse cell lines exhibited one or more additional bands. A single band was obtained in all cell lines derived from the Chinese hamster, Syrian hamster, mink, pig, frog, trout, and blue-striped salt-water grunt.

No variant forms of G6PD were detected within a species except in the human cell lines. The human cell lines can be separated into two groups based on their G6PD isoenzyme patterns. Of the 34 human lines assayed, 24 exhibited the faster (A) G6PD variant while 10 possessed the slow (B) band. Figure 2A shows the characteristic mobilities exhibited by the two different forms of G6PD in some human cell lines. All of the cell lines in which the A variant was found are heteroploid and have an apparent infinite life expectancy. The 10 lines (12) that exhibited the B band are either diploid or near-diploid and have an apparent finite life expectancy. A possible exception is the RPMI 2650 (CCL 30) cell line which was found to possess the B band yet has been carried beyond the 102nd serial subcultivation in our laboratory without change.

Five LDH bands with mobilities characteristic of human tissues were retained in all of the human cell lines we examined. On occasion, some of the bands did not appear but were detectable after longer incubation. Although differences in activity of the bands were not quantitated, there is evidence of variation in the LDH patterns. Figure 2B shows LDH bands with the same mobility for five human cell lines; however, differences in intensity of the bands are evident. In almost all of the human cell lines the LDH activity was highest in the cathodal bands or in the LDH_3 band. Early passage cell lines of apparent finite life expectancy characteristically showed an intense LDH_5 band and little or no activity in the anodal band (Fig. 2B, zymogram 4). In contrast, the RPMI 2650 cells (CCL 30) exhibited the greatest activity in the anodal bands (Fig. 2B, zymograms 5 and 6). The electrophoretic mobilities of the human and monkey G6PD and LDH isoenzymes were virtually identical (Fig. 2A and B). It was observed that two monkey cell lines (LLC-MK_2 cells, CCL 7 and 7.1) adapted to growth in different media exhibited marked differences in the LDH patterns (Fig. 2B, zymograms 7 and 8).

We have compared the G6PD and the LDH patterns of permanent cell lines and fresh tissues from the Chinese hamster, Syrian hamster, and mouse. A single G6PD band was obtained from each of the three species (Fig. 1) and the mobility of the isoenzyme from the cell lines was the same as that from the fresh tissues. The LDH isoenzymes of the fresh tissues showed characteristic differences in cathodal or anodal intensities in the various tissues; however, the majority of the cell lines derived from these species exhibited

only a single LDH band with the same mobility as the tissue LDH_5 (Fig. 3). Five cell lines derived from C_3H mice were exceptions and exhibited two or more LDH bands. NCTC clone 929, Strain L, (CCL 1) and its derivatives, NCTC 2071 (CCL 1.1) and L-M (CCL 1.2), showed two bands with mobilities identical to the tissue LDH_4 and LDH_5. The same was true of NCTC 2472 (CCL 11), the high tumor-producing line (Fig. 3, zymogram 10). In contrast, NCTC 2555 (CCL 12), the low tumor-producing line, exhibited four bands corresponding to the tissue LDH_2, LDH_3, LDH_4 and LDH_5 (Fig. 3, zymogram 9).

