The Extraction of Brain Isozymes with Solvents of Varying Polarity (36355)

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Our laboratory has, for many years, been involved with brain cholesterol metabolism. In an effort to refine our technique, we thought that it would be worthwhile to investigate the metabolism of brain lipoproteins (proteolipids). As a means towards this end, we investigated various extracting procedures for brain protein. The object of these experiments was to solubilize as much brain protein as possible and, subsequently, fractionate it by electrophoresis into a variety of subfractions for further metabolic studies. In our attempt to evaluate protein denaturation by changes in enzyme activity in these extracts, we were provided with circumstantial evidence for the lipoprotein nature of certain isozymes. This report deals with this aspect of the problem.

Materials and Methods. All chemicals and solvents were reagent grade. Chemicals were used without further purification.

Aqueous solutions of different inorganic salts (NACl, KCl and $CaCl_2$) and at various concentrations (1–20%) were examined for their extraction qualities.

The choice of organic compounds ran a gamut of the usual alcohol solutions, detergents as well as more exotic solvents, such as trichloreothylene and tetrabromoethylene. These solvents were used singly and in combination. Again various concentrations (1–10%) of organic solvents and water were compared.

In a third series various combinations of organic solvents and inorganic salt solutions were investigated for their ability to extract brain protein (enzymes).

General extraction procedure. Normal Swiss male (SF-1) 8–10 week old mice were obtained from Stout Farms (Dexter, MI) and were killed by ether anesthesia. The brains were removed, rinsed in distilled water, blotted, quick frozen, weighed and homogenized (five brains per homogenate) with extracting solvent (g/ml) in glass microhomogenizers. The homogenizers were kept in ice baths during the actual grinding to avoid thermal inactivation of the enzymes. The homogenerates were then transferred to cellulose acetate centrifuge tubes which were inserted in metal tubes in which the homogenates were frozen and thawed six times in rapid succession in an acetone–dry ice mixture. After the sixth thaw, the homogenates were centrifuged in an International Refrigerated Centrifuge (Model PR-2) for forty minutes at a force of 20,000*g* and at a temperature of 0°.

The supernatate was then removed and the precipitate discarded. G-25 coarse Sephadex (0.5 g) was added to the supernate. This particular grade of Sephadex was used because it was feared that G-50, G-75, etc., might remove too much of the high molecular weight proteins from the samples. The samples were allowed to stand for 10 min after which the supernates were removed from the Sephadex by means of capillary pipettes.

Starch-gel electrophoresis. A modified Smithies method of horizontal starch-gel electrophoresis was used to evaluate the samples (1, 2). Poulik's (3) discontinuous buffer system was employed at the following concentrations: 0.034 *M* Tris–citrate, pH 8.65; 0.277 *M* borate, pH 8.4. Hydrolyzed starch (Lot #187-1) from Connaught Laboratories, Toronto, was used. Gels were prepared in the morning and were used 1–2 hr later. Samples were allowed to soak up onto 4-ply Whatman #1 filter paper wicks and were inserted in

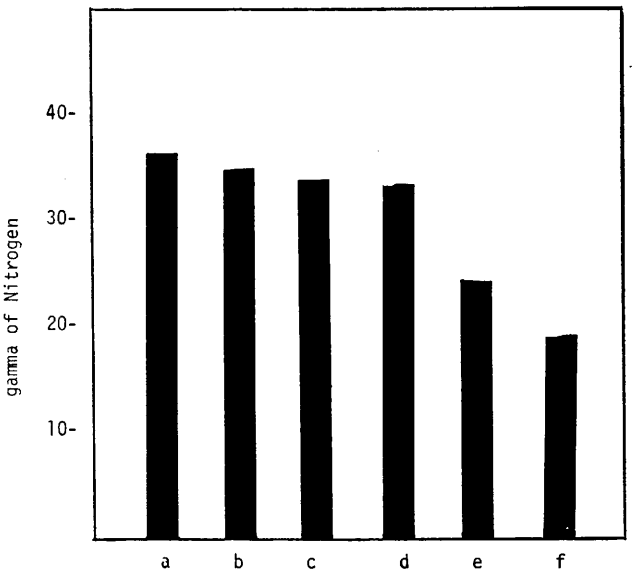

FIG. 1. Extraction of brain nitrogen by various solvent systems: a. 5% butanol; b. 5% KCl; c. 5% KCl, 5% butanol; d. water; e. 1% $CaCl_2$; f. 1% $CaCl_2$, 5% butanol.

the gel 2¾ in. from the cathode end. Samples were allowed to migrate at 6 V/cm until the brownish-yellow buffer line was at a distance of 3 in. from the point of insertion of the sample. Voltages were checked and readjusted to 6 V/cm after 1 hr running time. The electrophoresis usually required 3 hr of running time.

