Purification and Comparative Properties of Human Lactate Dehydrogenase Isozymes from Uterus, Uterine Myoma, and Cervical Cancer*

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ABSTRACT: Five isozymes of human lactate dehydrogenase (LDH) (I–V designated in order of increasing anodic mobility) have been found in differing proportions in normal uterus, its myoma, and cervical cancer, and a definite and consistent shift to LDH-I in the isozyme patterns has been found in uterine neoplasms. These isozymes have been isolated and purified from both normal uterus and its myoma, respectively, by fractionation of crude water extracts with alcohol, ammonium sulfate, and calcium phosphate gel followed by chromatography on DEAE-cellulose and hydroxylapatite. Four isozymes have been partially purified by fractionation of a water extract of cervical cancer with ammonium sulfate and by DEAE-cellulose chromatography. Electrophoretic and ultracentrifugal analyses of the highly purified isozymes showed them to be essentially homogeneous. The molecular weights of the major isozyme (LDH-III) of both normal uterus and its myoma, calculated from sedimentation and diffusion coefficients, are 152,000 and 143,000, respectively. The total numbers of SH groups of both purified isozymes were calculated to be 12 and 11 moles per mole of isozyme, respectively. Five isozymes of human uterine neoplastic lactate dehydrogenase exhibited higher heat stability than those of normal uterine isozymes. The rates of reduction of oxidized nicotinamide–adenine dinucleotide (NAD) analogs by the isozymes from cervical cancer were generally higher than those by the isozymes from normal uterus and its myoma. The inhibitory effects of oxalate and α-ketoadipate were larger upon the isozymes from cervical cancer than upon normal uterus and its myoma, and also LDH-V was more strongly inhibited than LDH-I. The mode of inhibition by α-ketoadipate was found to be competitive with lactate and to be noncompetitive with pyruvate. The isozymes from uterine neoplasms were strongly and competitively inhibited by pyridine-3-aldehyde–adenine dinucleotide and its K_i value was calculated to be 10^{-7} M, compared with K_m value of 10^{-4} M for NAD.

It is needless to say that human uterus plays an important role in reproduction through the course of pregnancy and labor, and the contraction of uterus muscle is one of the main sources of power in the course of labor. However, it is very important to know which metabolic processes supply the energy required for its contraction. For this reason we have been investigating the distribution of enzyme activities associated with energy metabolism in various states of human uterus including its neoplasms and pregnant tissue (Okabe *et al.*, 1965, 1966). It was concluded that energy production in uterine tissue is not as dependent on glycolysis as in skeletal muscle. There was, however, no significant difference of these activities between normal uterus and its neoplasms. These findings prompted us to investigate the molecular forms (isozymes) of human uterine lactate dehydrogenase.

Five isozymes of human lactate dehydrogenase have been found in differing proportions in normal uterus, its myoma, and cervical cancer. It was also found that normal uterus contained predominantly a hybrid-type isozyme, whereas the uterine neoplasms showed an apparent shift toward the muscle-type isozyme with no increase of the total activity. This paper describes improved isolation and purification procedures for each isozyme of lactate dehydrogenase[1] (I–V designated in order of increasing anodic mobility) from normal human uterus, its myoma, and cervical cancer, and some physicochemical properties of these isozymes. The comparative properties in the physical and kinetic studies of the

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[1] Abbreviations used: LDH, lactic dehydrogenase; α-NAD, oxidized α-nicotinamide–adenine dinucleotide; NADH, reduced NAD; NADP, nicotinamide–adenine dinucleotide phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-hydroxymercuribenzoic acid; TNAD, thionicotinamide–adenine dinucleotide; NHXD, nicotinamide–hypoxanthine dinucleotide; 3-AcPyAD, 3-acetylpyridine–adenine dinucleotide; 3-PyAlAD, pyridine-3-aldehyde–adenine dinucleotide; 3-AcPyHXD, 3-acetylpyridine–hypoxanthine dinucleotide.

isozymes from the normal uterus and its neoplasms are also described.

Materials and Methods

Materials. NAD, NADH, α-NAD, NADP, pyruvic acid, 2-oxoglutaric acid, α-ketobutyric acid, α-hydroxybutyric acid, crystalline bovine serum albumin, phenazine methosulfate, Tris, and PMB were obtained from Sigma Chemical Company. Potassium pyruvate was prepared by the method of Korkes *et al.* (1951). Cyanogum 41 gel was obtained from Seikagaku Kogyo. Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956). DEAE-cellulose and CM-cellulose were purchased from Brown Co. Synthetic 5,5′-dithiobis-(2-nitrobenzoic acid) was a generous gift of Dr. L. J. Reed. D-Lactic acid was generously supplied by Kyowa Hakko Kogyo, Tokyo. Synthetic α-ketoadipic acid was kindly furnished by Dr. Y. Nishizuka. Oxamate was prepared by the method of Sah and Chien (1931). Normal human uterus, uterine myoma, and cervical cancer tissue were obtained at operation, and samples were subjected to assay for various enzyme activities (Okabe *et al.*, 1965). The remainder was frozen at −20° until use.