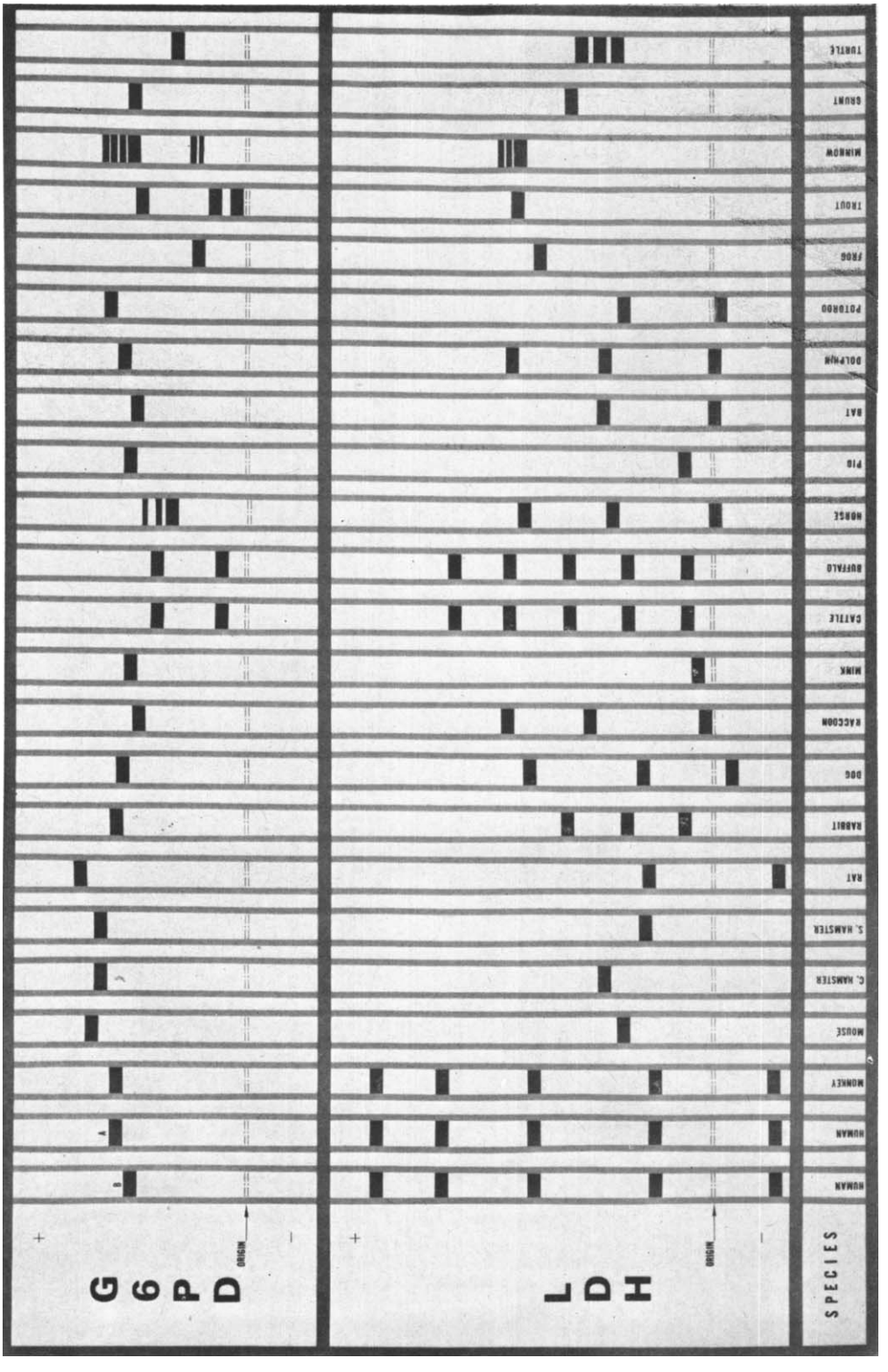
Discussion. As was anticipated by Vesell *et al.* (4), enzyme polymorphisms provide an effective method for the identification of cells cultured *in vitro*, at least as far as the generic, and possibly the species level. We have shown that cell cultures representing 20 out of 22 taxonomic groups can be easily distinguished from each other by comparison of their G6PD and LDH isoenzyme patterns (Fig. 1). Of the two exceptions, similarity in the patterns of the two isoenzymes exists even at the family level in one case and in the other, at the generic level. There may be significant differences in the G6PD and LDH isoenzymes that we were unable to detect with our present methods. The study of additional polymorphic enzymes may be required in order to clearly differentiate cells from these taxonomic groups.

