Table 2. Emission spectroscopic data of pigmented rat skin and albino rat skin

Ash of rat skins	Mean result (ppm) ± SD				
	Pb	As	Ni	Co	Cu
Albino	150 ± 13	500 ± 17	30 ± 4	10 ± 1	600 ± 20
Pigmented	70 ± 11*	500 ± 13 (NS)	10 ± 1*	10 ± 1 (NS)	120 ± 12*

n = 6; *p < 0.001; NS = not significant.

case of these 2 metals only. Nickel, cobalt and copper which have also been detected in toad's skin show an insignificant change after depigmentation as well as after recovery. Considering the intermediate status of the mobility of skin lead, as compared with those of bones and soft tissues[7], the variation of lead concentration under different experimental conditions could be expected. The higher concentration of lead found in albino rat skin (table 2) is parallel with our observations during experimental depigmentation (table 1). The observation of the larger amount of copper in albino rat skin (table 2) is analogous to the observations of Genov et al.[8]., regarding a 30% higher total copper level with the concomitant higher oxidase activity of ceruloplasmin (130%) as compared with normal subjects. Albino rat skin also contains more nickel than the pigmented skin. Both the varieties of rat contain a significant amount of arsenic in their skins, although the difference between the two is not significant. This arsenic which could normally be present in rat hair might come from endogenous sources, or from exogenous sources by adsorption in the hair. In our experiments, arsenic was not detected in toad skin.

It is well documented that lead inhibits tyrosine hydroxylase activity by reducing the tetrahydrobiopterin level in serum[7]. Lead has also been suggested to alter the metabolism of tryptophan[9]. Kurbanov et al.[10] as well as Roy Chowdhury et al.[11] have shown that abnormal tryptophan metabolism may be involved in vitiligo. Again, schizophrenia, which has been related to abnormal tryptophan metabolism also produces elevated lead levels in hair[12]. The increased concentration of skin lead in experimental depigmentation are therefore interesting, and calls for further investigations. From our results it appears that apart from copper, other trace metals may be involved in melanogenesis affecting the activity of the oxygenase enzymes involved in it.

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Lactate dehydrogenase activity in cell subpopulations of 7,12-dimethylbenz(a)anthracene-induced mammary tumors[1]

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Summary. Heterogeneity of cells in a solid tumor prevents direct assessment of enzyme activity of cell subpopulations by conventional homogenization techniques. Lactate dehydrogenase (LDH) activity before and after ovariectomy and following estrogen supplementation in density-defined cell subpopulations from DMBA-induced mammary tumors was determined. Different levels of LDH activity were found in different cell subpopulations. After ovariectomy the level of LDH activity declined. Restoration of the circulatory estrogen level resulted in increased enzyme activity. The highest level of LDH was found in a band which consisted mainly of poorly differentiated cells. This cell subpopulation also tended to be more responsive to endocrine manipulation.

Mammary tumors induced by 7,12-dimethylbenz(a)anthracene contain extremely high lactate dehydrogenase (LDH) activity, specifically LDH isozyme 5, and ovariectomy of tumor-bearing rats causes tumor regression and a decrease in LDH isozyme[5]. The estrogen dependency of LDH has also been shown in the MCF-7 human breast cancer cell line[4]. Solid tumors such as breast neoplasms consist of multiple epithelial and stromal cell types[5]. The heterogeneity of the tumor cells within a given tumor makes it impossible to pinpoint the cellular source of LDH activity after tissue homogenization. A higher content of estrogen receptors in better differentiated cell subpopulations of the DMBA-induced mammary tumor has been reported[6]. Similarly, the existence within a single solid tumor of multiple

cell subpopulations, each with characteristic physical and molecular properties, has been demonstrated[5,7].

To ascertain the cellular source of LDH activity, density-defined cell subpopulations from DMBA-induced mammary tumors obtained after tissue dispersion and Ficoll gradient separation were used for enzyme determination and ultrastructural examination. The estrogen control of LDH was further tested by manipulation of the endocrine millieu by estrogen implants and other means.

Materials and methods. Mammary tumors were induced in 50-day-old female Sprague-Dawley rats by gastric intubation of DMBA[8]. Density-defined cell subpopulations were obtained after mechanical and enzymatic dispersion of solid tumor followed by isopycnic centrifugation on a Ficoll gradient[6]. 4–6 distinct cell bands were routinely obtained. Bands 1 and 2 (the lightest) contained few cells and large amounts of broken membranes; the lowest cell band consisted mainly of erythrocytes, fibroblasts and cellular debris, and was discarded. Lactate dehydrogenase was determined only on cells in bands 3, 4 and 5. Cells were suspended in 10 mM Tris HCl buffer, pH 7.4, and homogenized in a Polytron (Brinkman Instruments) with a 6–10 sec burst. The homogenate was then centrifuged at 40,000 × g for 45 min at 2 °C. The supernatant was used for LDH activity determination according to Balinsky et al.[9]. The reaction mixture for LDH assay contained 0.1 M Tris HCl buffer, pH 7.4, 2.9 mM pyruvate, and 0.15 mM NADH.

