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Factors Affecting the Use of Lactate Dehydrogenase as a Means of Bloodstain Differentiation

Lactate dehydrogenase (LDH), a ubiquitous enzyme present in human tissues and body fluids, catalyzes the reversible oxidation of lactate to pyruvate. Electrophoretically, LDH can be separated into five distinct isozyme fractions commonly designated as LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5 [*1*].

Varying levels of the five LDH isozymes have been found to be present in specific human tissues and organs [*2*]. For example, cardiac tissue contains greater levels of the LDH-1 and LDH-2 isozymes while the predominate fraction in hepatic tissue is the LDH-5 isozyme [*3*]. In normal whole blood the LDH-2 isozyme is present in the highest concentration, followed closely by LDH-1 and LDH-3, while the LDH-4 and LDH-5 isozymes are present in the lowest concentrations [*4*].

Asano et al [*4*], using a Cellogel electrophoretic system, demonstrated elevated levels of LDH-4 and LDH-5 isozymes in menstrual blood over the levels present in normal whole blood and proposed a forensic method for the identification of menstrual bloodstains. Another forensic system for menstrual bloodstain identification using an LDH cellulose acetate electrophoretic system has been described by Dixon [*5*].

The purpose of this report is to introduce a modified forensic electrophoretic technique for the analysis of LDH on cellulose acetate and to determine the suitability of using LDH for the differentiation of dried bloodstains. Menstrual, cadaveric, umbilical cord, and normal peripheral bloodstains as well as seminal and saliva stains were characterized electrophoretically. In addition, a time-effect study on LDH isozyme patterns from dried menstrual, cadaveric, umbilical cord, and normal peripheral bloodstains was performed. Modifications in the assay and quantification procedure used in LDH isozyme level determination on cellulose acetate are also introduced.

Methods and Materials

Preparation of Samples

Peripheral blood taken by finger puncture, menstrual blood, semen, and saliva samples were collected from selected donors in our laboratory onto clean white cotton sheeting. Liquid cadaveric blood samples were provided by the District of Columbia Medical Examiner's Office and were collected within 24 h after death. Liquid umbilical cord blood and peripheral blood samples were provided by a metropolitan Washington, D.C., hospital.

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All liquid blood samples were collected into a citrate anticoagulant and subsequently transferred to sample sheeting. Each stain prepared on the sample sheeting was allowed to dry completely in air at room temperature, placed into a separate envelope, and stored at room temperature (25 °C).

Extracts for electrophoresis were prepared by placing a 1-cm^2 portion of the stained material into a 12 by 75-mm test tube. Three drops of the barbital cell buffer [pH 8.6, ionic strength 0.05, 1.84 g barbital (Sigma) and 10.3 g sodium barbital (Sigma) made up to 1 litre of distilled water according to Ref *6*] were added to each tube. The stained cloth in buffer was ground with a glass rod and subsequently allowed to soak for 10 min. Each extracted sample was removed with a Pasteur pipette, placed into a 400-μl polyethylene Microfuge® tube, and centrifuged for 30 s in a Beckman Model B Microfuge. The supernatant of each sample was then pipetted onto the surface of a small sheet of Para-film® and covered with a small beaker. The samples were transferred onto the cellulose acetate membrane by using a Beckman single-sample applicator.

Electrophoresis

Electrophoresis for this study was carried out on the Beckman Model R-101 Microzone® Electrophoresis System.

A cellulose acetate membrane was floated onto a portion of the cell buffer until the buffer had been uniformly absorbed into the membrane. The membrane was then immersed in the buffer for 30 s, withdrawn with forceps, blotted between filter papers, and placed onto the spring-loaded membrane holder. The holder and membrane were placed into the electrophoresis cell, which had been previously filled with 4 °C cell buffer. After an equilibration period of 1 min, the extracted samples were applied to the membrane from the B slot position with the single-sample applicator. Separate multiple quantity deposits of 0.75, 0.50, and 0.25 μl were applied for each sample.

Electrophoresis was performed at a constant voltage of 250 V for 30 min. Starting amperage was approximately 2.5 mA and slowly rose to 3.5 mA at the completion of the run.

Assay for LDH Activity

Prior to the electrophoretic run, an LDH assay plate was prepared by adding the following reagents to a 25-ml, aluminum-foil-wrapped, Erlenmeyer flask: 0.4 ml of 60% sodium lactate syrup (Fisher), 4 mg 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT) (Sigma), 4 mg phenazine methosulfate (Sigma), 20 mg nicotinamide adenine dinucleotide (Sigma), and 6 ml of distilled water. This solution was thoroughly mixed to bring all the dry ingredients into solution and then added to 14 ml of a 2% agarose solution at 55 °C [agarose (Swartz/Mann) was dissolved in a pH 8.3, 0.2*M* tris (hydroxymethyl)-aminomethanehydrochloric acid (Tris-HCl) buffer by boiling and subsequently cooled to 55 °C]. The entire assay mixture was quickly stirred and then poured into a 9.5-cm^2 disposable petri dish and allowed to solidify. The entire preparation of the assay plate just described was carried out in the dark. Five assay plates were routinely prepared at one time and could be stored up to one week at 4 °C in the dark with no loss of activity.

