Fractionation of Serum Lactic Dehydrogenase by Salt Concentration Gradient Elution and Paper Electrophoresis.* (23580)

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The enzyme, lactic dehydrogenase (LDH), has been reported previously to be present at an elevated level in the serum of many individuals with neoplastic diseases(1,2). Although sedimentation studies have indicated the presence of only one component (3), fractionation with ammonium sulfate has revealed at least 2 components with enzymatic activity to be present. Two electrophoretic components with LDH activity have been reported in heart muscle(4,5). In the present work an attempt was made to determine whether the elevation in LDH found in the serum of some individuals with leukemia is reflected in an increase in one or more of the detectable components.

Materials and methods. Enzymatic assay. Lactic dehydrogenase activity was determined by a spectrophotometric method previously described by Hill and Levi(1). *Separation of enzyme.* Two technics have been employed in the attempts to fractionate the LDH activity: (1) *Concentration gradient elution.* The ammonium sulfate concentration gradient elution technic is a modification (6) of the method of Schwimmer(7). Serum proteins were precipitated by the addition of 9 ml of saturated ammonium sulfate solution to 1 ml of serum. The precipitated proteins were suspended in an 85% saturated ammonium sulfate solution and applied to the top of an 8 cm column of Hyflow Supercel. The suspended proteins were eluted by a steadily decreasing concentration of ammonium sulfate achieved by gradual dilution with water of an 85% saturated ammonium sulfate solution in a mixing chamber which was kept thoroughly mixed by means of a magnetic stirrer mounted beneath it. The fractions were collected in test tubes by means of an automatic fraction collector which collected 120 drops in each tube. Ammonium sulfate concentration in individual tubes was determined by nesslerization of suitable aliquots. Approximate protein concentration of all fractions was estimated by reading the optical density at 280 mμ in a Beckman DU spectrophotometer. For more precise determinations of protein concentration, the 280/260 absorption ratio was determined and protein concentration calculated according to the method of Warburg and Christian(8). (2) *Continuous flow paper electrophoresis.* Electrophoretic separation of components was carried out in the Spinco continuous flow paper electrophoresis apparatus, model CP. Prior to application sera were diluted 1:10 (for high activity serum) or 1:5 (for low activity serum) with barbiturate buffer, pH 8.6, 0.02 ionic strength. The diluted serum was then dialyzed against the same buffer overnight at 4°C. The diluted, dialyzed serum was fractionated by electrophoresis employing a current of 30 milliamperes at 450 volts. LDH activity was determined for all fractions. Upon completion of each run the paper was dried at 110° for 20 minutes, stained for 4 hours with Spinco dye B-1, washed twice with 5% acetic acid, fixed with Spinco Fixative B-2, and dried again at 110°C.

Results. Fig. 1A shows a typical column fractionation pattern of the enzyme components from the serum of a leukemic patient (high activity serum). Four peaks of enzymatic activity were obtained. In this particular experiment the eluates in tubes 8 through 39 were combined and refractionated. Again (1B) the 2 major peaks were observed but with an apparent shift of the second peak to the first. This shift has been characteristic of all results of similar experiments. Tubes 18 through 26 from the second pass of this material were combined, reprecipitated and passed through the column. The major por-

* This investigation was supported by research grants CY-3126 and C-2568 (C2) from Nat. Cancer Inst., USPHS.

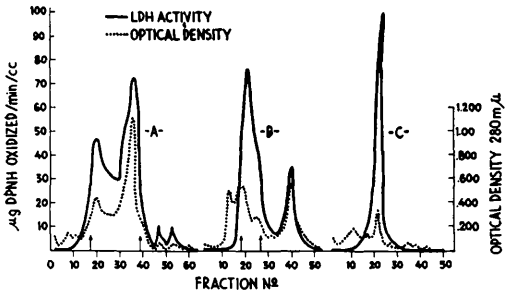

FIG. 1. Gradient elution purification of serum lactic dehydrogenase. The material used for this separation was the precipitate from 1.5 ml serum having activity equivalent to oxidation of 2.83 mg DPNH per min per ml serum. A. First pass through column; B. Second pass; C. Third pass, (see text).

tion of LDH activity now was found in 3 tubes and a protein peak was coincident with the LDH peak. The fractionation patterns obtained were essentially the same whether or not the pH of ammonium sulfate was adjusted to neutrality.

The importance of protein-protein interactions on separations of enzymes(9,10) is well known. The results in Fig. 2 show that when the highly active purified fraction is added to normal serum the activity is spread out over a larger number of tubes on a subsequent column run suggesting that such interactions may also be taking place under the conditions employed by our column procedure.

Lactic dehydrogenase has been shown to be a sulfhydryl enzyme(11,12). Since it was suspected that the shifts in peaks observed during successive fractionations might be the result of sulfhydryl interactions, fractionations were carried out on aliquots of the same serum in the absence and presence of reduced glutathione (GSH) at concentrations of 10^{-4}M. It can be seen in Fig. 3 that the separations with and without glutathione differed in that the major portion of activity was obtained in the first peak with GSH, whereas a greater spread was observed in the absence of GSH. It is of interest to note that the total activity recovered in both cases is approximately the same as determined by the areas beneath the two curves.