Procedures. Protein chromatography on columns of DEAE- and CM-cellulose and hydroxylapatite was carried out as described by Peterson and Sober (1962) and Tiselius *et al.* (1956), respectively. Commercial DEAE-cellulose was pretreated by the method described by Peterson and Sober (1962). After equilibration with starting buffer the cellulose was transferred to the container for storage. The ratio of enzyme protein to DEAE-cellulose was 1:100. The cellulose was packed into a column (4 × 45 cm) by the application of air pressure of 4–5 lb/cm². Zone electrophoresis on 5% acrylamide gel (Raymond and Wang, 1960) was carried out in a horizontal electrophoresis cell devised in this laboratory, giving a gel 15 × 11 × 0.3 cm divided into four strips. The gel was made up in 0.05 M barbital buffer (pH 8.6) and then soaked in 0.05 M barbital buffer (pH 8.6) for at least 24 hr before use. Sample solutions (0.03 ml), containing enzyme activity optimal for gel analysis (2–3 μmoles of NADH oxidized/min per ml of enzyme solution), were introduced into the sample slots by means of Pedersen pipets. Electrophoresis was conducted at 4° for 8 hr with a voltage of 360 and a current of 20 ma. After the separation, enzyme activity was located by a modification of the method of Fine and Costello (1963). The gel strip was incubated for 1 hr at 38° in a solution containing 5×10^{-2} M DL-lactate, 2.8×10^{-4} M NAD, 0.02% Nitro Blue tetrazolium chloride, 0.005% phenazine methosulfate, and 0.05% sodium cyanide. Quantitative determination of each isozyme of lactate dehydrogenase was performed by the following procedure. Equal amounts of the same sample were run on adjacent gel strips. The unstained strip of gel was compared with the stained strip, and appropriate segments were taken. These segments were added, together with 1 ml of 0.2 M phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin, to a small hand-operated homogenizer equipped with a Teflon pestle. After standing for 2 hr in an ice bath, this suspension was poured in a medium grain, sintered-glass filter and was filtered by application of gentle suction. Recovery of the total activity applied to the gel was found to be over 80%, as measured by the NADH–pyruvate assay system. Protein was located by staining with bromophenol blue as described by Durrum (1950). Protein was estimated by the phenol method of Lowry *et al.* (1951) or by the biuret method as described by Layne (1957). Chloride ion concentration was measured with a Beckman Model 76 pH meter equipped with a chloride ion–silver electrode. Sulfhydryl content was determined by the method of Ellman (1959). The calcium phosphate gel was prepared by the method of Keilin and Hartree (1938).

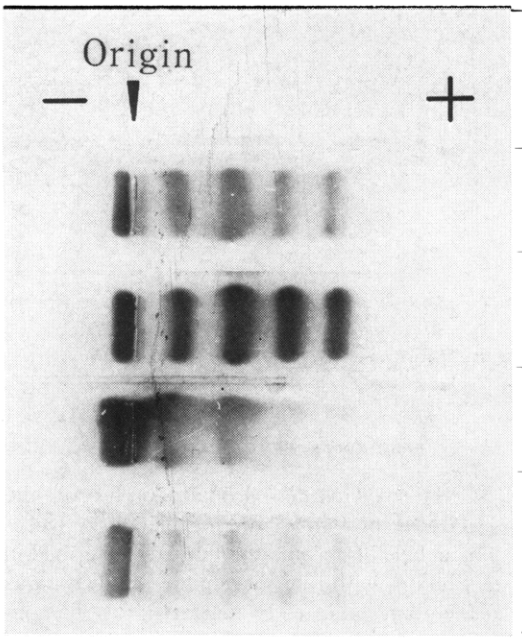
Enzyme Assays. The reaction with pyruvate and NADH was measured, according to the method of Kornberg (1955), with 0.2 μmole of NADH and 2 μmoles of potassium pyruvate in 2 ml of 0.05 M phosphate buffer (pH 7.4). The reaction was initiated by addition of enough enzyme solution to give a decrease in optical density of 0.03–0.1/min at 340 mμ. The change in absorbance was followed with a Beckman Model DB spectrophotometer and a Sargent SRL recorder, or with a Shimadzu Model QR-50 spectrophotometer and a Shimadzu Model ARP recorder. The reverse reaction was measured according to the method of Neiland (1955) with 0.2 μmole of NAD or its analogs and 100 μmoles of DL-lactate in 2 ml of 0.1 M Tris-HCl buffer (pH 9.2). The reaction was started by addition of enough enzyme solution to give an increase in optical density of 0.02–0.06/min at the appropriate wavelength for each nucleotide. To prepare a low concentration of enzyme solution (1 μg or below), stock solution was diluted with 0.1 M phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin. Specific activity of lactate dehydrogenase is expressed as micromoles of NADH oxidized or formed per minute per milligram of enzyme protein.

Experimental Results

Electrophoretic Patterns of the Isozymes of Human Lactate Dehydrogenases in Normal Uterus and Its Neoplasms. As indicated in Figure 1, five isozymes of human lactate dehydrogenase from uterus and its neoplasms have been found in differing proportions. It can be seen that normal uterus contains a high level of hybrid-type lactate dehydrogenases (LDH-III and -IV), whereas uterine neoplasms showed a consistent shift to M-type lactate dehydrogenase (LDH-I). There was a significantly higher percentage of LDH-I in cervical cancer and myosarcoma with no increase of the total activity.

Purification of Human Lactate Dehydrogenase Isozymes from Normal Uterus. All operations were carried out at 0–5°, and the solutions were made up with deionized water. All of the phosphate buffers used for chromatography contained 1 mM EDTA.

STEP 1. EXTRACTION. About 1.7 kg of frozen normal



Uterine Tissues	Percentage of Isozymes I	II	III	IV	V
Normal	4	15	45	30	6
Leiomyoma	13	32	35	16	4
Leiomyosarcoma	38	34	20	5	4
Cervical cancer	42	29	17	9	3

FIGURE 1: Electrophoretic patterns and percentages of the isozymes of human lactate dehydrogenase in water extracts of normal uterus and its neoplasms. Electrophoretic procedures and the quantitative determination of each isozyme are described in the text.

human uterus was diced and ground in a mechanical meat grinder. The frozen mince was again diced and homogenized for 2 min in a Waring Blendor with 5.1 l. of ice-cold, deionized water. The homogenate was frozen and thawed once and then centrifuged at 13,000*g* for 30 min. No significant amount of activity was found in the pellet. The clear supernatant fluid was filtered through cotton to remove fat.

STEP 2. ETHANOL FRACTIONATION. The extract was cooled to 0°, and to each 300 ml were added 1.5 ml of 0.8 M acetate buffer (pH 4.0) and 150 ml of 95% ethanol (precooled below −20°). The ethanol was gradually added with efficient stirring and the temperature of the solution was not allowed to rise above −3° after the first addition of ethanol. After centrifugation at 13,000*g* for 5 min, as much as possible of the supernatant liquid was removed. The precipitate was suspended in 50 ml of 0.1 M phosphate buffer (pH 7.0), and the mixture was homogenized with a Teflon pestle. The insoluble material was removed by centrifugation at 13,000*g* for 15 min. It was found impracticable to attempt fractionation of a large volume (more than 300 ml) of the extract in one operation. Therefore, a maximum of 300 ml of the extract was taken, and the fractions from nine such lots were pooled before proceeding to the next step.

