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Lactic Dehydrogenase Isozymes During Development of Azo Dye Tumors. (29728)

EDWARD S. KLINE AND C. C. CLAYTON (Introduced by E. S. Higgins)
Department of Biochemistry, Medical College of Virginia, Richmond

According to current thinking the various molecular forms of lactic dehydrogenase (LDH) in most animal tissues are the result of random tetrameric associations of 2 sub-units, the synthesis of each sub-unit being under the control of a separate gene(1,2,3). In tissues one may expect to find up to 5 distinct forms (isozymes) of LDH(2,4). The relative distribution of the isozymes is tissue specific, and in various diseases changes in serum LDH reflect the isozymic pattern of the affected tissue(5,6,7). That the isozyme pattern is not "static" throughout the life of an organism is indicated by the fact that the distribution in fetal tissue is usually different from that of the adult tissue(4,8,9). In this connection Kaplan and his co-workers have advanced the thesis that the various isozymes, although having the same catalytic specificities, possess different physiological functions(8,10). Several lines of evidence have led these workers to postulate that LDH-1 (the isozyme with the greatest mobility toward the anode) more readily allows for the further aerobic oxidation of pyruvate than does LDH-5 (the isozyme with the least mobility toward the anode). On the other hand, LDH-5 seems to be more involved in anaerobic processes by more effectively catalyzing pyruvate reduction. Developmental changes as well as the observed tissue specificity of LDH isozymes tend to support this thesis.

In view of the above findings it was of interest to ascertain whether changes occurred in the distribution of LDH isozymes during the induction of liver tumors in the rat. For this purpose, the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) was employed. This compound, at the concentration fed, must be administered for a considerable period (about 60 days) before evidence of tumor appears (11,12,13,14).

Methods. Young adult male rats (Holtzman), weighing approximately 300 g, were fed the semi-synthetic diet employed by Clayton and Abbott(15) to which 0.064% 3′-Me-DAB was added. Control animals were fed the same diet without the dye. Both food and water were given *ad lib.* At stated time intervals animals were killed by decapitation and tissues were removed and rinsed. They were then cut into small pieces, thoroughly washed, homogenized, and centrifuged. The supernatants were used for the reported studies.

LDH was assayed by measuring the rate of NADH oxidation at 340 mμ in a Zeiss PMQII spectrophotometer. The reaction mixture of 3.0 ml contained the following: 200 μmoles of potassium phosphate buffer, pH 7.4, 0.26 μmoles of NADH, 0.96 μmoles of sodium pyruvate, and the tissue extract. Measurements were carried out at 30°.

Vertical starch gel electrophoresis was based on the procedure of Smithies(16). The gels were buffered at pH 8.6 according to the procedure of Boyer, Fainer and Naughton(17), and the resulting zymogram developed with the reaction mixture employed by Markert and Ursprung(18). For each electrophoresis experiment all extracts were diluted to contain the same total LDH activity and were developed under the same conditions.

Results and discussion. Table I shows the LDH activity in rat liver during the time course of tumor induction by 3′-Me-DAB.

TABLE I. Effect of Tumor Induction on Rat Liver Lactic Dehydrogenase.

Days after start of experiment	Activity (μmoles/mg wet wt/min) Basal diet	Basal diet plus dye* (a)	(b)	(c)
0	371(N)	—	—	—
4	354(N)	389(N)	—	—
13	455(N)	272(N)	—	—
21	288(N)	275(N)	—	—
33	363(N)	226(C)	—	—
42	262(N)	268(C)[1]	296(C)[1]	—
53	338(N)	197(C)[1] 199(C)	341(N)[2]	—
116	—	146(T)	302(N)[2]	290(T)† 76
131	297(N)	—	357(N)	238(T)

* Animals in Group (a) were maintained on basal diet plus dye for duration of experiment, those in Group (b) were placed on basal diet after 36 days, those in Group (c) were placed on basal diet after 57 days.

Gross examination of the livers revealed certain changes during the course of tumor induction. The letter (N) indicates normal appearing liver, (C) cirrhotic liver, and (T) tumor-containing liver. At 116 days numerous animals were sacrificed, all in Group (c) had tumors while none in Group (b) had tumors. The livers of most Group (b) animals appeared normal.

† Low value represents the activity of tumor tissue and high value shows activity in adjacent tissue of the same liver, without gross indication of tumor. For all other cases, representative areas of entire liver were assayed.

[1] Microscopic examination of sections from these livers showed the presence of varying degrees of bile duct proliferation, and in most cases, cirrhosis and other pathological changes. No tumors were found.