Small quantities of minced tumor tissue were implanted s.c. in the abdominal region of the rats to produce autotransplanted tumors for use in experiments on the estrogen control of LDH activity. When autotransplanted tumors reached a diameter of 1.5–2 cm, bilateral ovariectomy was performed. Tumor sizes were monitored and after a 50% decrease in size was observed, 1 tumor was removed for LDH determination. Subsequently the circulating estrogen level was restored through a subcutaneous implant of estrogen-containing Silastic tubing[10]. Tumor size continued to be monitored. When the remaining autotransplanted tumors in the same animals regrew to 100% of their original size, they were removed for enzyme determination. Analysis of variance was conducted by factorial design.

For ultrastructural analysis cells were collected in a 1.5 ml microcentrifuge tube, fixed, and processed for electron microscopy as described by Fink and Tseng[11]. Thin sections were cut on a MT-2 ultramicrotome, stained with uranyl acetate and lead citrate before being examined in a Philips 300 electron microscope.

Results. The distribution of the cells from a single DMBA-induced mammary tumor after velocity sedimentation on a continuous Ficoll gradient is presented in the table. Lactate dehydrogenase levels were different in these cell subpopulations (fig. 1). The highest LDH activity was found in band 4 which has an activity 5 times that of band 5 and about 12 times that of band 3. Ultrastructural observation of band 3 showed a relatively homogeneous population of well-differentiated epithelial cells (fig. 2). Bands 4 and 5 on the other hand contained mixed populations of well and poorly differentiated cells, as well as some fibroblasts (fig. 3).

After bilateral ovariectomy tumors regressed and the level of LDH activity in bands 3, 4 and 5 dropped respectively to 20%, 2.1% and 12% of original levels (fig. 1). Two ultrastructural changes predominated in these tumors: a generalized

The distribution of cells from a single DMBA-induced mammary tumor after velocity sedimentation

Cell Bands	Cell No./bands
B_1	2.8×10^6
B_2	10.5×10^6
B_3	19.0×10^6
B_4	17.0×10^6
B_5	16.0×10^6
B_6	9.0×10^6

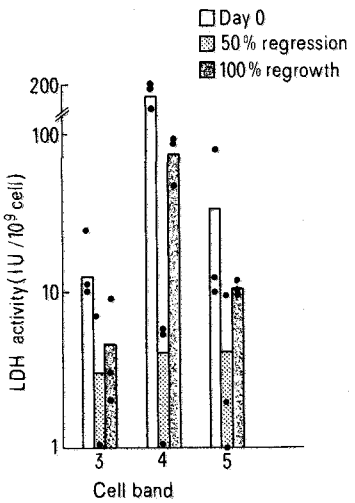

Figure 1. LDH activity in cells of bands 3, 4 and 5 of DMBA-induced mammary tumors: before ovariectomy (day 0), during tumor regression, and after regrowth. Data presented reflect results from 3 rats. Statistical analysis was conducted by analysis of variance factorial design ($p < 0.001$).

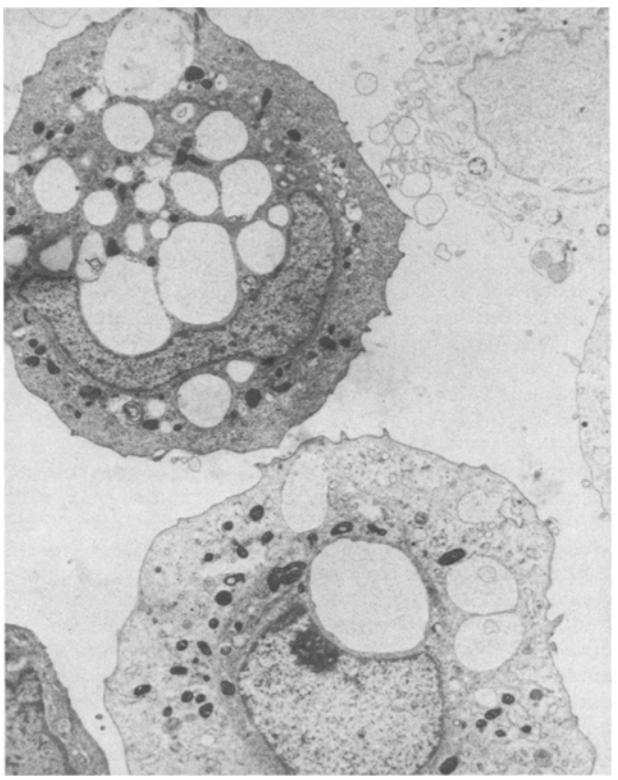

Figure 2. Cells typical of higher cell bands (i.e. bands 2, 3). The cytoplasm contain numerous large clear vacuoles. × 3900.