A number of immunological techniques are employed routinely for the identification of the species of origin of cells cultured *in vitro* (16–19). These techniques are effective; however, they either require the time-consuming procedure of injecting large numbers of the test cells into laboratory animals in order to prepare a specific antiserum (16), or they require the prior preparation of a relatively large number of expensive antisera (17–19). The antisera often must be rendered species-specific via absorption techniques and it is sometimes difficult to obtain adequate quantities of effective antisera. A single unknown must be assayed against an array of antisera for each individual test.

Isoenzyme analysis of cells provides a means of complementing or, in some cases, supplanting the immunological techniques. As we have shown, once the electrophoretic patterns for several isoenzymes are determined for a large number of species and a "fingerprint" identification chart constructed (see Fig. 1.), the species or genera of an unknown can, in most cases, be readily pinpointed. If the isoenzyme analysis shows that the species of the cells is *other* than that which was originally supposed, then it is not necessary to examine the cells via immunological techniques. This conserves both time and valuable antisera. Another advantage of isoenzyme analysis is that a permanent photographic record of the zymogram may be obtained for future comparisons and study. Also, as pointed out by Gartler (9), the technique is relatively simple and the processing of approximately 20 samples can be carried out within 1–2 days.

In addition, isoenzyme analysis has been useful in determining the species of presumably "transformed" cells that have been submitted to us for identification. In all cases examined thus far, determination of the G6PD and LDH patterns has shown that such cultures contained cells predominantly (if not all) from another species. We find that cultures containing cells from two different species exhibit the isoenzyme patterns characteristic of both species but we have not as yet determined the lower level of sensitivity of the isoenzyme tests. For detection of incipient low-level contaminations with cells

FIG. 1. Diagrammatic comparison of the LDH and G6PD zymograms of 86 animal cell lines representing 22 taxonomic groups. For the human cells the B band of G6PD represents 10 cell lines and the A band represents 24 cell lines. The zymograms of the monkey were obtained from 6 cell lines; the mouse from 16; the Chinese hamster from 5; the Syrian hamster and the rat from 3; and the potoroo from 2. All of the zymograms for the remaining species were obtained from a single cell line. Note that all cells may be readily differentiated from each other except for those originating from the human (A) and monkey, and the buffalo and cattle.