Acrylamide disc electrophoresis. Gels were prepared fresh on the day of the electrophoretic run. One milliliter of lower gel (Canalco 601P) was added to each column (Canalco 1102-1) and allowed to polymerize in high intensity light for 40 min. A ⅜-in. layer of spacer gel (Canalco 603P, concentrated) was then layered over the lower gel (pH 8.7). The spacer gel was polymerized for 15 min. Three microliters of extract were mixed with 0.15 ml of upper gel (Canalco 603P, diluted 5:1). Sample layer was allowed to polymerize for 20–30 min.

Electrophoresis was carried out at a constant current of 5 mA per tube. Running time was approximately 1 hr.

Evaluation of extracts. Initially, the evaluation of our extracting procedure was carried out by measuring the amount of nitrogen (micro-Kjeldahl procedure) and protein (Biuret).

For disc gels the protein bands were immediately stained for one hour with a solution of 1% Amido Schwarz in 7% acetic acid. The gels were than destained at 15 mA per tube in 10% acetic acid instead of buffer solution.

After starch electrophoresis, the gel was sliced into two halves. One half was placed in methanol for 1 min to fix the protein and then covered with saturated Acrilan black 10B dye (Verona Dyestuff, Union, NY) solution prepared in methanol–distilled water–glacial acetic acid (50:50:10). The gel was stained for 3 min and decolorized by a series of three 5% acetic acid wash solutions.

The second half of the starch gel was stained for nonspecific esterase (4) or for lactic acid dehydrogenase (5).

The stained bands (protein or isozymes) were photographed using Polaroid film Type 42.

Results. Extraction of brain tissue by different solvents. While a great variety of solvents were examined, only those that proved to be of special importance are reported. In Fig. 1, the relative amount of nitrogenous material being solubilized was measured. The amount of protein extracted by a given solvent system was approximately the

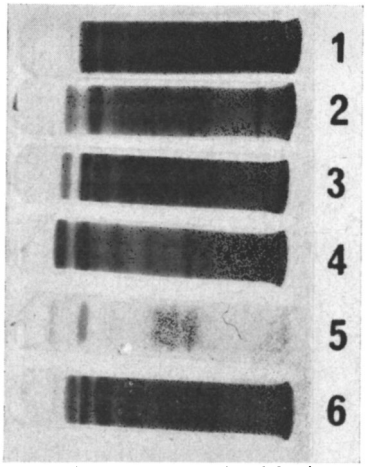

FIG. 2. Protein electrophoresis of brain protein extracted by various solvent systems. Note the lack of staining by the 1% $CaCl_2$ in butanol. 1. H_2O; 2. 1% $CaCl_2$; 3. 5% KCl; 4. 5% butanol; 5. 1% $CaCl_2$ in butanol; 6. 5% KCl in butanol.

same whether nitrogen or peptide linkage (Biuret reaction) was measured. Variation in the extraction procedure of the amount of nitrogen extracted varied between 7 and 10%.

Although different solvent systems solubilized similar amounts of protein, electrophoresis was used to qualify the kind of protein extracted. In Fig. 2 a select composite of our findings are presented. In general, while nitrogen and Biuret values of the extracts were similar, different proteins or altered migration of the same protein are seen.

A highly unexpected finding was the lack of protein staining in brain protein samples extracted with 1% calcium chloride in 5% butanol (Fig. 2). Lack of staining was not due to low protein concentration.

Effect of solvent system on brain isozyme. The extracting qualities of various concentrations of sodium chloride solutions on the isozyme pattern of enzyme activity between supernate and pellet were compared with distilled water. In these experiments freshly homogenized tissue as well as homogenates which were kept frozen for 24 hr were compared. There was some but not highly significant difference between LDH patterns obtained with 0.1% sodium chloride and 5.0% sodium chloride as compared to distilled water. The water extracts seem to give greater resolution and less smearing of the isozyme. At the end of a 24 hr period, the samples were rerun and except for a slight decrease in LDH activity (staining quality) at high salt concentration, approximately the same results were noted as for the fresh extracts.