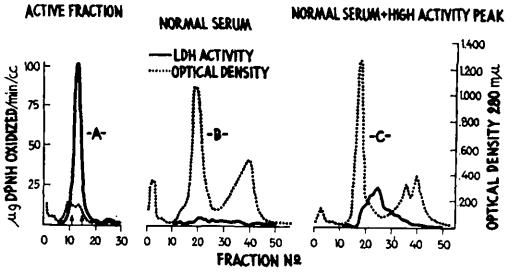

FIG. 2. Effect of serum proteins upon gradient elution fractionation. A. Active fraction from third pass through column (as in Fig. 1); B. Low activity serum (1.5 ml serum with activity equivalent to oxidation of 0.17 mg DPNH per min per ml serum); C. Active fraction (from A) and low activity serum (B) combined, (see text).

Fig. 4 shows a typical electrophoretic pattern obtained from the high activity serum of a leukemic patient. The enzymatic (LDH) activity is shown for each fraction. The same type of pattern was obtained on electrophoresis of low activity serum from apparently healthy individuals. Upon recombination of the active peaks which were rerun under the same conditions, the same electrophoretic pattern was obtained for LDH activity. This is in contrast to the results obtained with the column in which the peaks all tend to migrate to a single large peak upon repeated fractionation. The single sharp peak obtained from the column reverts to two peaks upon electrophoresis.

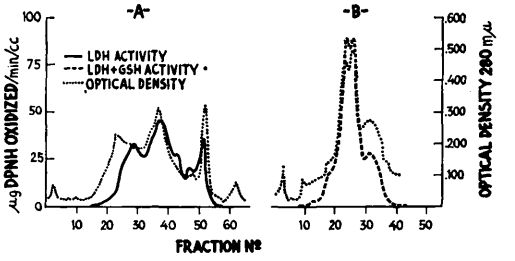

FIG. 3. Effect of glutathione upon components of LDH obtained by gradient elution fractionation of the precipitate from 1.5 ml serum having activity equivalent to oxidation of 2.83 mg DPNH per min per ml. A. No glutathione added; B. Glutathione added (1×10^{-4} M in all solutions).

Serum fractionated electrophoretically in the presence and in the absence of GSH (10^{-4}M) gave identical patterns. However, crystalline rabbit muscle LDH (Worthington Biochemical Corp.) showed a shift from a major and 2 minor components to a single major component in the presence of added GSH (10^{-4}M).

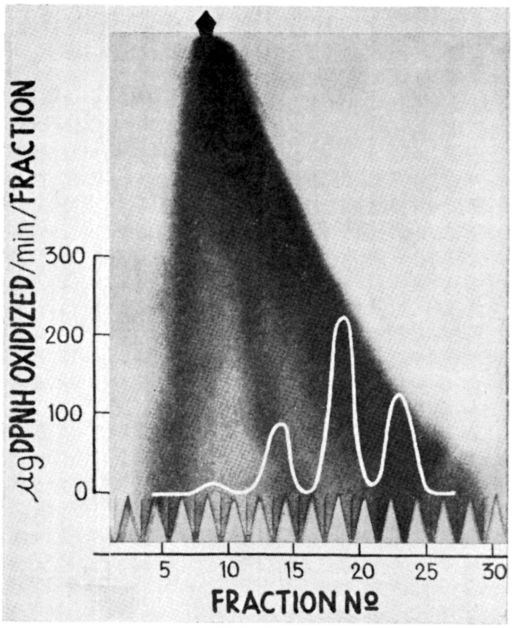

FIG. 4. Electrophoretic separation of 2 ml high activity serum (2.83 mg DPNH per min per ml). LDH activity per tube is shown superimposed on dyed paper which shows protein distribution.

Discussion. Vesell and Bearn(13) recently have reported fractionation of serum LDH into 3 distinct peaks of activity by zone electrophoresis using starch or polyvinyl as a supporting medium. These authors describe different ratios of activity in the various peaks in myocardial infarction and in leukemia. They also postulate that this may be suggestive of different forms of LDH elaborated from different sites.

All of the untreated cases of leukemia studied in our laboratories have shown a general elevation of LDH, reflected to some extent in all of the detectable constituents rather than in elevation of a single component.

At the present time it seems probable that LDH may consist of a single molecular species which can be observed as a number of components representing different states of aggregation as a result of sulfhydryl or other protein interactions. Ledoux(14) has shown in the case of ribonuclease that new forms appear during oxidation of the protein and that this can be prevented by reduced GSH. Morton and Deutsch(15) have observed similar phenomena with serum macroglobulins. We feel that perhaps we are dealing with an analogous situation with LDH.

Summary. Serum lactic dehydrogenase from leukemic and non-leukemic individuals has been separated into a number of components by ammonium sulfate concentration gradient elution and by paper electrophoresis. The effect of glutathione and of other proteins upon the fractionation patterns obtained by these two technics is described.

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Received September 19, 1957. P.S.E.B.M., 1957, v96.