STEP 3. FIRST AMMONIUM SULFATE FRACTIONATION. About 680 ml of the supernatant solution from step 2 was brought to 0.65 saturation with solid ammonium sulfate. After 15-min of stirring, the precipitate was collected by centrifugation at 15,000*g* for 20 min. The loosely packed precipitate was dissolved in 150 ml of 0.1 M phosphate buffer (pH 7.0), and the solution was dialyzed overnight against 0.005 M phosphate buffer (pH 7.0).

STEP 4. ADSORPTION ON CALCIUM PHOSPHATE GEL. The enzyme solution from step 3, containing 19 g of protein, was mixed with centrifuged calcium phosphate gel (47.5 g dry wt). The mixture was stirred for 1 hr at 0° and then centrifuged at 6000*g* for 10 min. The loosely packed calcium phosphate gel was eluted three times with a total volume of about 1550 ml of 0.2 M phosphate buffer (pH 7.2).

STEP 5. SECOND AMMONIUM SULFATE FRACTIONATION. The combined eluates from step 4 were brought to 0.40 saturation with solid ammonium sulfate, and the mixture was centrifuged at 13,000*g* for 20 min. To the clear supernatant fluid, solid ammonium sulfate was added with slow stirring to give 0.60 saturation. After 2-hr stirring at 0°, the precipitate was collected by centrifugation as before. The loosely packed reddish precipitate was suspended in a small volume of 0.1 M phosphate buffer (pH 7.0). The suspension was dialyzed overnight against 0.001 M phosphate buffer (pH 7.0), and insoluble material was removed by centrifugation.

STEP 6. FIRST DEAE-CELLULOSE COLUMN CHROMATOGRAPHY. The dialyzed solution, containing 2 g of protein, was introduced onto a DEAE-cellulose column (4.0 × 45 cm), which had been equilibrated with 0.001 M phosphate buffer (pH 7.0). The column was washed with 0.001 M phosphate buffer (pH 7.0), to elute LDH-I, and then with 0.005 M phosphate buffer (pH 7.0). The other isozymes were eluted with the usual concave-downward gradient. The 2-l. mixing chamber contained

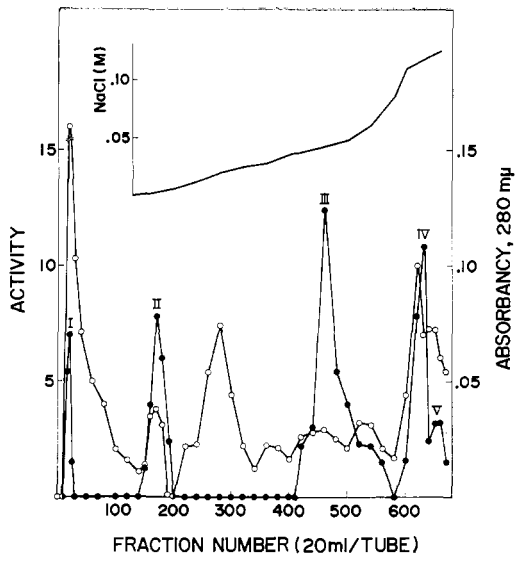


FIGURE 2: DEAE-cellulose chromatography of isozymes of normal human uterine lactate dehydrogenase. The details of chromatography are described in the text. Protein concentration is represented by open circles. Activity (solid circles) is in terms of μmoles of NADH formed/min per 20 ml of the eluate. NaCl gradient (moles) is represented by the solid line.

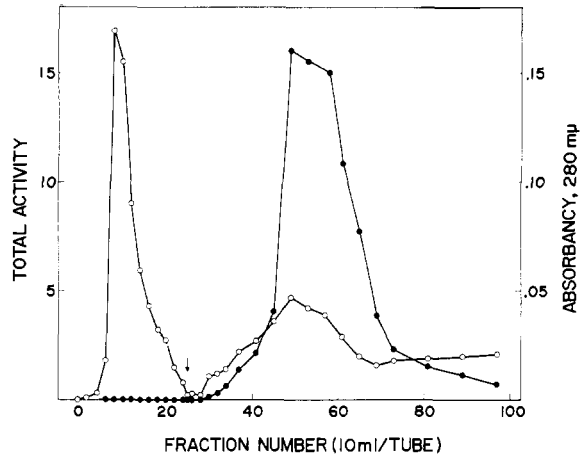


FIGURE 3: Hydroxylapatite chromatography of the isozyme LDH-III of human uterine lactate dehydrogenase. The details of chromatography are described in the text. Elution with the phosphate gradient was started at the point indicated by an arrow. Protein concentration is represented by open circles. Activity (solid circles) is in terms of μmoles of NADH formed/min per 10 ml of the eluate.

initially 0.01 M phosphate buffer (pH 7.0). The reservoir contained initially 2 l. of 0.01 M sodium chloride in 0.01 M phosphate buffer (pH 7.0). Portions (2 l.) of 0.05, 0.08, 0.1, and 0.15 M sodium chloride in 0.01 M phosphate buffer (pH 7.0) were added successively to the reservoir. A typical elution pattern is shown in Figure 2. The activity peaks designated I–V correspond to the isozymes detected by electrophoresis on acrylamide gel (*cf.* Figure 1). The fractions corresponding to each isozyme were combined and stirred with centrifuged calcium phosphate gel (10 mg dry wt/mg of protein) for 2 hr. The gel was collected by centrifugation and was eluted three times with a minimum volume of 0.5 M phosphate buffer (pH 7.0). The eluates were combined and dialyzed overnight against 0.001 M phosphate buffer (pH 7.0). Insoluble material was removed by centrifugation.

STEP 7. SECOND DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF LDH-III FRACTION. About 20 ml of concentrated LDH-III fraction was rechromatographed on a DEAE-cellulose column (2.0 × 30 cm) by the procedure described above. This step leads to some further purification.