G6PD
LDH
SPECIES
HUMAN
HUMAN
MONKEY
MOUSE
C. HAMSTER
S. HAMSTER
RAT
RABBIT
DOG
RACCOON
MINK
CATTLE
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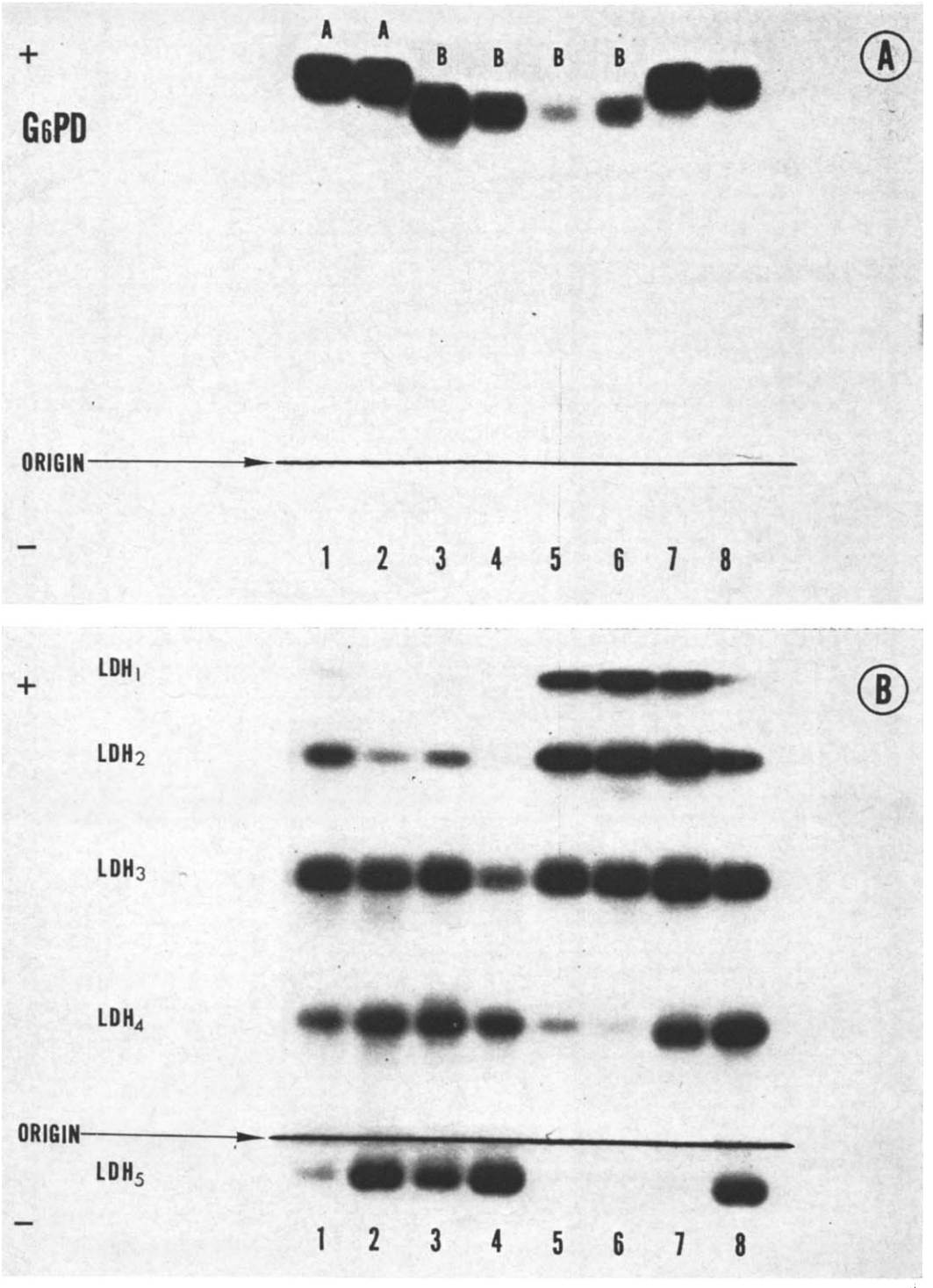

FIG. 2. Starch gel zymograms of extracts from cultured human and monkey cells: (A, top), Gel stained for G6PD; (B, bottom), Gel stained for LDH. Slots as follows: (1) HeLa, CCL 2.1 and (2) AV_3. CCL 21, are permanent human heteroploid cell lines. (3) WI-38, CCL 75, and (4) Detroit 529,

are human diploid or near-diploid cell lines with a finite life expectancy. (5) and (6) are the quasi-diploid human cell line RPMI 2650, CCL 30, from a frozen ampule (passage 22) and from a culture (passage 90), respectively. (7) Rhesus monkey LLC-MK_2, CCL 7, cells cultured in medium containing horse serum. (8) Derivative of LLC-MK_2, CCL 7.1, cultured in medium containing bovine serum.

of another species more elaborate tests employing immunological techniques would be necessary, but even these techniques have their limitations (18).

Our examination of all the human cell lines in the Animal Cell Culture Collection showed that 24 cell lines exhibited the faster (A) variant of G6PD while only 10 cell lines possessed the slow (B) band. These results, as well as similar studies (20, 21) by the reference laboratories which cooperate with us in characterizing cells for the Animal Cell Culture Collection, confirm Gartler's experimental results (9, 10) and, thus far, are consistent with his interpretation. These findings have focused the attention of cell culturists on the importance of the positive identification of cells within a species.