Immediately after the completion of the electrophoretic run the holder and membrane were removed from the cell. The ends of the cellulose acetate membrane (3 to 4 cm) were cut off. The membrane was then removed from the spring bridge assembly and placed facedown onto the surface of the gel-assay plate that had previously been incubated in the dark for 30 min at 37 °C. Care was taken not to entrap any air bubbles under the membrane, thus enabling complete contact between membrane and assay gel. The cover was then placed over the gel and the entire petri dish was inverted and incubated at 37 °C, in the dark, for exactly 10 min.

After the incubation period the membrane was carefully lifted from the reaction plate and gently washed one time with successive 2-min rinses of distilled water, 5% glacial acetic acid, and distilled water. The rinsed membrane was placed in a drying frame between two filter paper blotters and incubated at 37 °C for 2 to 3 h until completely dry. The dried membrane was then carefully placed into a clear plastic envelope for densitometric reading.

Calculation of Percentage of LDH-4 Plus LDH-5

Densitometric readings were carried out on the Beckman R-112 Densitometer. The cellulose acetate membrane was scanned at the slow chart speed with a slit width setting of 0.4 by 5 mm and a 600-nm filter. The percentage of LDH-4 plus LDH-5 was calculated by the following formula:

$$\text{Percentage of LDH-4 plus LDH-5} = \frac{\text{sum of integrated area of LDH isozyme Peaks 4 and 5}}{\text{sum of integrated area of LDH isozyme Peaks 1, 2, 3, 4, and 5}} \times 100$$

Results

Characteristic LDH zymograms were obtained from extracts of one-day-old menstrual, cadaveric, umbilical cord, and normal peripheral bloodstains (Fig. 1) by the electrophoretic procedure described previously. The LDH zymograms for seminal and saliva stains as well as mixtures of semen and saliva with peripheral blood are also shown in Fig. 1.

It is apparent that the zymograms (Fig. 1), densitometric tracings, and the percentages of LDH-4 plus LDH-5 obtained from the extracts of menstrual (66.1%) and cadaveric bloodstains (35.2%) and saliva stains (96.0%) are distinct and can be easily differentiated from normal peripheral (7.6%) and umbilical cord bloodstains (13.3%). It should be noted, however, that the zymogram, densitometric tracing, and percentage of LDH-4 plus LDH-5 obtained from the saliva and peripheral bloodstain mixture (25.7%) are similar to those obtained from the cadaveric bloodstain. Additionally, similarities in the profiles obtained from umbilical cord and normal peripheral bloodstains are also apparent (Fig. 1).

Spermatozoa contain the LDH_x isozyme [*7,8*], which migrates between LDH isozymes 3 and 4. The percentages of LDH-4 plus LDH-5 calculated from a densitometric tracing containing the LDH_x isozyme would not be equal in value to the calculation of the percentages of LDH-4 plus LDH-5 of an identical densitometric tracing less the LDH_x isozyme. Therefore, the percentage of LDH-4 plus LDH-5 was not calculated on either seminal or mixed blood and seminal stains (Fig. 1).

The range, mean, and standard deviation values of the percentages of LDH-4 plus LDH-5 for various bloodstains and saliva were calculated to determine if these percentages could be used as an aid in stain differentiation. The stains examined in this study were one day old when they were run electrophoretically. The results of this study are presented in Table 1. The ranges of the percentages of LDH-4 plus LDH-5 obtained from the examined peripheral (4.2 to 10.2%) and umbilical cord (8.4 to 15.4%) bloodstains and saliva stains (93.4 to 100.0%) are narrow; however, the ranges obtained from menstrual (8.9 to 73.0%) and cadaveric (2.9 to 61.4%) bloodstains are extremely broad. Moreover, the range exhibited by menstrual bloodstains overlaps the ranges obtained from cadaveric, umbilical cord, and peripheral bloodstains.

Although a distinct mean percentage of LDH-4 plus LDH-5 could be calculated for each type of stain examined (7.5% for peripheral, 12.1% for umbilical cord, 47.5% for menstrual, 17.1% for cadaveric, and 98.4% for saliva), the large standard deviation of the

