benzene. The $\beta$ + $\gamma$-zone and the $\delta$-zone were eluted separately and reapplied to 20 × 20 plates for 2-dimensional separation. The $\beta$ + $\gamma$ sample gave 2 distinct quenching spots with $R_f$'s similar to standard $\beta$- and $\gamma$-tocopherols. When eluted and determined colorimetrically, the $\gamma$-tocopherol corresponded to 56 $\mu$g/100 ml serum and the $\beta$-tocopherol to 10 $\mu$g/100 ml. Thus, the $\gamma$-tocopherol comprised about 85% and the $\beta$-tocopherol 15% of the reducing material in the combined $\beta$ + $\gamma$ zone from one-dimensional TLC.

The $\delta$-zone from the one-dimensional TLC when run in 2 dimensions produced no detectable spot where $\delta$-tocopherol should be, either when viewed under ultraviolet light or when sprayed with $FeCl_3$-bipyridyl. This confirmed the absence of $\delta$-tocopherol as noted above by GLC.

Forty sera from normal, adult employees of the National Institutes of Health were analyzed by the one-dimensional TLC method and also by the "direct" colorimetry procedure, following saponification, as reported in a previous study(2). The results (Table III) with TLC gave for $\alpha$-tocopherol 0.916 mg/100 ml serum and for $\beta$ + $\gamma$-tocopherols, 0.133 mg/100 ml. Thus, $\alpha$-tocopherol comprised about 88% of the total tocopherols. The total tocopherols by TLC were 10% lower than by the "direct" procedure. Examination of the $\alpha$-tocopherol values showed none below 0.5 mg/100 ml and 70% between 0.7-1.2 mg/100 ml. The values for $\alpha$-tocopherol are somewhat higher than those reported recently by Dayton *et al*(4) and by Herting and Drury(5), both using TLC methods.

TABLE III. Serum Tocopherols Determined by TLC or by Direct Colorimetry (40 Samples).*

| Determined by TLC | | | | |
|---|---|---|---|---|
| $\alpha$ | $\beta+\gamma$ | Total | $\alpha$ as % of total | Total direct |
| .916 ±.304 | .133 ±.075 | 1.049 ±.360 | 87.6 ±4.4 | 1.165 ±.351 |

* Values are means ± S.D., expressed as mg/100 ml serum.

*Summary.* Forty normal adult sera were analyzed by one-dimensional TLC. The averages in mg/100 ml were: $\alpha$-tocopherol, 0.916; combined $\beta$ + $\gamma$-tocopherols, 0.133. No $\delta$-tocopherol was found. The procedure was checked by comparing with GLC analyses and also with 2-dimensional TLC.

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## Lactic Dehydrogenase Isozymes in Rat Tissues, Tumors, and Precancerous Livers. (30589)

HERMAN L. JOHNSON AND RALPH F. KAMPSCHMIDT
*Biomedical Division, Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.*

Lactic dehydrogenase (LDH) activity has been extensively studied in both animal and human tumor bearers. Numerous investigators have reported elevations of enzymic activity in the plasma of animals bearing either primary or transplanted tumors, and recent evidence indicates that this increased activity can be at least partially attributable to a decreased clearance rate of the enzyme from the blood stream(1-4). Starch gel electrophoresis of LDH has revealed at least 5 isozymes, and these are thought to be tetramers of 2 subunits of the enzyme. Synthesis of each of the polypeptide subunits appears to be controlled by a different gene(5-8), and in different tissues each gene shows varying

degrees of activity so that each organ has a characteristic zymogram, *i.e.*, starch gel electrophoretic pattern(9,10). Kinetic studies indicated that isozymes, although having the same catalytic role, possess different physiological functions which are affected by the oxidative condition present(6,11). Various pathological conditions result in a release of LDH into the blood stream which will alter isozyme distribution in the plasma(12-14). These alterations could be used to determine which tissue was affected. Kline and Clayton (15) have reported that the liver zymogram was altered during the feeding of a carcinogenic azo dye.

These studies were initiated to determine if the plasma zymogram was affected by a growing tumor, if different transplantable rat tumors had similar LDH zymograms, and if primary azo dye hepatoma zymograms resembled those of the liver.