Except for the A type electrophoretic variant of G6PD in human cells we have not detected, within a species, any other electrophoretic variant of G6PD (or LDH) isoenzymes in cell lines thus far examined. In some instances, however, consistent differences in the relative intensities of the LDH isoenzymes suggest that some types of cells can be differentiated from one another. For example the early-passage cell lines with a finite life expectancy can be distinguished from permanent heteroploid cell lines in that the cathodal LDH components are more intensely expressed, especially LDH_5, and the anodal LDH components are more severely suppressed (Fig. 2B). A notable exception to the general predominance of cathodal bands in human cell lines is the quasi-diploid cell line, RPMI 2650 (CCL 30), which exhibits the greatest activity in the anodal band (Fig. 2B). It has been suggested (22) that the high lactic acid production and the predominantly anaerobic metabolism characteristic of cells cultured *in vitro* enhances the production of the LDH_5 type subunits and inhibits the LDH_1 type subunits. The characteristically different LDH pattern exhibited by the RPMI 2650 (CCL 30) cells may be related to a more aerobic type of metabolism, to the high mucopolysaccharide production of these cells, or to other causes.

Among the mouse cell lines examined there was some suggestion that cells derived from the C3H mouse can be distinguished from cells derived from other strains of mice. Five cell lines of C3H origin exhibited two or more LDH bands whereas cells derived from all other strains of mice exhibited only a single LDH_5 band. It is interesting to note that the high tumor-producing cell line, NCTC clone 2472 (CCL 11) and the low tumor-producing cell lines are of common parental lineage and were derived from NCTC clone 1328. These cell lines not only differ in their tumor-producing capacities but also in their rates of anaerobic glycolysis (23). The high line, CCL 11, was found to have a higher rate of anaerobic glycolysis than the low line, CCL 12. We have found that the low line, CCL 12, exhibits two more anodal bands than the high line, CCL 11. With the retention of four LDH isoenzymes *in vitro* the low tumor-producing cells, CCL 12, exhibit a pattern similar to that obtained from fresh normal lung tissue of the C3H mouse. The significance, if any, of this observation is not known.

There are several factors which should be taken into consideration in evaluating the differences observed in the distribution and expression of the LDH isoenzymes. The subunits of LDH isoenzyme extracted from tissues have been shown to disassociate and reassociate upon freezing and thawing (24). Changes in the distribution of LDH isoenzymes have also been reported to occur in certain types of cells after freezing and thawing (25). There is also some evidence for the occurrence of differences in LDH patterns of a cell line when different culture media are employed (26). We have observed shifts in the distribution of LDH isoenzymes and de-