When extraction solutions were made to the same salt concentration but with the addition of 5% butanol, a more dramatic effect on the brain isozymes was apparent. As can be seen from Fig. 3, the addition of 5% butanol had a significant effect on the isozyme pattern. The addition of 5% butanol to sodium chloride solutions caused increasing effects at high salt concentrations. Isozymes which migrate the fastest were least affected by this operation. The salt–alcohol extracts kept for 24 hr in the frozen state showed more marked effects when compared to the 5% butanol system alone. Under these conditions, only the 0.5% sodium chloride–5% butanol extract had any demonstrable brain lactic dehydrogenase activity in the fastest running component. After 24 hr only the 5% butanol group showed any activity. Samples tested for brain esterase activity with

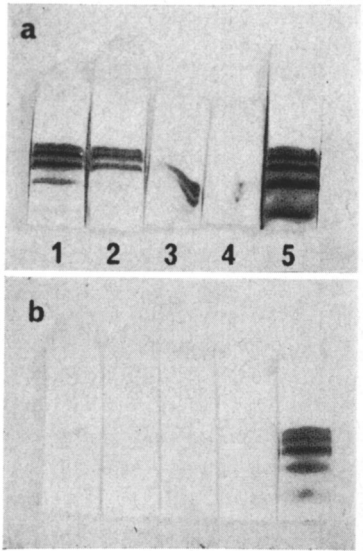

FIG. 3. Brain lactic dehydrogenase: a. fresh; b. after 24 hr. Extracting solvents: 1. 0.5% NaCl + 5% butanol; 2. 1.0% NaCl + 5% butanol; 3. 2.0% NaCl + 5% butanol; 4. 5.0% NaCl + 5% butanol; 5. 5% butanol.

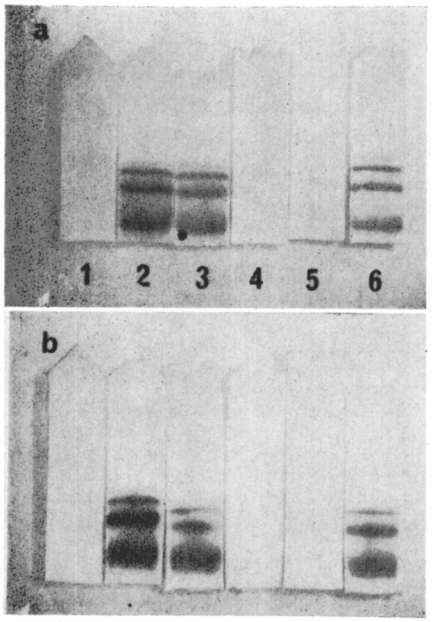

FIG. 4. Brain lactic dehydrogenase: a. fresh; b. after 24 hr. Extracting solvents: 1. 1% $CaCl_2$ + 5% butanol; 2. 1% $CaCl_2$ + 5% methanol; 3. 1% $CaCl_2$ + 5% ethanol; 4. 1% $CaCl_2$ + 5% propanol; 5. 1% $CaCl_2$ + 5% dioxane; 6. 1% $CaCl_2$.

these same solvents showed similar but less marked qualitative changes.

Similar experiments were carried out comparing 1% calcium chloride solutions alone or 1% calcium chloride with various alcohol or dioxane solutions (Fig. 4). These experiments indicated that the presence of calcium ion helped split LDH-5 (slowest bands) into at least four more subunits with enzymatic activity. While even fresh samples showed loss of LDH-1, -2, splitting of LDH-5 was more evident upon storage. Solvents containing higher alkyl chain alcohols were more effective in effecting lactic dehydrogenase isozyme patterns than the lower chain alcohols. The fact that the faster moving bands were affected more than the slower bands is opposite to that found for NaCl–butanol solvents.

Under the same experimental conditions (Fig. 5), brain esterases showed little if any differences in fresh tissue samples. It is only after storage of 24 hr that the effect of butanol and propanol could be seen. The general affect of solvents on esterase activity followed that of lactic dehydrogenase and will not be further discussed.