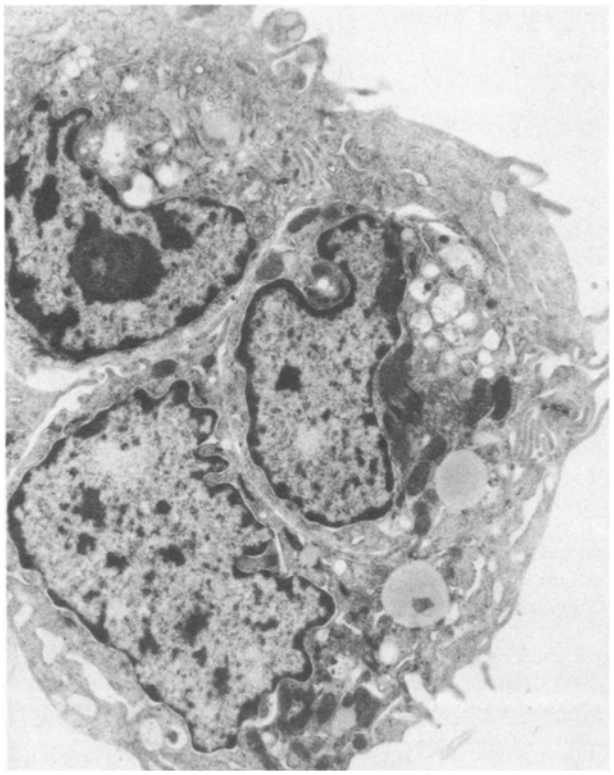

Figure 3. A cell aggregate in the lower cell bands (i.e. bands 4, 5). These cells have a higher nuclear-cytoplasmic ratio and sparse cytoplasmic organelles. × 6000.

cellular detachment and selective degeneration of more differentiated cells.

Restoration of the endocrine milieu by estrogen implant permitted tumors to regrow to a size equal to or greater than their original size. Histology of the restored tumors was indistinguishable from that of the original tumors. Concomitant with the tissue regrowth, LDH activity was also increased. LDH activities in cell bands 3, 4 and 5 were 30.6, 43.3 and 31.5%, respectively, of original levels (fig. 1).

Discussion. Our data demonstrated a highly significant ($p < 0.001$) difference in LDH activity among the different cell subpopulations from a single tumor before and after endocrine manipulations. Most of the enzyme activity resides in cells found in band 4. Whether elevated levels are due to increased synthesis rates or to decreased intracellular metabolism is not known. Using an identical approach, we reported earlier a relatively small amount of estrogen receptor in the same cell subpopulation[6]. This lack of positive correlation between estrogen receptor content and LDH activity nevertheless appears to be in agreement with findings in some human breast tumors[12].

Several studies have been conducted on the dehydrogenase enzymes, particularly glucose-6-phosphate dehydrogenase (G6PDH) in hormone-dependent mammary tumors because these enzymes may relate to tumor growth and regression[15,16]. It has been shown that administration of estradiol-17β to ovariectomized Fisher rats bearing R3230AC mammary adenocarcinoma results in 2-fold increase in G6PDH activity which is preceded by a 5-fold increase in synthesis of the enzyme[15]. In addition, the injection of dibutyryl-cAMP into rats bearing MTW9 mammary tumors resulted in early disappearance of microsomal G6PDH activity[16]. Prolonged treatment of rats bearing 5123 hepatoma with dibutyryl-cAMP significantly decreased G6PDH[16]. Such inhibitory effect of cAMP on LDH activity in DMBA-induced mammary tumors remains to be found. It has been suggested that the regulation of hormone-dependent tumor growth may depend on the antagonistic action between estrogen and cAMP[17].

The hormone dependence of LDH activity has invited speculation on the value of LDH as a marker for hormone-sensitive tumors[13,14]. Our findings on the estrogen dependency of LDH in the DMBA-induced tumor is consistent with those reported for the MCF-7 cell line[4]. The elevation of LDH activity after estrogen administration and the decrease in enzyme activity following ovariectomy support the earlier data of Lee et al.[3] and suggest the value of LDH as a marker protein for hormone-dependent mammary tumors.

This study supports the concept of inherent cellular heterogeneity in solid tumors and suggests that cells of different densities are physiologically and ultrastructurally distinct. Furthermore, the exquisite sensitivity to estrogen manipulation in band 4 cells suggests that this subpopulation may be a more precise indicator of hormone dependence.

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