STEP 8. HYDROXYLAPATITE COLUMN CHROMATOGRAPHY. About 6 ml of concentrated LDH-III fraction from step 7 was introduced onto a hydroxylapatite column (1.5 × 10 cm), which had been equilibrated with 0.001 M phosphate buffer (pH 6.8). The column was washed with 0.06 M phosphate buffer (pH 6.8) until about two-thirds of the total protein was eluted. Active material was then eluted with the usual concave-downward gradient employing a 200-ml mixing chamber containing 0.06 M phosphate buffer (pH 6.8). The reservoir contained 0.08 M phosphate buffer (pH 6.8). A typical elution pattern is shown in Figure 3. The most active fractions were mixed with a small amount of calcium phosphate gel and dialyzed against 0.001 M phosphate buffer (pH 7.0). After complete adsorption of activity the gel was eluted with an appropriate volume of 0.5 M phosphate buffer (pH 7.0). The eluate was brought to 0.90 saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at 151,000*g* for 1 hr in a Beckman Model L-2 ultracentrifuge and was dissolved in a minimum volume of 0.1 M phosphate buffer (pH 7.0). A summary of the purification is presented in Table I. A 480-fold purification of LDH-III isozyme was achieved.

Purification of Human Lactate Dehydrogenase Isozymes from Uterine Myoma. About 1.8 kg of frozen human uterine myoma was diced and ground in a meat grinder at 4°. The myoma mince was stored at −20° and then homogenized with 5.4 l. of ice-cold, deionized water. The homogenate was frozen and thawed once, and centrifuged at 13,000*g* for 30 min. The clear supernatant fluid was filtered through cotton to remove fat.

Further purification of the crude extract was accomplished by the same procedure used for the isolation of five isozymes of human lactate dehydrogenase from normal uterus. About 25 ml of the concentrated LDH-III fraction was further purified by chromatography on a hydroxylapatite column (3.5 × 5 cm) as described above. About 420-fold purification of LDH-III isozyme from the water extract was achieved.

Purification of Human Lactate Dehydrogenase Isozymes from Cervical Cancer. Zone electrophoresis of the water extract of human cervical cancer tissue on

TABLE I: Summary of Purification of the Isozymes of Human Lactate Dehydrogenase from Normal Uterus.

Purification Step	Vol (ml)	Total Protein (g)	Sp Act. (μmoles of NADH formed/ min per mg)	Total Act. (μmoles of NADH/min)	Recov (%)
1. Water extract[a]	5260	68.60	0.16	10,980	100
2. Alcohol precipitate	680	24.02	0.38	9,180	83.7
3. First ammonium sulfate, 0–0.65 satd	480	19.01	0.49	9,320	85.0
4. Calcium phosphate gel eluate	1550	12.54	0.71	8,910	81.2
5. Second ammonium sulfate, 0.4–0.6 satd	88	4.59	1.53	7,030	64.0
6. First DEAE-cellulose				3,352	30.3
Isozyme I	16	0.091	2.52	229	
Isozyme II	15	0.090	7.60	680	
Isozyme III	20	0.134	14.10	1,890	
Isozyme IV	12	0.086	5.82	498	
Isozyme V	10	0.020	2.80	55	
7. Second DEAE-cellulose Isozyme III[b]	6	0.047	26.40	1,241	11.3
8. Hydroxylapatite Isozyme III	1.2	0.009	76.70	700	6.4

[a] This extract was derived from 1.7 kg (wet wt) of normal human uterus. [b] Derived from step 6, first DEAE-cellulose, isozyme III fraction.

TABLE II: Summary of Purification of the Isozymes of Human Lactate Dehydrogenase from Cervical Cancer.

Purification Step	Vol (ml)	Total Protein (mg)	Sp Act. (μmoles of NADH/min per mg)	Total Act. (μmoles of NADH/min)	Recov (%)
1. Water extract[a]	11	156	0.30	46.65	100
2. Ammonium sulfate, 0.35–0.65 satd	1.5	29	1.39	40.03	86
3. DEAE-cellulose		5		29.81	64
Isozyme I	2.5	1.35	3.82	5.16	
Isozyme II	2.1	0.20	10.32	2.06	
Isozyme III	2.5	0.65	8.43	5.48	
Isozyme IV	2.1	0.11	1.19	0.13	

[a] This extract was derived from 4.5 g (wet wt) of cervical cancer tissue.

acrylamide gel showed five isozymes of lactate dehydrogenase. About 4.5 g of combined tissue was homogenized with three volumes of ice-cold water in a Polytron 20ST homogenizer (Kinematica G.m.b.H., Switzerland) for 2 min. The mixture was centrifuged at 16,000*g* for 30 min, and a fraction which precipitated between 0.35 and 0.65 ammonium sulfate saturation was obtained from the supernatant fluid. The precipitate was dissolved in a minimum volume of 0.1 M phosphate buffer (pH 7.0) and dialyzed against 0.001 M phosphate buffer (pH 7.0). The dialyzed solution was centrifuged and the clear supernatant fluid was introduced onto a DEAE-cellulose column (2.0 × 17 cm) which had been equilibrated with 0.001 M phosphate buffer (pH 7.0). The column was washed with 0.001 M phosphate buffer (pH 7.0) and eluted with the usual concave downward gradient employing a 500-ml mixing chamber containing 0.001 M phosphate buffer (pH 7.0). The reservoir