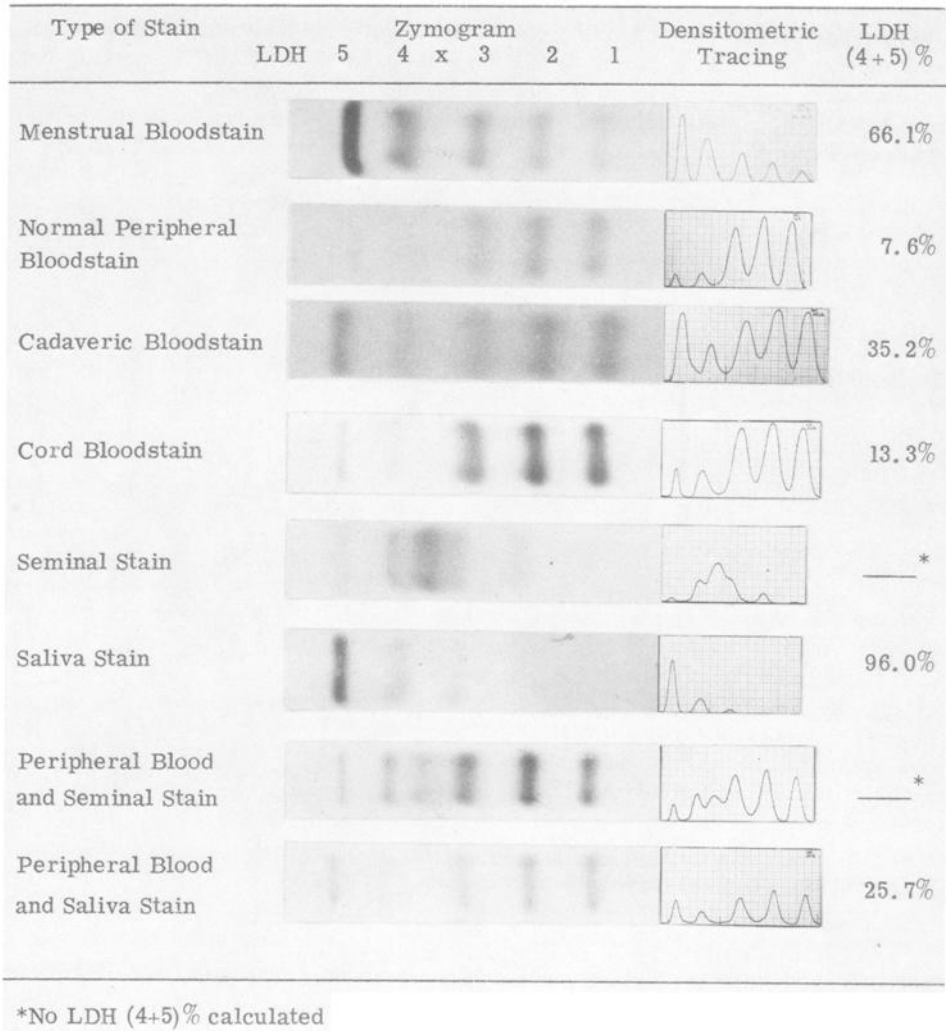

FIG. 1—*Representative LDH isozyme patterns and LDH-4 plus LDH-5 percentages obtained by cellulose acetate electrophoresis of extracts of dried stains from several sources.*

TABLE 1—*Calculated range, mean, and standard deviation of percentages of LDH-4 plus LDH-5 in extracts from saliva and bloodstains of different origins.*

Type of Stain	Stains Examined, *n*	Range of Percentages of LDH-4 plus LDH-5	Mean, %	SD
Normal peripheral	29	4.2 to 10.2	7.5	±1.11
Cord	22	8.4 to 15.4	12.1	±1.71
Cadaveric	30	2.9 to 61.4	17.1	±11.34
Menstrual	11	8.9 to 73.0	47.5	±21.31
Saliva	10	93.4 to 100.0	98.4	±2.69

mean experienced with cadaveric and menstrual bloodstains would preclude the use of the mean percentage as an aid in stain differentiation (Table 1).

The wide variation of the percentages of LDH-4 plus LDH-5 obtained during the examination of individual cadaveric bloodstains was found to be related, in part, to the circumstances that contributed to the death of the individual. The percentages of LDH-4 plus LDH-5 and corresponding LDH zymograms for eight individual cadaveric bloodstains are shown in Fig. 2. Also shown is the cause of death for each individual as deter-