The effect of 1% calcium chloride solutions as well as various concentrations of calcium chloride solutions and 5% butanol were studied (Fig. 6). As can be seen, the higher concentration of calcium chloride in butanol seems to change the isozyme pattern to a greater extent than butanol alone. As indicated by previous experience, it is evident that the calcium chloride by itself, or the butanol alone, had little inactivating effect on brain lactic dehydrogenase isozyme. Also of interest and contrary to NaCl salt effects, the most forward moving bands were inactivated by calcium chloride salts.

This differential effect of salt solutions was studied by the addition of NaCl to the usual butanol–calcium systems (Fig. 7). Under these experimental conditions, the addition of 2% sodium chloride spares the inactivation of the brain lactic dehydrogenase. At higher salt concentrations (3–5%) isozyme changes due to too high a salt concentration becomes manifest. Potassium chloride at concentrations of 2–3% also reversed the effect of 1%

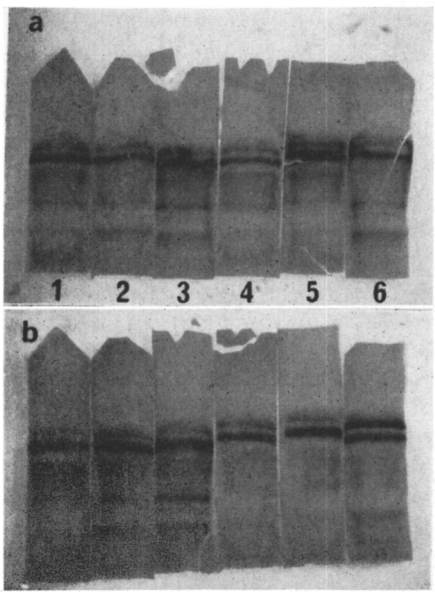

FIG. 5. Brain Esterase: a. fresh; b. after 24 hr. Extracting solvents: 1. 1% $CaCl_2$ + 5% butanol; 2. 1% $CaCl_2$ + 5% methanol; 3. 1% $CaCl_2$ + 5% ethanol; 4. 1% $CaCl_2$ + 5% propanol; 5. 1% $CaCl_2$ + 5% dioxane; 6. 1% $CaCl_2$.

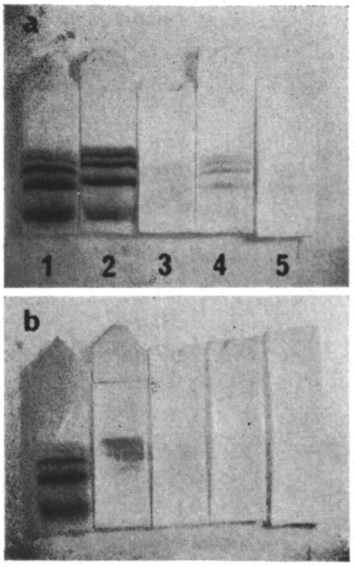

FIG. 6. Brain lactic dehydrogenase: a. fresh; b. after 24 hr. Extracting solvent: 1. 1.0% $CaCl_2$; 2. 0.1% $CaCl_2$ + 5% butanol; 3. 0.5% $CaCl_2$ + 5% butanol; 4. 1.0% $CaCl_2$ + 5% butanol; 5. 2.0% $CaCl_2$ + 5% butanol.

calcium chloride–1% butanol solution.

Effect of lipids on brain extracts. The protective effect of alkali metal ions on the effect of the calcium–butanol extracting solvent gave impetus to study the possible role of lipids on this phenomenon.

Mouse brains were extracted with a solvent using 1% calcium chloride and 1% butanol. Crude lipid extracts from 0.5, 1.0 and 2.0 g of liver tissue were then added to aliquots of homogenized brain tissue. The homogenates prepared in the usual way were tested at the end of 24 hr incubation. As can be seen from Fig. 8, the addition of crude lipids from mouse liver offered protection to solvent inactivation. Lipid extracts equivalent to 1.5–2.0 g of liver demonstrated the greatest protective effect.