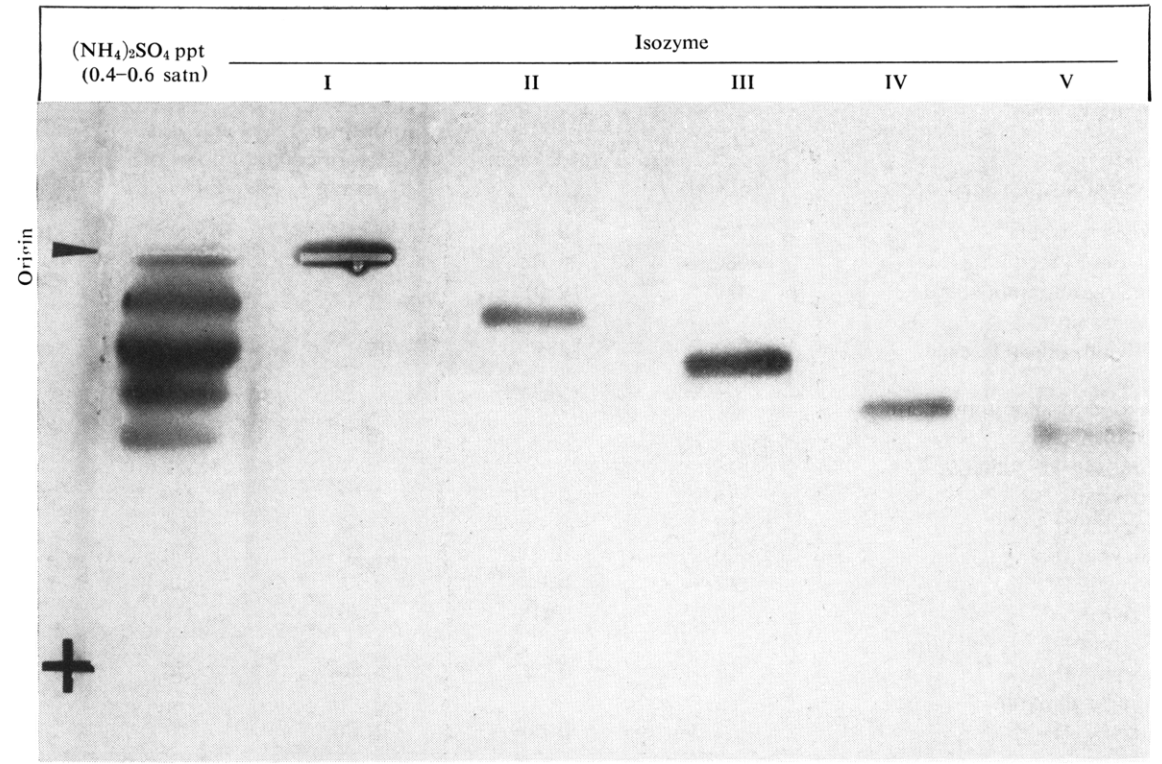


FIGURE 4: Electrophoretic patterns of the five purified isozymes of human lactate dehydrogenase from normal human uterus. The 0.4–0.6 saturated ammonium sulfate fraction (step 5, Table I) was fractionated on DEAE-cellulose (step 6, Table I).

contained 0.1 M sodium chloride in 0.01 M phosphate buffer (pH 7.0). Fractions of each active peak were pooled, concentrated with calcium phosphate gel, and then precipitated with ammonium sulfate as described above. A summary of the purification is given in Table II.

Physicochemical Properties of Purified Isozymes

Electrophoresis. Each of the five purified isozymes of human lactate dehydrogenase from normal uterus showed a single band of enzymatic activity after electrophoresis on acrylamide gel (Figure 4). Highly purified LDH-III from both normal uterus and its myoma were homogeneous with respect to both protein and enzymatic activity.

Ultracentrifugal Analysis and Molecular Weight. Sedimentation velocity experiments were performed at 2° in a Beckman Model E ultracentrifuge equipped with a phase plate and an RTIC temperature unit. The movement of the boundaries was traced on graph paper, with the use of an Omega D_3 type photographic enlarger, and measured. Sedimentation coefficients were calculated and corrected as described by Schachman (1957). The sedimentation patterns obtained with the highly purified preparations of LDH-III from normal uterus and its myoma showed a single component (Figure 5). The values extrapolated to infinite dilution ($s^0_{20,w}$) of these enzymes are 7.56 and 7.78 S, respectively.

Diffusion study was performed in a synthetic boundary cell at 10,573 rpm in an An-J rotor. Temperature was controlled at 6°, and the measurements were made within 3720 sec after boundary formation. The values $(2\ (D/\omega^2)sX_0^2)$ and $(2\omega^2 st)$ were about 0.03 and 0.008, respectively. Therefore, the system satisfied both eq 2 $(D/\omega^2)sX_0^2 \ll 1$ and $2\omega^2 st \ll 1$. Accordingly Faxén's equation (Faxén, 1929) was used for the conventional determination of the diffusion constant. The values extrapolated to infinite dilution ($D^0_{20,w}$) of LDH-III of both uterine tissues were 4.45 and 4.88 $\times 10^{-7}$ cm^2 sec^{-1}, respectively.

With the use of these values for the diffusion constant, the $s^0_{20,w}$ values of 7.56 and 7.78 S, and assuming a partial specific volume of 0.73 ml/g, the molecular weights of both LDH-III were calculated to be 152,000 and 143,000, respectively. The data obtained from ultracentrifugal analysis mentioned above are summarized and compared with several enzymes derived from various human tissues in Table III.

Number of SH Groups in Purified Isozymes. The total number of SH groups per mole of enzyme was determined by the modified method of Ellman (1959) with DTNB. The reaction mixture contained 10 μmoles of potassium phosphate buffer (pH 8.0), 45.6 μg of purified normal uterine LDH-III or 52.9 μg of uterine myoma LDH-III, and 2 μmoles of DTNB in a final volume of 1 ml. Cuvets with a 1-cm light path were used. The absorbance at 412 mμ was determined 2 hr after addi-

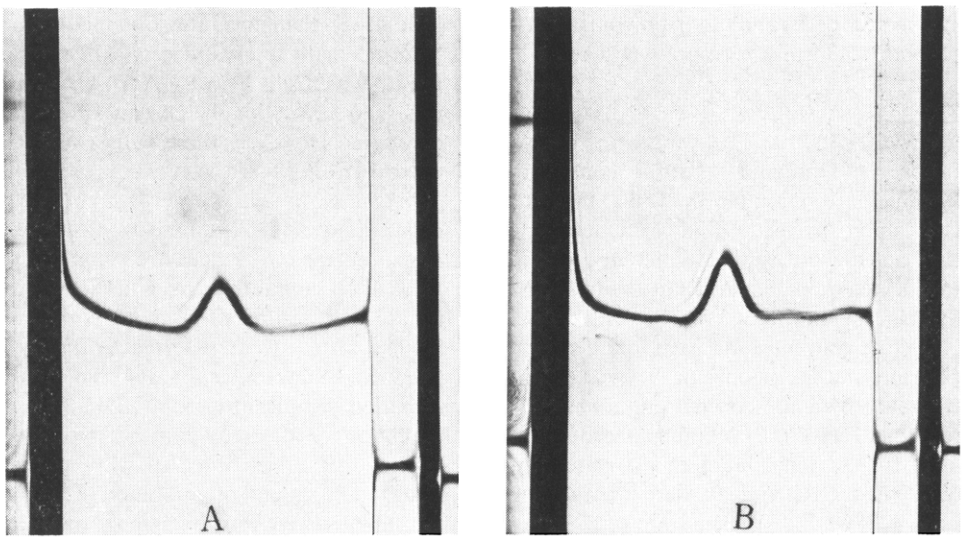


FIGURE 5: Sedimentation patterns obtained with purified isozymes LDH-III of human lactate dehydrogenase from normal uterus (A) after 79 min at 59,780 rpm at 3°, and uterine myoma (B) after 73 min at 59,780 rpm at 5°. Protein concentrations were 0.2 and 0.46 g/100 ml of 0.05 M potassium phosphate buffer (pH 7.0), and the phase-plate angles were 45 and 50°, respectively.