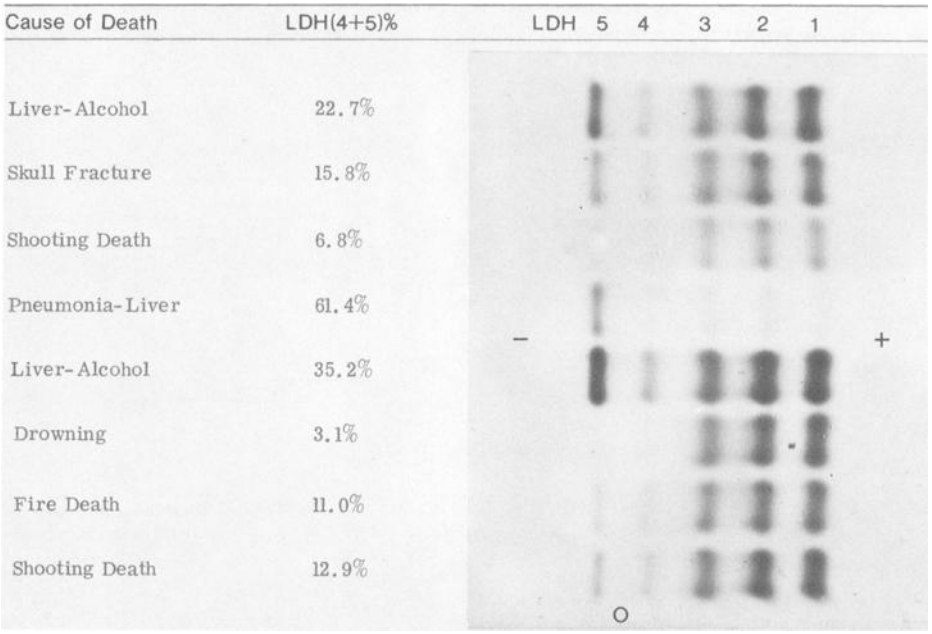

FIG. 2—*Variation in LDH isozyme patterns and LDH-4 plus LDH-5 percentages observed in cadaveric bloodstains obtained from different individuals.*

mined by the District of Columbia Medical Examiner's Office. The percentages of LDH-4 plus LDH-5 obtained from the bloodstains of those individuals whose death were considered instantaneous (shooting, drowning, and fire) fall within or near the percentages of LDH-4 plus LDH-5 exhibited by peripheral blood (4.2 to 10.2%, Table 1). In contrast, the percentages of LDH-4 plus LDH-5 obtained from the bloodstains of individuals whose deaths involved tissue damage were elevated. Deaths involving extensive liver damage showed the highest percentages of LDH-4 plus LDH-5, ranging from 22.7 to 61.4% (Fig. 2).

During the examination of menstrual bloodstains it was observed that a wide variation in the percentages of LDH-4 plus LDH-5 could be obtained between individual bloodstains (Table 1). Interestingly, this same variation also occurred when four random cuttings were examined from the same menstrual bloodstain (Fig. 3). The 9.1% LDH-4 plus LDH-5 value obtained from Cutting 4 (Fig. 3) is representative of peripheral blood (range, 4.2 to 10.2%, Table 1), while Cutting 2 shows a 33.3% LDH-4 plus LDH-5 value, which is greatly elevated over that of peripheral blood.

The percentages of LDH-4 plus LDH-5 for mixed stains of saliva and peripheral blood range from 6.5 to 25.7%.

The percentages of LDH-4 plus LDH-5 calculated from zymograms of four different seminal stains and stains containing a mixture of seminal fluid and peripheral blood

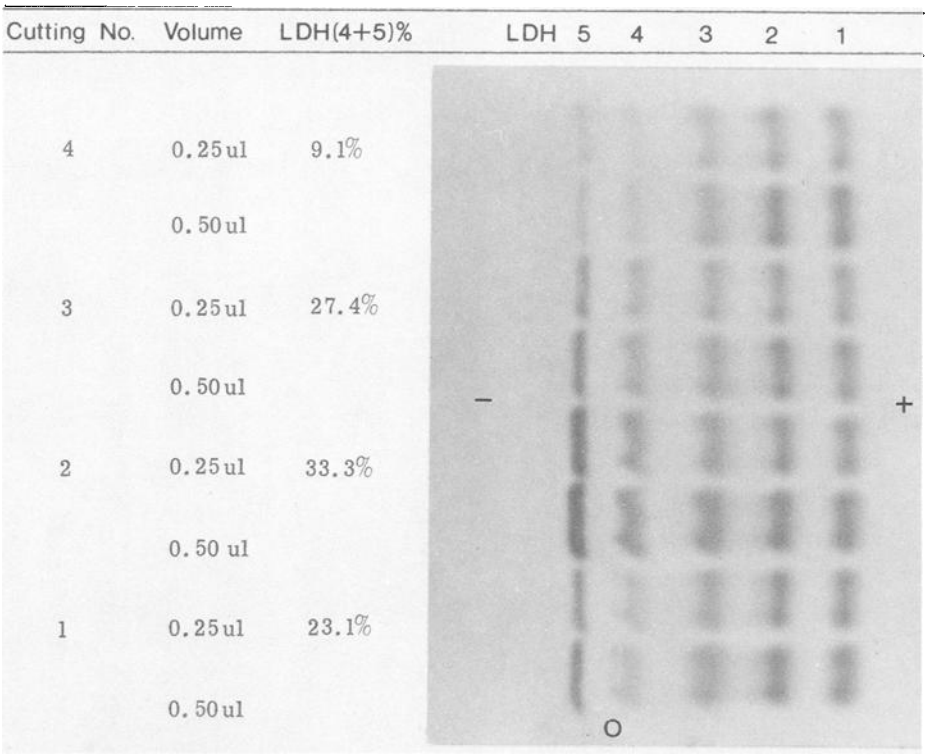

FIG. 3—*Variation in LDH isozyme patterns and LDH-4 plus LDH-5 percentages observed in four different cuttings from the same menstrual bloodstain.*

showed a great deal of variation. This variation was due, in part, to the presence or absence of the LDH_x isozyme. Seminal stain cuttings that did not exhibit an LDH_x band produced mean values of LDH-4 plus LDH-5 of 28.0%. Stains containing mixtures of peripheral blood and semen that did not show the LDH_x band gave percentages of LDH-4 plus LDH-5 high as 15.8% and as low as 10.1%.