[2] Microscopic examination of sections from these livers showed them to be normal.

Although the normal range of activity from one animal to another was fairly great it seemed clear that there were no large changes in total activity during the induction period. Pre-tumor changes(14) did not drastically alter activities, although we observed that animals with severe cirrhosis appeared to have significantly reduced activities. In animals with tumors the activity was inversely related to the extent of the tumor in most of the animals studied.

The electrophoretic pattern of normal liver (fed "basal diet" or "rat chow") is shown in Fig. 1. The isozymes found in kidney are shown for comparison. These patterns are similar to those reported previously for such tissues(5,7,18). In addition to the strong LDH-5 band many of the zymograms exhibited a faint indication of a band in the LDH-4 position, the definition of which was not altered by changing the development time. There were no isozyme changes in normal livers during this study.

Fig. 2 shows the electrophoretic pattern of livers from rats fed the dye. Up to 21 days after the start of the experiment there were no changes in isozyme distribution. At 33 days a definite isozyme appeared in position 4. For the remainder of the study this band was found in the liver of all animals which were kept on the dye-containing diet (Group a). Occasional livers also contained a band in the LDH-3 position. Animals in Group (a) subsequently developed tumors. Some animals were removed from the carcinogenic diet after 36 days (Group b). Throughout this study none of these animals developed gross evidence of tumors. This is consistent with studies previously cited. Within 17 days after removal from the carcinogenic diet the liver pattern reverted to that found in normal animals in all but one of the numerous livers studied thus far. Other animals (Group c) were removed from the carcinogenic diet after 57 days. These animals developed liver tumors. In the few tumor-bearing animals studied, the liver isozyme pattern remained the same as in the animals kept on the dye. Tumor tissue in these livers appeared to have more of the isozyme in position 4 than did tissue which did not exhibit gross evidence of tumor. After removal of this group of animals from the carcinogenic diet the first electrophoretic study was made after tumors had already developed. Thus, it is not known whether the observed altered pattern prevailed during the period that the animals were fed the non-carcinogenic diet prior to the appearance of tumors.

In this study certain changes have been observed during the induction of tumors, which persist in animals that eventually develop liver tumors. We do not know whether the observed changes are intimately related to eventual formation of tumor or whether they are related to other changes caused by

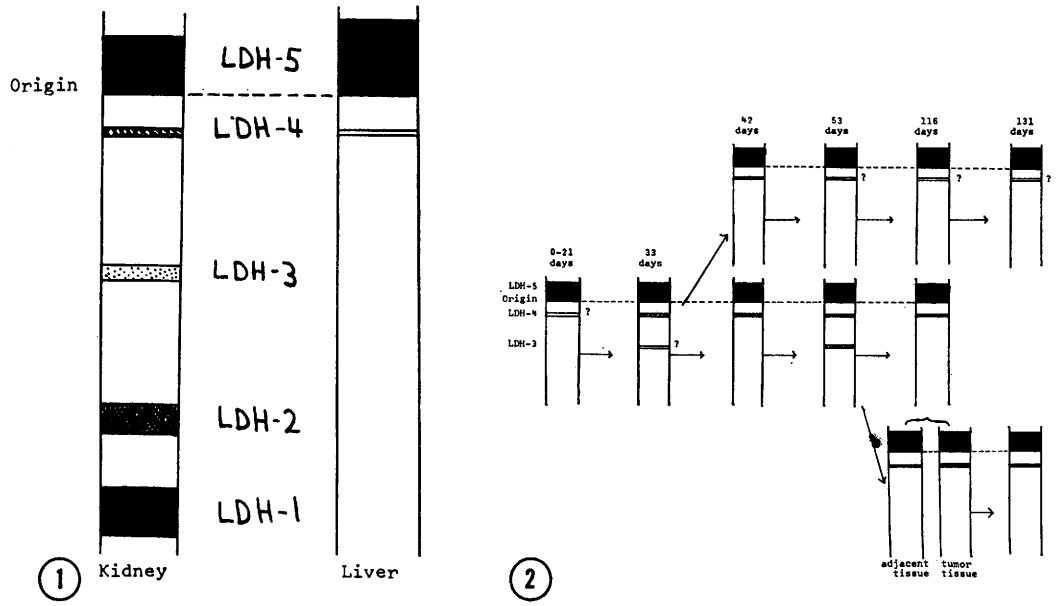

FIG. 1. Lactic dehydrogenase isozyme patterns of tissues from normal rats.