*Materials and methods.* Female Holtzman rats, weighing 160-180 g, were used as hosts for the transplanted tumors. The rats were maintained in a 73-75°F, 40-50% humidity environment of 12 hours of light and 12 hours of darkness. Water and Rockland Rat and Mouse Diet were provided *ad libitum*. The Walker 256 carcinoma, Jensen sarcoma, and MDAB hepatoma(16) were grown intramuscularly (IM) by injecting both *recti femora* with 0.2 ml of a 50% suspension of tumor cells. The suspension was prepared by excising an IM tumor, separating the viable tumor tissue, and forcing it through a 30 mesh stainless steel wire cloth in the barrel of a syringe. The cells were then suspended in an equal volume of saline containing 0.2% sodium penicillin G and 0.2% streptomycin sulfate.

For intraperitoneal (IP) injection of either 3'- or 4'-methyl-4-dimethylaminoazobenzene (-Me-DAB), a 2.5% suspension of the dye was prepared in olive oil, warmed at 37°C and 2 ml of the suspension or oil alone was injected per rat. The rats tolerated this dose fairly well and survived for 13-15 days. Intraperitoneal edema appeared at about 10 days and dye was still present in the cavity at 13 days.

Tissues were prepared for electrophoresis by homogenizing with an equal volume of 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol-0.1% disodium ethylenedinitrilotetraacetate buffer adjusted to pH 8.8 with HCl in a Potter-Elvehjem all glass homogenizer. After centrifuging 1 hour at 30,000 $\times$ *g*, a 0.05 ml aliquot was pipetted into the slot in the starch block. Blood was obtained by heart puncture with heparin as an anticoagulant, centrifuged, and 0.05 ml plasma was used for the zymogram.

Electrophoresis was run for 16 hours with 200 v, 30-40 Ma on the vertical starch block of Smithies(17). Starch was obtained commercially from Connaught Medical Research Laboratories and distributed by Fisher Scientific Co. The buffer was the same as that used to homogenize the tissue. Zymograms were stained by the method of Dewey and Conklin(18) as modified by Fine and Costello(19).

*Results.* Table I depicts the zymograms of various tissues, tumors and plasma from the host animal. The plasma LDH zymogram of the host is altered as the tumor becomes

TABLE I. Lactic Dehydrogenase Isozymes in Normal Tissues, Transplanted Tumors, and Host Plasma.

| Source | LDH isozymes* 5 | 4 | 3 | 2 | 1 |
|---|---|---|---|---|---|
| Muscle | +++ | ++ | ++ | + | ++ |
| Kidney | ++ | ++ | + | + | +++ |
| Spleen | +++ | +++ | ++ | + | + |
| Liver | +++ | ++ | (+) | | |
| Plasma | +++ | ++ | | | |
| Erythrocytes | ++ | | | | |
| Walker carcinoma | ++ | ++ | ++ | | + |
| plasma | +++ | ++ | ++ | | + |
| Jensen sarcoma | ++ | ++ | | | |
| plasma | +++ | +++ | | | |
| Novikoff hepatoma | +++ | ++ | ++ | ++ | ++ |
| plasma | +++ | ++ | ++ | + | + |
| MDAB hepatoma | +++ | ++ | ++ | + | + |
| plasma | +++ | ++ | ++ | ++ | ++ |

* LDH-1 → LDH-5. Isozymes number from the cathode toward the anode end of starch (LDH-1 migrates toward cathode and the others toward the anode). Number of crosses indicates intensity of the band when starch is stained.

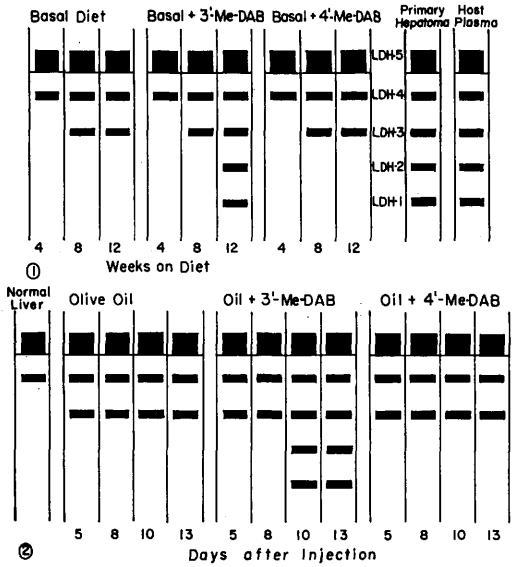


FIG. 1. Lactic dehydrogenase isozyme patterns of livers of rats fed azo dyes for varying times. Cathode is at top and origin is represented by line under LDH-5.