TABLE III: Comparison of Physicochemical Characteristics of Purified Lactate Dehydrogenase Isozymes.

	Human			Beef[b]	
Tissue Isozyme Type	Normal Uterus (LDH-III)	Uterine Myoma (LDH-III)	Heart[a] (LDH-V)	Heart (LDH-V)	Muscle (LDH-I)
Sedimentation coefficient ($s^0_{20,w} \times 10^{-13}$ cm sec^{-1})	7.56	7.78	7.6	7.45	7.32
Diffusion coefficient ($D^0_{20,w} \times 10^{-7}$ cm^2 sec^{-1})	4.45	4.88		5.47	4.47
Molecular weight from s and D	152,000	143,000	140,000 (±4,000)	131,000	126,000
Content of SH group (mole/mole of enzyme protein)	12	11		17[c]	

[a] By Nisselbaum and Bodansky (1961). [b] By Pesce *et al.* (1964). [c] By Sabato and Kaplan (1963).

tion of DTNB. The value of the molecular extinction coefficient of DTNB, 1.36×10^4, was used. From this measurement the total number of SH groups of both purified LDH-III's from normal uterus and its myoma were calculated to be 12 and 11 moles per mole of isozyme, respectively.

Physical Characteristics of Five Isozymes from Normal Uterus and Its Neoplasms. All five isozymes of human lactate dehydrogenase from normal uterus, its myoma, and cervical cancer are stable for at least several days at 0–2° in 0.1 M phosphate buffer (pH 7.0) containing 0.2% crystalline bovine serum albumin. These isozymes, however, are completely inactivated at pH 4.0 within 30 min. To determine heat stability of these isozymes, each purified preparation was diluted with 0.1 M phosphate buffer (pH 7.0) containing 0.2% crystalline bovine serum albumin. The enzyme solution was heated at 50, 55, and 60° in a water bath for 10 min and immediately cooled and assayed. It is interesting to note that LDH-I was very heat labile, whereas LDH-V retained its activity even though heated at 60°. LDH-II, -III, and -IV showed intermediate stability between LDH-I and -V. No significant differences between the normal uterine and its neoplastic isozymes were observed at 50 or 60°. However, it is evident that the neoplastic isozymes, even LDH-I, in contrast to the normal uterine isozymes, are very stable at 55°.

The optimum pH in the pyruvate–NADH system was 7.0–7.5 and that of lactate–NAD system was 9.5–10. The ultraviolet absorption spectra of 0.1% solutions

of both highly purified uterus and uterine myoma LDH-III yielded 1.23 and 1.24 for $E_{280}^{0.1\%}$, respectively.

Kinetic Properties of Five Isozymes from Normal Uterus and Its Neoplasms

Substrate Affinities. Optimum substrate concentrations and K_m values are presented in Table IV. The isozymes acted upon pyruvate and α-ketobutyrate (Meister, 1950), but did not show any activity toward 2-oxoglutarate, α-ketoadipate, D-lactate, and α-hydroxybutyrate. No significant differences with respect to optimum substrate concentration were found among the normal uterine and its neoplastic isozymes. For LDH-I the optimum pyruvate concentration was 2×10^{-3} M, whereas for LDH-V the optimum concentration was $0.25–0.4 \times 10^{-3}$ M. The optimum pyruvate concentrations for LDH-II, -III, and -IV were intermediate between those of LDH-I and -V. The ratio of the reaction rate with 0.25×10^{-3} M pyruvate to that with 2×10^{-3} M pyruvate was not significantly different among the isozymes from the three states of uterus. However, the data indicate that this ratio gradually increased from LDH-I to -V. The K_m values of LDH-I for pyruvate and α-ketobutyrate were the largest and decreased toward LDH-V. The K_m value for lactate was dependent on the concentration of NAD, and was a little larger at low NAD concentration than at high NAD concentration. The K_m values of the various isozymes for lactate were quite similar to those for pyruvate. These K_m values for substrates were very similar to those reported by Nisselbaum and Bodansky (1963) and Balinsky (1966) for the human liver and heart enzymes.

Relative Rates of Reduction of NAD and Its Analogs by the Isozymes. In each series of assays the reaction rates of lactate with NAD or its analogs were usually measured in the presence of the same concentration of each isozyme. NADP and α-NAD were inactive in this assay system, but NAD analogs, especially TNAD and NHXD, did show good activity.

The relative rates of reduction of TNAD and NHXD by LDH-V were significantly higher than those obtained with LDH-I. However, essentially no differences among the reduction rates of 3-AcPyAD, 3-PyAlAD, and their deamino forms were observed with the isozymes from the three states of uterus. The rates of reduction of TNAD by the isozymes from cervical cancer were higher than those obtained with the isozymes from normal uterus and its myoma.

No significant difference between the K_m values for NAD was observed with the isozymes from normal uterus and its myoma. However, the K_m value of LDH-I with respect to NAD and its analogs is significantly different from that of LDH-V. The K_m values of LDH-II, -III, and -IV for the nucleotides were intermediate between those of LDH-I and -V. The K_m values of the four isozymes from cervical cancer for NAD, TNAD, and 3-PyAlAD were somewhat smaller than the K_m values obtained with the isozymes from normal uterus and its myoma. The rates of reduction of 3-PyAlAD by the isozymes from uterine myoma were a little lower than the rates obtained with the isozymes from normal uterus and cervical cancer. The ratio of the rate of reduction of 3-AcPyAD to that of TNAD was very similar for the isozymes from the three states of uterus. However, these values tended to gradually decrease from LDH-I to -V.