Additional factors such as length of incubation time during LDH assay, enzyme concentration, and the age of bloodstained material were studied to determine if these factors could affect the appearance of the LDH isozyme patterns and the calculation of the LDH-4 plus LDH-5 percentages.

The effect of incubation time during LDH assay on the calculated percentages of LDH-4 plus LDH-5 is shown in Fig. 4. In freshly prepared peripheral bloodstains the sum of activities in LDH isozymes 4 and 5 increased at a linear rate with time from 0 to 40 min. However, the sum of the activities in LDH isozymes 1, 2, and 3 became nonlinear after 5 min and continued at a nonlinear rate through 25 min, when the rate began to reach a maximum. Since the percentage of LDH-4 plus LDH-5 is calculated according to the formula previously described, the nonlinearity of the sum of the activities in Fractions 1, 2, and 3 would cause a slight constant increase in the percentage of LDH-4 plus LDH-5 at the longer incubation times. The increase is dependent, although not directly proportional to, the intensity of activity present in the LDH-4 and LDH-5 fractions. Therefore, if excessive incubation periods are allowed, artificially high percentages of LDH-4 plus LDH-5 could be produced.

Incubation periods under 5 min resulted in low percentages of LDH-4 plus LDH-5 be-

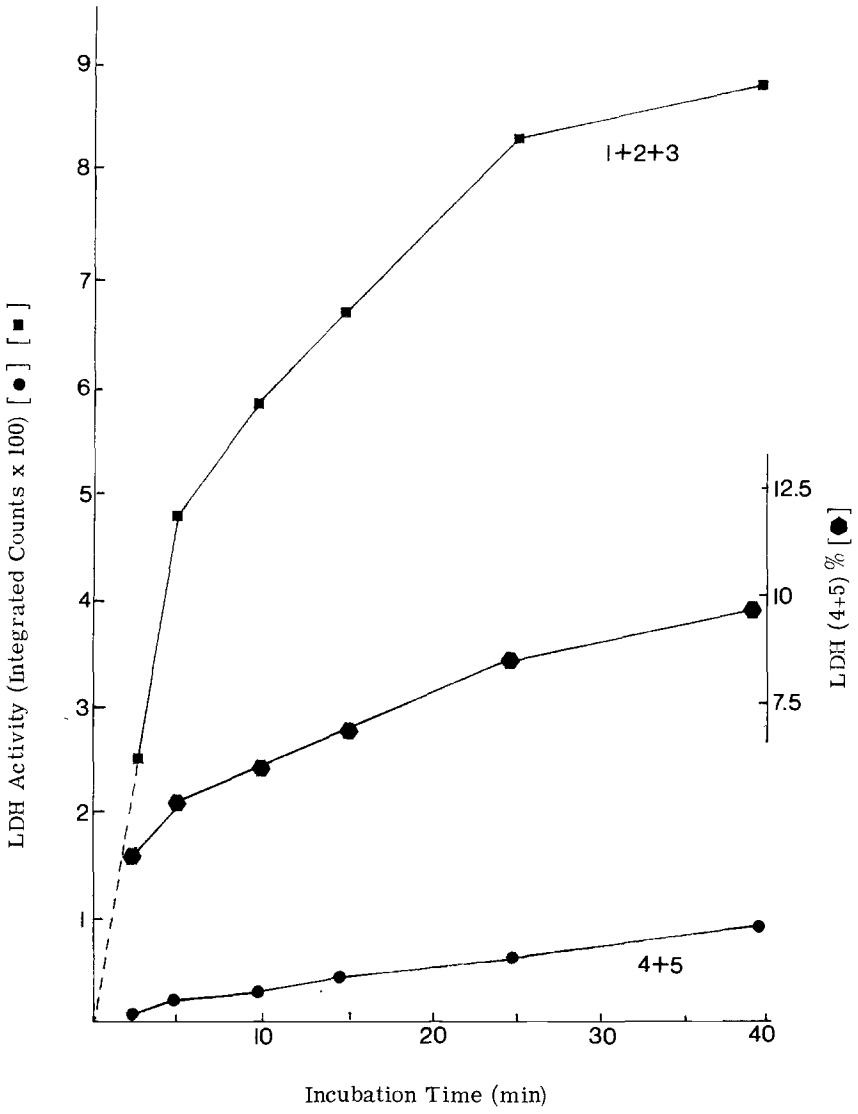

FIG. 4—*The effect of incubation time during LDH assay on the calcuation of the LDH-4 plus LDH-5 percentages from a dried peripheral bloodstain. The LDH activity is expressed as the integrated area under the densitometrically traced peaks.*

cause the activity in the LDH isozyme fractions of lowest concentration was often absent or of low intensity.