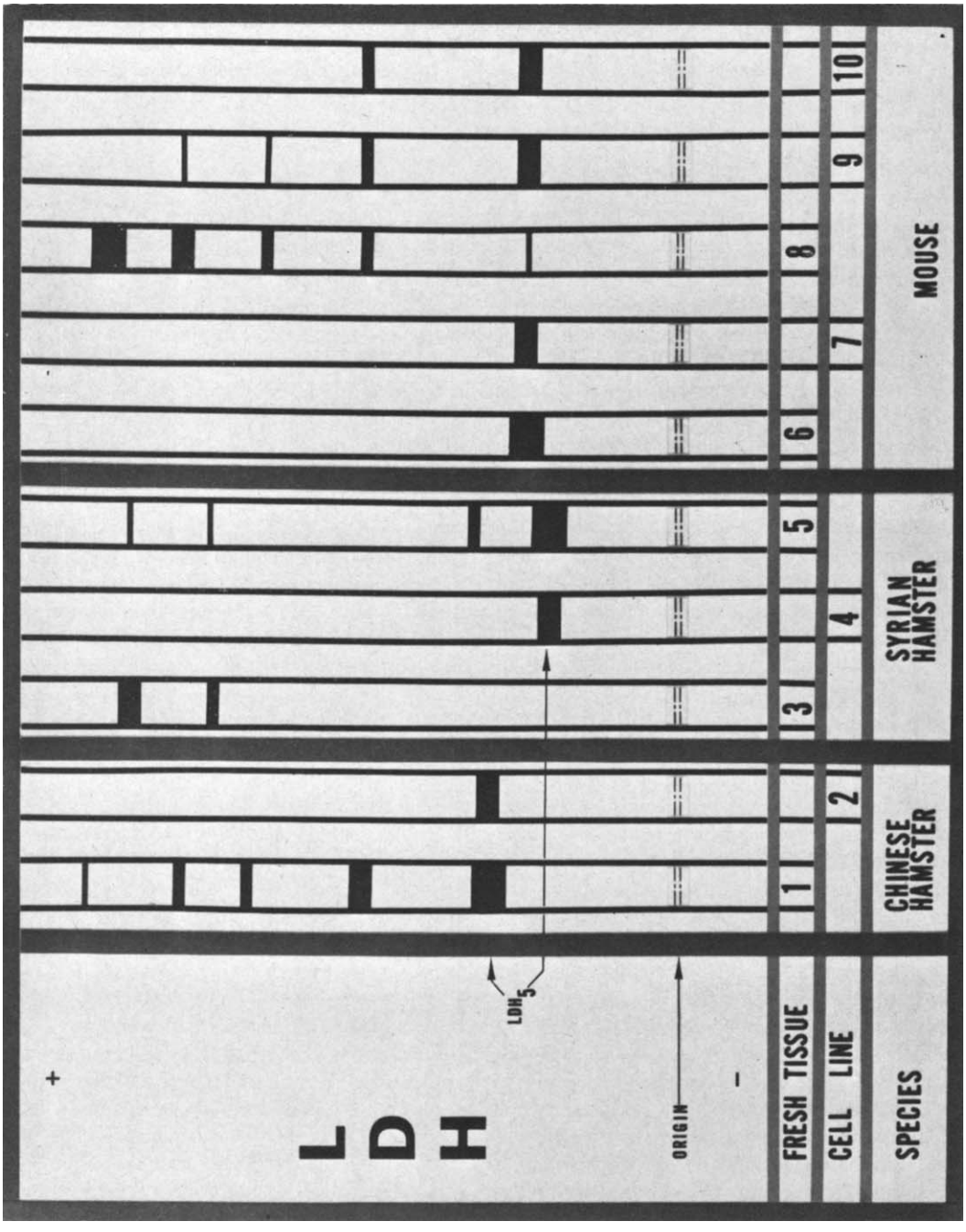

FIG. 3. Diagrammatic comparison of LDH zymograms obtained from fresh tissue and cultured cells of Chinese hamster, Syrian hamster and C3H/HeN mouse: (1) lung; (2) lung, Don, CCL 16; (3) kidney; (4) kidney, BHK-21, CCL 10; (5) liver, (6) liver; (7) liver, NCTC 1469 derivative, CCL 9.1; (8) kidney; (9) connective tissue, NCTC 2555 low tumor-producing line, CCL 12; (10) connective tissue, NCTC 2472 high tumor-producing line, CCL 11.

creased activity in extracts prepared from some of the cell lines after the extracts were subjected to storage by freezing; however, extracts prepared from most of the cell lines remained remarkably stable.

Summary. We have found the isoenzyme analysis of cells to be a significant aid in the characterization and identification of animal cell cultures from a variety of species. The G6PD and LDH patterns of 86 animal cell lines were examined using starch gel electrophoresis. From the data accumulated it was possible to construct a "fingerprint" identification chart for ready identification of animal cells from 20 out of 22 different taxonomic groups. Although there are limitations to the technique the systematic study of additional polymorphic enzymes should provide valuable information for the more precise characterization of animal cells.

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