Inhibition of the Isozymes by Oxalate, Oxamate, and α-Ketoadipate. Inhibitory effects of these α-keto acids and their K_i values are summarized in Table V. It was observed that oxalate was competitive with lactate and oxamate was competitive with pyruvate. Similar results were reported before by Novoa *et al.* (1959) and Emerson *et al.* (1964). These inhibitory effects increased significantly from LDH-I to -V. Significant inhibition of the reduction of pyruvate by synthetic α-ketoadipate was observed with normal uterine isozymes. The extent of inhibition was about one-third of that observed with oxalate or oxamate. α-Ketoadipate acted competitively with lactate and noncompetitively with pyruvate. LDH-V was more strongly inhibited than LDH-I by this α-keto acid. The inhibitory effects of oxalate and α-ketoadipate were larger upon the isozymes from cervical cancer than upon the isozymes from normal uterus and its myoma.

Inhibition of the Isozymes by NAD Analogs. The reduction of NAD by lactate was strongly inhibited by 3-AcPyAD and 3-AcPyHXD in a competitive manner. Inhibition by TNAD was also competitive with NAD. This inhibition was greater with LDH-V than with LDH-I. 3-PyAlAD inhibited significantly and competitively all isozymes from three states of uterus, but especially the isozymes from cervical cancer. K_i values ranging from 3 to 4×10^{-7} M for 3-AcPyAD obtained with the uterine lactate dehydrogenases. Similar results were reported by Nisselbaum and Bodansky (1961) for the human heart enzyme. No inhibition was observed with either α-NAD or NADP.

Effect of Other Inhibitors. The three major isozymes of lactate dehydrogenase from normal human uterus were inhibited 80 and 100%, respectively, by 20-min preincubation with 2.5×10^{-5} and 1×10^{-4} M PMB. The inhibition was reversed completely by 1×10^{-3} M L-cysteine. These data and the SH content indicate involvement of SH groups of lactate dehydrogenase in the mechanism of action of these isozymes. The isozymes from normal uterus were inhibited 30% by 1×10^{-2} M and 40–60% by 5×10^{-2} M idodacetate in the oxidation of lactate, and the inhibition was prevented completely in the presence of 0.1 M L-cysteine.

Discussion

In order to minimize the possibility of artifactual modification of the protein during isolation, the mildest procedure was chosen at the expense of yield. Alcohol fractionation at step 2 avoided the considerable loss of activity associated with acetone fractionation. The use of DEAE-cellulose column chromatography as an initial purification step rather than a final one may have general application in enzyme preparation. This may be particularly true for those proteins which have

TABLE IV: Substrate Affinities of Human Lactate Dehydrogenases.

Tissue Isozyme Type[a]	Normal Uterus					Uterine Myoma					Cervical Cancer				Liver[b]	Heart[b]
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	I	V
Substrates																
Optimum pyruvate concentration ($\times 10^{-3}$ M)	2.0	0.5	0.5	0.4	0.25	2.0	0.5	0.4	0.4	0.25	2.0	1.0	0.8	0.4		
$\frac{V_L\ (2.5 \times 10^{-4}\ M)}{V_H\ (2.0 \times 10^{-3}\ M)}$	0.8	1.1	1.4	1.5	1.6	0.8	1.2	1.4	1.4	1.6	0.7	0.8	1.0	1.0		
K_m (pyruvate)[c] ($\times 10^{-4}$ M)	1.3	0.9	0.5	0.4	0.3	1.1	1.0	0.7	0.6	0.5	1.5	1.5	1.3	0.8	3.1	0.7
Optimum α-ketobutyrate concentration ($\times 10^{-2}$ M)	3.0	2.5	1.0	1.0	1.0	2.5	2.5	2.0	1.0	1.0	2.5	2.5	2.0	1.0		
$\frac{V_L\ (2.5 \times 10^{-3}\ M)}{V_H\ (2.5 \times 10^{-2}\ M)}$	0.5	0.6	0.7	0.7	0.9	0.3	0.4	0.6	0.7	0.7	0.4	0.5	0.6	1.0		
K_m (α-ketobutyrate)[c] ($\times 10^{-4}$ M)	3.8	3.8	2.4	1.8	1.2	5.9	4.5	3.5	2.7	2.3	5.0	3.6	2.2	1.7		1.3[d]
Optimum L-lactate concentration ($\times 10^{-1}$ M)	1.0	1.0	1.0	0.5	0.25	1.5	1.0	1.0	0.5	0.5	1.0	1.0	0.5	0.5		
K_m (L-lactate)[e] ($\times 10^{-4}$ M) with NAD (1×10^{-4} M)	1.7	0.9	0.6	0.5	0.4	1.7	1.1	0.9	0.9	0.7	1.7	1.7	1.3	0.9	1.6	0.6

[a] V_L and V_H designate velocities obtained, respectively, with low and high concentrations of pyruvate. [b] Nisselbaum and Bodansky (1963). [c] K_m (pyruvate) and K_m (α-ketobutyrate) were determined at 1×10^{-4} M NADH, and at pyruvate and α-ketobutyrate concentrations ranging from 5×10^{-5} to 1×10^{-3} M and from 2×10^{-3} to 2.5×10^{-2} M, respectively. [d] Balinsky (1966). [e] K_m (L-lactate) was determined at 1×10^{-4} M NAD and at L-lactate concentrations ranging from 2.5×10^{-3} to 2×10^{-1} M.

TABLE V: Inhibition of Human Uterine Lactate Dehydrogenases by Oxalate, Oxamate, and α-Ketoadipate.