An incubation time of 10 min allowed for sufficient development of activity in the less intense LDH isozyme fractions and fell on the portion of the curve (Fig.4) where all LDH isozyme fractions were increasing at a nearly linear rate.

Figure 5 shows the effect of enzyme concentration on the calculation of the percentages of LDH-4 plus LDH-5 in freshly prepared peripheral bloodstains. Enzyme concentration was expressed as a dilution of the neat extract and was prepared by adding drops of diluting buffer to a drop of neat extract. A constant amount of each dilution (0.25 μl) was deposited on the cellulose acetate membrane for electrophoresis.

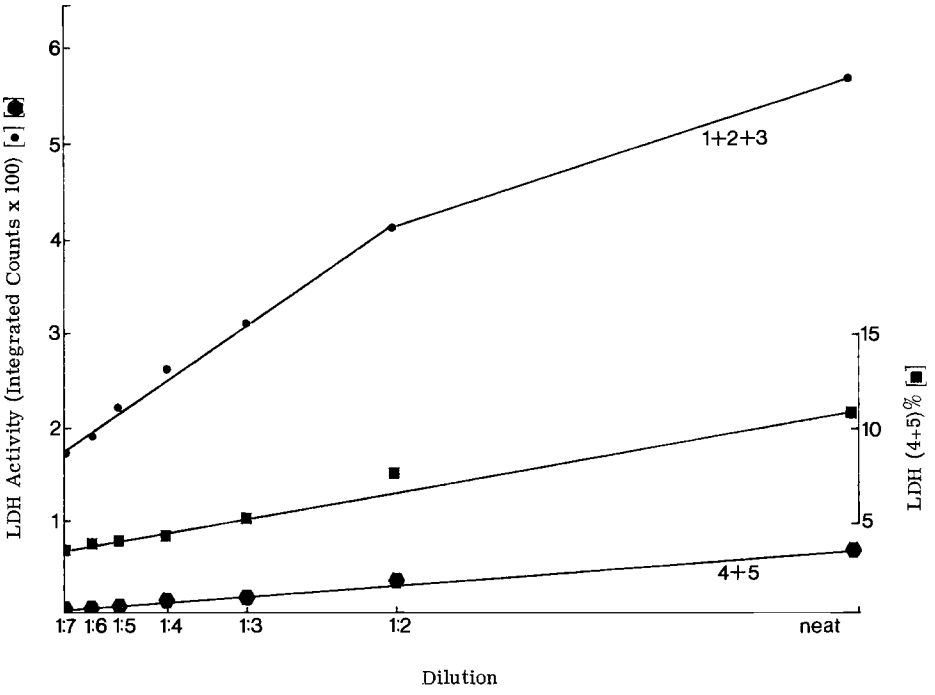

FIG. 5—*The effect of increasing enzyme concentration on the calculation of LDH-4 plus LDH-5 percentages from a dried peripheral bloodstain. The LDH activity is expressed as the integrated area under the densitometrically traced peaks.*

The sum of the LDH-4 plus LDH-5 isozyme activities increased at a linear rate with increased enzyme concentration while the sum of the activities in LDH isozymes 1, 2, and 3 became nonlinear at a dilution of 1:2 or less. The percentages of LDH-4 plus LDH-5 gradually increased from 3.4 to 10.1%. The elution and extraction procedure described in the Methods section gave a sample concentration in the range of 1:2 to 1:3 dilution. The calculated percentages of LDH-4 plus LDH-5 from three sample volumes of 0.25, 0.50, and 0.75 μl would reduce the possibility of misinterpreting an artificially high percentage of LDH-4 plus LDH-5 from a too-concentrated sample.

The effect of extraction time on the recovery of LDH isozyme activity in dried bloodstains was examined and the results are shown in Fig. 6. One-day-old peripheral bloodstains extracted for over 10 min did not show a significant increase in the total LDH activity recovered. Stains extracted overnight at 4°C showed a decrease in total LDH activity recovered as well as consistently lower percentages of LDH-4 plus LDH-5. The reason for this loss of activity may be the relative instability of the LDH enzyme, specifically isozymes 4 and 5 [*9*].

The calculated percentages of LDH-4 plus LDH-5 were constant for extraction times from 2.5 to 20 min, indicating that LDH isozymes 4 and 5 were no less soluble in the extraction buffer than LDH isozymes 1, 2, and 3.

In all cases the percentages of LDH-4 plus LDH-5 decreased as the bloodstain aged (Fig. 7). During the first 15 days, percentages of LDH-4 plus LDH-5 in peripheral bloodstains decreased from an initial value of 6.8 to 4.8%, umbilical cord bloodstains from 15.5 to 8.9%, and cadaveric bloodstains from 26.2 to 14.4%. The largest decreases in percentages of LDH-4 and LDH-5 were observed in all stains between Day 1 and Day 7.