FIG. 2. Lactic dehydrogenase isozyme patterns of livers after intraperitoneal injections. Controls received 2 ml of olive oil and the treated rats received 50 mg of dye in same volume of oil.

larger (5-10% of the body weight) until it closely resembles that of the tumor. Different tumors have different isozyme patterns, and it is interesting that tumor patterns do not resemble those of the tissue of origin (*e.g.*, Jensen *vs* muscle; Novikoff and MDAB *vs* liver).

Since the tumor LDH zymogram was altered in comparison to the tissue of origin we decided to study the effect of feeding azo dyes upon the liver isozyme patterns. Fig. 1 shows alterations in the liver zymogram during the feeding period. After 8 weeks, as many as 3 isozymes were observed in the livers of basal diet-fed rats. The 3 bands were consistently detectable in the livers of rats receiving either 3'- or 4'-Me-DAB diets for 8 weeks. Only 3'-Me-DAB diets produced rat livers with 5 distinct bands of LDH activity after 12 weeks of feeding, and these zymograms corresponded to those of induced hepatomas which had grown for 2 to 3 months after the 90-day dye feeding period. The hosts' plasma zymograms also displayed all 5 isozymes.

Certain alterations which require weeks of feeding have been observed within a few days after dye injections(20), so liver LDH zymograms were made after injection of 3'- and 4'-Me-DAB and of olive oil. Fig. 2 shows 3 bands of activity were present 5 days after injection of either dye or oil alone. After 3'-Me-DAB injections, bands 1 and 2 appear on the 10th day, and this is the typical pattern which we obtained from the primary tumors induced by feeding this dye. The weaker carcinogen, 4'-Me-DAB, which is more toxic, did not cause the liver to display more than 3 bands of activity during the 13-day period.

*Discussion.* As enzymatic alterations occur in tumors, the changes are generally convergent so that various tumors resemble each other more than they resemble other tissues (21). These changes appear to give the malignant cell a competitive advantage in the struggle with the host cell as emphasized by Weber(22) and Shrivastava and Quastel (23). It was anticipated that the LDH isozyme patterns of the tumors would either resemble each other or each would retain the pattern of the parent tissue; therefore, it was interesting to observe that each tumor had its own characterisic LDH zymogram.

All variations in the isozymes have occurred in bands 1, 2, and 3, so if the current theory that synthesis of the 2 subunits of the LDH molecule is controlled by 2 different genes is correct, then only 1 gene is affected. In hepatomas, the gene's action is stimulated so that isozymes 1, 2, and 3 are formed; in the sarcoma it is inhibited. Van Wijhe *et al* (24) have reported that human white muscle fibers contained almost exclusively isozymes 4 and 5, but red fibers contained all 5 and were richest in 1, 2, and 3. If this can be applied to the rat, then the zymogram of the Jensen sarcoma would be identical to that of the white fibers suggesting that these fibers were the tissue of origin.

Kaplan(6,11) has proposed that LDH-5 is involved in anaerobic reduction of pyruvate, although LDH-1 is more active under conditions of high oxygen tension, and that the subunits of the LDH molecule are synthesized accordingly. Since the oxygen supply to tumors is generally more limited than it

is to other tissues, the synthesis of the subunits of LDH-1 should be depressed in the tumor. It appears, therefore, as if oxygen tension does not exert a significant effect upon this gene's action in the primary tumor because LDH-1 and -2 are present in the tumor but not detectable in normal liver.

Dietary 3'-Me-DAB produces tumors of both biliary and parenchymal cell origin. Most of the tumors studied had all 5 bands present; however, occasionally tumors occurred with only isozymes 3, 4, and 5. This could indicate that the tumors originated from different cell types, that the dye caused a stepwise alteration, or that it affected different cells differently. The 5 isozymes could be detected in the livers of dye-fed animals before any tumors were apparent. Kline and Clayton(15) reported that in normal liver, LDH-5 was very strong and that a questionable band of activity was detected at the LDH-4 position. In these studies, both LDH-5 and LDH-4 were clearly seen in normal livers and LDH-3 could be detected after about 2 months of feeding the basal diet.