Tissue Isozyme Type Inhibitors	Percentage Inhibition											
	Normal Uterus					Uterine Myoma					Cervical Cancer	
	I	II	III	IV	V	I	II	III	IV	V	I	III
Oxalate (1×10^{-3} M)												
Pyruvate → lactate[a]	74	81	91	92	94	76	79	85	89	91	88	96
Lactate → pyruvate[b]	21	23	32	35	40	0	8	14	23	41	22	43
Oxamate (1×10^{-3} M)												
Pyruvate → lactate[a]	85	87	89	89	90	77	78	82	86	88	72	79
Lactate → pyruvate[b]	43	45	52	54	56	23	34	46	50	63	35	55
α-Ketoadipate												
Pyruvate → lactate[a] (1×10^{-3} M)	28	36	55	61	62	48	54	57	63	71	58	72
Lactate → pyruvate[b] (5×10^{-3} M)	15	27	46	60	64	16	18	26	56	62	32	58
K_i (oxalate)[c] ($\times 10^{-4}$ M)	25		8		2	38		15		5	14	6
K_i (oxamate)[c] ($\times 10^{-6}$ M)	58		18		15	44		25		20	48	40
K_i (α-ketoadipate)[c] ($\times 10^{-4}$ M)	82		13		4	68		35		16	23	7

[a] The reaction from pyruvate to lactate was determined for each isozyme at 1×10^{-4} M NADH and optimal pyruvate concentrations ranging from 2.5×10^{-4} to 2×10^{-3} M (*cf.* Table II). An appropriate amount of purified enzyme was used to give a rate of NADH oxidation of 0.08–0.12 μmole/min. [b] The reverse reaction, lactate to pyruvate, was determined at 1×10^{-4} M NAD and 5×10^{-2} M L-lactate, and with three times the amount of enzyme used in the forward reaction (see *a*). [c] K_i values were determined at 1×10^{-4} M NADH, 5×10^{-4} M inhibitor, and pyruvate concentrations varying from 5×10^{-4} to 1×10^{-3} M.

been enriched in part by the use of a hydroxylapatite column. The LDH-I isozyme from uterine myoma has been purified further by CM-cellulose column chromatography with the usual linear pH gradient elution. This latter procedure resulted in an increase of specific activity of about fivefold above that obtained with the first DEAE-cellulose column chromatography step. Further purifications of two LDH-II's from normal uterus and its myoma were achieved by DEAE-cellulose column chromatography, giving a specific activity about 230-fold that obtained in the initial chromatographic step. Experiments are in progress to achieve further purification of LDH-I, -II, -IV, and -V from normal uterus and its myoma.

The molecular weights of normal uterine LDH-III and its myoma LDH-III are in good agreement with the values of human heart LDH-V (Nisselbaum and Bodansky, 1961), beef heart LDH-V, and beef muscle LDH-I (Pesce *et al.*, 1964). The total numbers of SH groups of both purified LDH-III's are in good agreement with that reported by Sabato and Kaplan (1963) for beef heart lactate dehydrogenase, and with those for lactate dehydrogenases from various species reported by Fondy *et al.* (1965), using PMB with 8 M urea. There are no significant differences of these values of the isozymes from normal uterus and its neoplasms, but it is apparent through the inhibition studies that the SH groups are involved in the mechanism of action of this enzyme.

As shown in Figure 1, a definite and consistent shift in the pattern of five isozymes of human uterine lactate dehydrogenase has been found in malignant human uterine neoplasms as compared with benign tumor and normal control. This has been associated with an absolute increase in LDH-I; however, there is no significant difference in its total activity among three states of uterine tissue. It was also noted that no significant changes in the pattern and total activities of other various enzymes associated with energy metabolism were accompanied by the increase of LDH-I in neoplasms.

It was found that LDH-I was very heat labile, whereas LDH-V retained its activity even at 60°. LDH-II to -IV showed intermediate behavior. However, it is particularly interesting that the neoplastic isozymes, even LDH-I, in contrast to the normal isozymes, are very stable at 55°, but not at 60°.

Comparative studies of the kinetic properties of the isozymes of human lactate dehydrogenase from normal and neoplastic uterine tissues have been achieved. No significant differences with respect to optimum substrate concentrations and K_m values among the isozymes from the three states of uterus were observed; however, the relative rates of reduction of TNAD by the isozymes from cervical cancer were higher than those with the isozymes from normal uterus and its myoma. The K_m values of the isozymes from cervical cancer for NAD, TNAD, and 3-PyAlAD were somewhat smaller than those of normal uterus and its myoma. The isozymes from three states of uterine tissue were inhibited by α-ketoadipate as well as oxamate and oxalate. These inhibitory effects increased significantly from LDH-I to -V. The inhibitory effects of oxalate and α-ketoadipate were larger upon the isozymes from cervical cancer than upon normal uterus and its myoma. α-Ketoadipate inhibited competitively with lactate and noncompetitively with pyruvate. The isozymes from cervical cancer were inhibited very strongly and competitively by NAD analogs, especially 3-PyAlAD. Its K_i value was calculated to be 10^{-7} M, whereas K_m value for NAD was 10^{-4} M. From these significant findings, it is readily apparent that the isozymes from benign tumor, especially uterine myoma, were essentially indistinguishable from its normal tissue of origin. Goldman *et al.* (1963, 1964) reported that a comparison of lactate dehydrogenase patterns in normal and tumor tissues revealed only a quantitative difference, characterized by the appearance of a relatively greater amount of M-type isozyme in tumors; however, he is lacking conclusive evidence in his findings. Our findings are still insufficient to explain the basic mechanism of an absolute increase in M-type isozyme in uterine noeplastic tissue with no increase of the total activity of lactate dehydrogenase.

There is a considerable amount of physical, catalytic, and immunochemical evidences that lactate dehydrogenase is composed of four subunits and that various multiple forms which are found native are hybrids consisting of varying proportions of different subunits (Cahn *et al.*, 1962; Appella and Markert, 1961). Strong supporting evidence was provided when Markert (1963) showed that freeze thawing of two electrophoretically distinct forms of lactate dehydrogenase in sodium chloride (1 M) causes the formation of five multiple forms in a binomial pattern. The nature of subunit–subunit interactions in lactate dehydrogenases has been further investigated by Chilson *et al.* (1965). A preliminary attempt has been made to demonstrate hybrid formation with myoma LDH-I and normal uterine LDH-V. Equal amounts of myoma LDH-I and normal uterine LDH-V were mixed, frozen, and thawed in 1 M sodium chloride solution. After this treatment an aliquot of the mixture was subjected to electrophoresis. The electrophoretic pattern suggested the formation of hybrids. No hybrid formation was observed with the isozymes from the same tissue, such as normal uterine LDH-I and -V. So far, there is no conclusive evidence to explain this phenomenon observed by human uterine isozymes. Amino acid analyses, finger print patterns of tryptic digests, and immunochemical studies of these isozymes are in progress. These experimental data might suggest the genetical control for the structure and its constituent subunit of each isozyme from these three states of human uterine tissue.

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