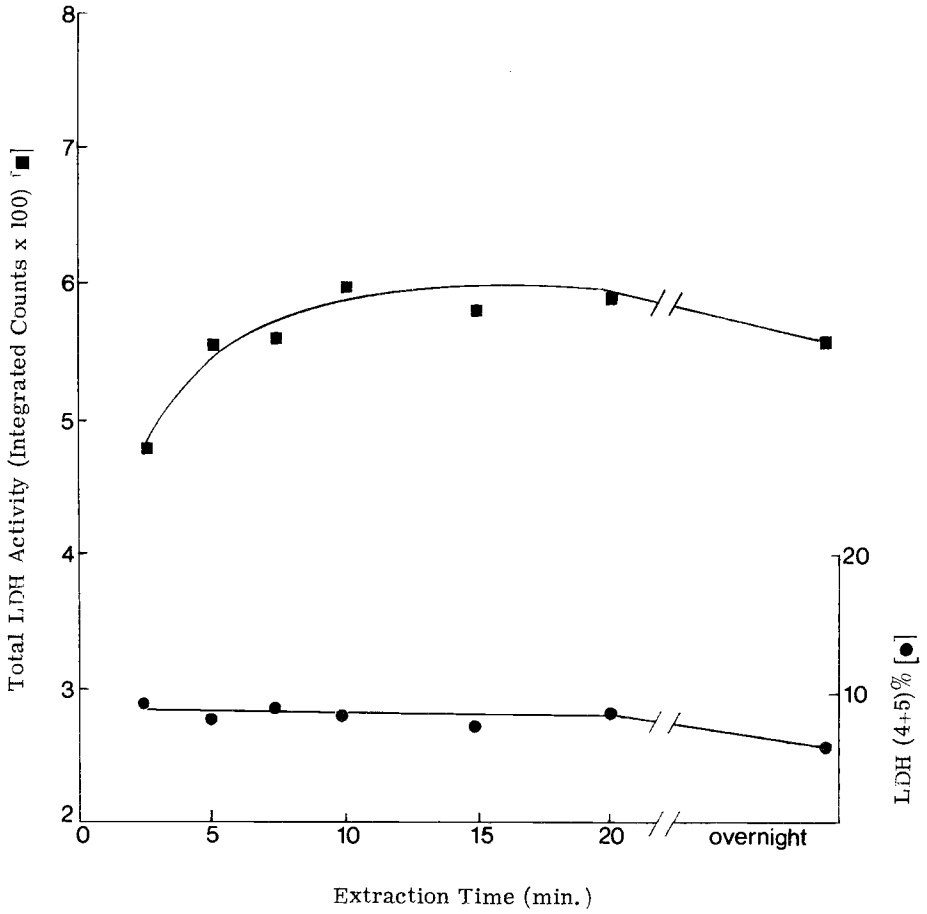

FIG. 6—*The effect of extraction time on the recovery of total LDH activity and the calculation of LDH-4 plus LDH-5 percentages from a dried peripheral bloodstain. The LDH activity is expressed as the integrated area under the densitometrically traced peaks.*

From the 15th day to the 40th day, the LDH-4 plus LDH-5 percentages showed only slight decreases.

In all stains studied the activity in the LDH-4 and LDH-5 isozymes was found to be the least stable. All five LDH isozymes were found in peripheral, cord, cadaveric, and menstrual bloodstains up to 1 month. Activity in the LDH-4 and LDH-5 isozymes began to disappear in peripheral bloodstains at 33 days and in cord and cadaveric bloodstains at 43 and 42 days, respectively. In menstrual bloodstains having very high LDH-4 and LDH-5 isozyme activities, the first LDH isozymes to disappear were 1, 2, and 3. Generally, those menstrual bloodstains exhibiting initially high percentages of LDH-4 plus LDH-5 showed activity in LDH isozymes 4 and 5 up to 50 days.

No attempt was made to plot the effect of age on menstrual bloodstains because of the heterogeneity experienced in the LDH isozyme levels present in separate cuttings taken from the same menstrual bloodstain.

Saliva stains showed no LDH activity after 10 days. Mixed saliva and peripheral bloodstains showed LDH isozyme stability similar to that of peripheral bloodstains.

The LDH_x isozyme present in seminal fluid as well as stains containing a mixture of semen and peripheral blood continued to show activity up to 15 days.