FIG. 2. Lactic dehydrogenase isozyme patterns of livers from rats fed 3′-Me-DAB. Stated times relate to "Days after start of experiment" as shown in Table I. Upper row of patterns are from Group (b) animals, middle row from Group (a) animals, bottom row from Group (c) animals. For details see Table I and *Methods*.

the dye. It is also possible that the altered nutritional status of the animals may have an effect, since the rats fed the dye do not eat as much or gain as much weight as do control animals. If the observed changes are related to tumor induction it would be of interest to relate such findings to the proposal of Kaplan's group that particular LDH isozymes are geared toward oxidative processes while others are better suited for anaerobic processes.

Summary. During the administration of the hepatocarcinogen 3′-methyl-4-dimethylaminobenzene changes occurred in the distribution of lactic dehydrogenase isozymes in rat liver. This new pattern persisted in those animals which were maintained on the carcinogenic diet until tumors developed. If animals were removed from the carcinogenic diet early enough to prevent the ultimate formation of tumors the altered pattern soon reverted to "normal" in most cases. However, if animals were removed from the carcinogenic diet too late to prevent the ultimate formation of tumors the altered pattern was still in evidence after these animals developed tumors.

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Metabolism of Glycerol by the Mammary Gland. (29729)

D. M. CARLSON, DIANE CRIDLER AND R. G. HANSEN
Department of Biochemistry, Michigan State University, East Lansing

Glycerol is known to undergo the following enzymatically catalyzed transformations: 1) phosphorylation by liver preparations to yield L-α-glycerophosphate(1) and 2) dehydrogenation to yield either glyceraldehyde(2, 3), or dihydroxyacetone(3,4).

When glycerol-1,3-C^{14} was injected *in vivo* unilaterally into the pubic artery of the bovine mammary gland, the galactose moiety of milk lactose from the injected side was labeled about 20 time greater than was the glucose moiety(5). Furthermore, 80% of the C^{14} of the galactose was distributed equally in C-4 and C-6 while the labeling of the glucose portion resembled that of blood glucose in that C-1, C-3, C-4, and C-6 had approximately equal activities. The distribution of label in glucose-6-phosphate, uridine diphosphate glucose and uridine diphosphate galactose isolated from the mammary gland tissue was similar to that of the galactose portion of lactose. A consideration of the isotope distribution in the hexose moieties of lactose after injection of labeled glycerol prompted this investigation of the metabolism of glycerol by mammary gland.

Methods. Rats that had been lactating 7-10 days were decapitated and the posterior mammary glands were excised and placed in ice. In most cases an acetone powder was prepared from the cold glands. The acetone powder was ground with 10 volumes of isotonic KCl in a pre-chilled mortar and then stirred for 30 minutes in an ice bath. The resulting extract was centrifuged at 5000 $\times$ *g* for 10 minutes and the precipitate discarded. The supernatant was used as the source of glycerokinase. When fresh tissue was used, it was homogenized in 2 volumes of isotonic KCl in a Waring blendor and centrifuged. The supernatant was filtered through cheesecloth to remove the fat layer and the filtrate used as the enzyme sources.

Results. For measuring the formation of L-α-glycerophosphate, the incubation conditions used were essentially those of Bublitz and Kennedy(1) with the following specific conditions: 25 μmoles KF, 25 μmoles glycerol, 1 μmole $MgCl_2$, 20 μmoles cysteine, 6 μmoles ATP, 61 μmoles Tris buffer, pH 7.4 and 0.2 ml enzyme (2.25 mg protein) in a total volume of 1 ml at 37°. Buffer was substituted for ATP in the control. After 30 minutes, the reaction was stopped by heating at 100° for 3 minutes and the coagulated protein removed by centrifugation.

L-α-glycerophosphate was measured in the incubation supernatant as follows: 0.25 μmoles DPN, 172 μmoles hydrazine buffer, pH 9.4, and 0.05 ml of supernatant were made to a volume of 0.49 ml. After an initial reading, 0.01 ml of L-α-glycerophosphate dehydrogenase was added and the optical density increase at 340 mμ measured. Both rat and bovine mammary gland extracts actively phosphorylated glycerol. Uridine triphosphate and ATP both serve as donors in the phosphorylation of glycerol by either tissue homogenates or acetone powders. This suggests direct phosphorylation by UTP or a phosphate transfer from UTP to endogenous ADP.

To identify the phosphorylated product glycerol-1,3-C^{14} was incubated with the en-