The differences in our observations regarding the presence of LDH-4 in the liver may be the result of different preparation methods. Recently, Vesell and Brody(25) showed that alterations in zymograms of a tissue can occur through differences in preparing the tissue homogenates. Our preparations were quite concentrated and Kline and Clayton(15) diluted theirs. The appearance of LDH-3 in our animals appears to be associated with the diet or with the age of the animal, since it was not observed until after 2 months of feeding. Other factors which differed in our experiments are size, age and sex of the rat employed. The detection of isozymes 1 and 2 in the precancerous liver after 12 weeks of feeding the dye and in the primary hepatoma probably requires a relatively concentrated homogenate, since these bands of activity are considerably less distinct than those of isozymes 4 and 5. We feel, however, that these bands may be more important than the others since the others can be observed after feeding the basal diet for 8 weeks or shortly after injecting 2 ml of olive oil into the intraperitoneal cavity of a rat.

Some effects of 3'-Me-DAB can be greatly accelerated by IP injections(20). All 5 isozymes appeared after more than 8 weeks of dye feeding, but one 50 mg injection of dye resulted in 5-band liver zymograms in 10 days. An IP injection of olive oil resulted in the appearance of isozyme 3 so that this may be the effect of a sterile injection. We have often observed the presence of the 3rd isozyme in normal rat livers, indicating its presence in so low a concentration that it is difficult to observe routinely. The 4'-Me-DAB isomer was used as a control to see if appearance of additional bands could be attributed to a general toxicity syndrome of azo dyes. Since only 3 isozymes were observed with 4'-Me-DAB through 13 days after injection, it appears that the synthesis of the other 2 isozymes was specifically stimulated by 3'-Me-DAB.

The similarity of the host's plasma zymogram to that of its tumor indicates that substantial amounts of the enzyme must be released from the tumor into the blood stream, and this may contribute significantly to the elevation of plasma activity. Burgess and Sylvén(26) came to this same conclusion in their studies on tumor-bearing mice.

The differences in the zymograms of the tumor LDH activity appear to indicate that the isozyme patterns of this enzyme are not intimately related to the malignant process. Although the isozyme pattern of the tumor appears to be altered in comparison to the tumor's tissue of origin, this alteration may be either an addition or deletion of certain isozymes.

*Summary.* Starch gel electrophoretic patterns of LDH of different transplantable tumors revealed that each type of tumor had a characteristic zymogram. Plasma zymograms from the hosts closely resembled those of their respective tumors, indicating that tumor LDH was released into plasma and contributed significantly to the elevated level found there. Feeding 3'-Me-DAB resulted in an altered liver LDH isozyme pattern after 12 weeks, and the altered pattern was similar to that found in primary tumors induced by this dye. Intraperitoneal injections of 3'- and 4'-Me-DAB indicated that the alteration

of isozyme patterns was specific for the 3'-isomer.

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## Monoamine Oxidase Inhibition by a New Carcinostatic Agent, N-Isopropyl-α-(2-Methylhydrazino)-p-Toluamide (MIH). (30590)

VINCENT T. DE VITA, MARY ANNE HAHN AND VINCENT T. OLIVERIO
(Introduced by David P. Rall)
*Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Md.*

In 1963, a new class of carcinostatic agents was introduced by Bollag and Grunberg(1). One compound in this class, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (MIH; RO-4-6467/1, NSC-77213), has been subjected to extensive clinical trials in Europe and has been shown to possess considerable activity against Hodgkin's disease (2,3). In addition to an overall 60% response rate, including 30% complete remissions, there appears to be no cross resistance between this drug and others used in the treatment of this disease. The major toxicity, like most other antitumor agents, has been hematopoietic. In addition, troublesome central nervous system toxicity in the form of hyperirritability, euphoria, ataxia and nystagmus has been noted, and the drug also potentiates the effects of chloropromazine derivatives, barbiturates and alcohol.

Because of its hydrazine structure and the presence of the above side effects, we considered the possibility that some of these symptoms might be due to inhibition of the enzyme, monoamine oxidase (MAO). The latter was determined by measurement of the