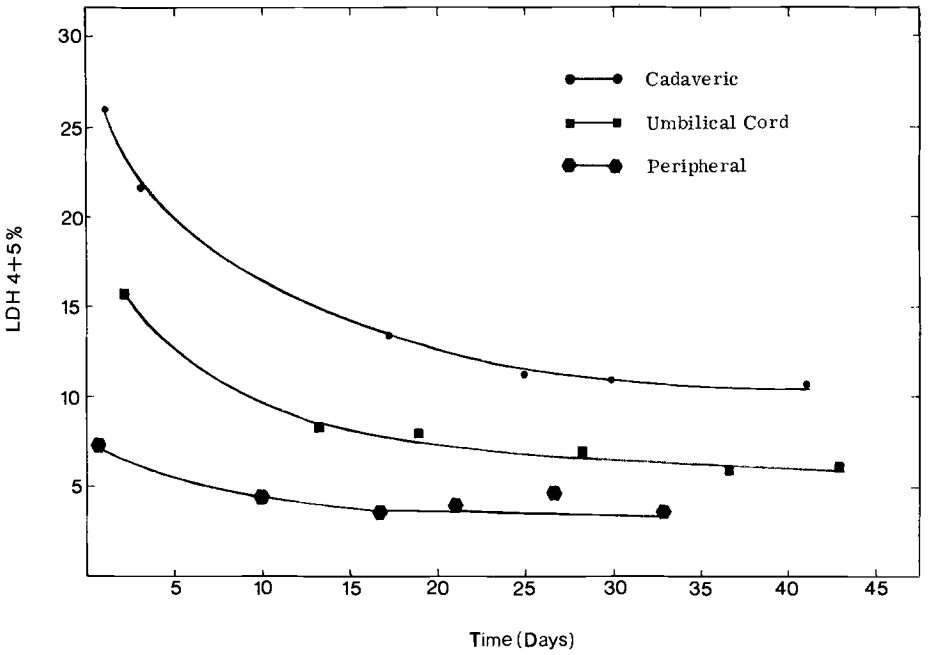

FIG. 7—*The effect of age on the calculated LDH-4 plus LDH-5 percentages obtained from extracts of dried cadaveric, umbilical cord, and peripheral bloodstains. All bloodstains were stored at room temperature (25 °C).*

The disappearance of the LDH-4 and LDH-5 isozymes as the bloodstain aged was not the only limiting factor that led to the inability to distinguish between bloodstains from various sources. As the bloodstain aged and began to deteriorate, a diffuse LDH background activity began to appear between all the isozyme bands on the cellulose acetate strip. Because of the diffuse background activity in most dried stains over 40 days old the ability to distinguish between the separate isozyme bands was difficult and the calculation of the LDH-4 plus LDH-5 percentages was not possible.

Discussion

The use of LDH isozyme patterns, obtained from bloodstains after electrophoresis, and the percentages of LDH-4 plus LDH-5, calculated from densitometric tracings of the isozyme patterns, has been described as a means to differentiate menstrual, cadaveric, parturition, and peripheral bloodstains [*4,5*]. However, in the present study, with a modified but highly sensitive cellulose acetate electrophoretic method and the densitometer for quantification, LDH isozyme patterns and their corresponding percentages of LDH-4 plus LDH-5 have proven to be of limited use in bloodstain differentiation.

Only peripheral and umbilical cord bloodstains provided uniform LDH isozyme patterns and LDH-4 plus LDH-5 percentages. Even then, the slight differences that could be observed in the LDH isozyme patterns and LDH-4 plus LDH-5 percentages from peripheral and umbilical cord bloodstains were so small that to differentiate between them was difficult.

During the examination of individual menstrual bloodstains, a wide variation in the LDH isozyme patterns and LDH-4 plus LDH-5 percentages was observed. More important was the observation that similar variations occurred when random multiple cuttings were examined from the same menstrual bloodstain.

Cadaveric bloodstains produced a wide variation of observable LDH isozyme patterns and LDH-4 plus LDH-5 percentages. From a study of known cadaveric bloodstains it was determined that the circumstances that contributed to the death of the individual greatly influenced the resulting LDH isozyme pattern obtained from the blood after death.

The variation in the LDH isozyme patterns observed in menstrual and cadaveric bloodstains produced broad ranges of LDH-4 plus LDH-5 percentages that not only overlapped each other but also overlapped the LDH-4 plus LDH-5 percentages obtained from peripheral and umbilical cord bloodstains.

Dried stains containing a mixture of peripheral blood with seminal fluid or saliva produced LDH isozyme patterns and LDH-4 plus LDH-5 percentages that could have been misinterpreted as having come from menstrual or cadaveric bloodstains.

Additional factors were found that affected the LDH isozyme patterns and LDH-4 plus LDH-5 percentages obtained from bloodstains. High percentages of LDH-4 plus LDH-5 were obtained when high enzyme concentrations and long incubation times were allowed during the LDH assay following electrophoresis. Low percentages of LDH-4 plus LDH-5 were obtained when extraction times became extensive or as the bloodstain aged.

Various pathological conditions such as necrosis of the liver [*10*], acute arthritis [*11*], myocardial infarction [*12*], muscular dystrophy [*13*], and neoplastic diseases [*3*] have also been shown to influence the levels of the individual isozymes present in the blood. Although an example of each of these conditions was not examined directly in this study, cadaveric bloodstains taken from individuals having liver damage produced high levels of the LDH-4 and LDH-5 isozymes.

After considering all of the above observations, the authors have come to the opinion that LDH alone should not be used as a means of specific bloodstain identification.

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