This action by crude lipid extracts led to experiments with specific lipid fractions. In these experiments, the effect of phospholipids and neutral lipid fractions were compared (Fig. 9). The recombination of the two fractions was also compared. Under these conditions, it was seen that a small amount of phospholipid gave some protection in maintaining brain lactic dehydrogenase activity. Of greater interest, however, was the comparison of gels 4 and 5. The one (gel 4) had the phospholipid and neutral lipid combined after going through silicic acid chromatography, and the other (gel 5) being the crude lipid extract before chromatography. A marked difference in effect was noted between the crude lipids, fractionated and recombined crude lipids.

"Lipoprotein" nature of LDH isozymes. A commercial source (Sigma Chemicals) of lactic dehydrogenase LDH-1 and LHD-5 was studied for their lipid composition. While these samples were pure protein fractions as determined by electrophoresis, small amounts of impurities could still have been present without detection.

In these preliminary studies the individual enzymes were extracted for their lipid content with chloroform:methanol (2:1). The crude lipid extracts were divided and chromatographed in two different solvent systems. Hexane:diethylether:acetic acid (80:20:1.5) was used to chromatograph the neutral lipids while phospholipids were chromatographed using *n*-propanol:ammonium hydroxide (70:30).

Only one lipid spot was ascertained in either solvent system. The position of the spot (Figs. 10, 11) did not correspond to any known standard model compound. Further chemical analysis needs to be undertaken be-

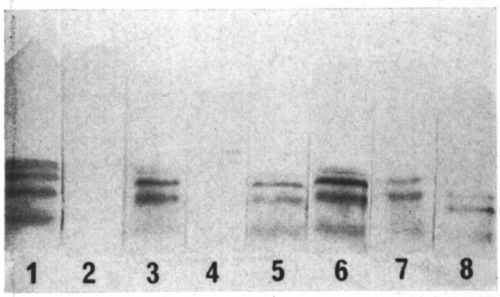

FIG. 7. Brain lactic dehydrogenase: 24 hr. Extracting solvents: 1. 1% butanol; 2. 1% butanol + $CaCl_2$ 3. 1% butanol + 5% NaCl; 4. 1% butanol + 1% $CaCl_2$ + 1.0% NaCl; 5. 1% butanol + 1% $CaCl_2$ + 2.0% NaCl; 6. 1% butanol + 1% $CaCl_2$ + 3.0% NaCl; 7. 1% butanol + 1% $CaCl_2$ + 4.0% NaCl; 8. 1% butanol + 1% $CaCl_2$ + 5.0% NaCl.

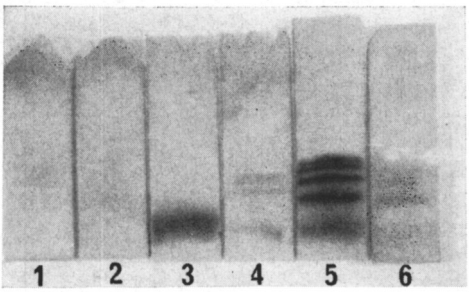

FIG. 8. Brain lactic dehydrogenase: 24 hr. Extracting solvents: 1. 1% $CaCl_2$ + 1% butanol + 0.5 g lipid equivalent; 2. 1% $CaCl_2$ + 1% butanol + 1.0 g lipid equivalent; 3. 1% $CaCl_2$ + 1% butanol + 1.5 g lipid equivalent; 4. 1% $CaCl_2$ + 1% butanol + 2.0 g lipid equivalent; 5. 1% butanol; 6. 1% $CaCl_2$ + 1% butanol.

fore identification can be made.

Discussion. The resolution of esterase and lactic dehydrogenase to more than one molecular species was treated with a great deal of skepticism by many biochemists. Isozymes, however, have proved to be quite stable through a variety of manipulations. This phenomenon of isozymes might be considered to have only esoteric biochemical significance, but the generality of the phenomenon suggests a broader significance. Each tissue within the animal contains characteristic assortment of isozymes.

Chemical models which help explain multiple enzyme forms have been proposed (7). The present study gives more details as to their chemical nature. The differential effect of alkali salts vs. calcium salts suggest a lipoprotein micelle. It was shown by Wohlman and Weiner in 1965 (8) that the effect of sodium and calcium on lipoproteins is to change the colloid from an oil–water pattern to a water–oil pattern. In other words, one assumes that the protein portion of a proteolipid is exposed in a sodium colloid milieu whereas a lipid surface is exposed in a calcium medium.

The period of profound changes in zymogram pattern and increased enzyme activity coincide with the initiation of myelin deposition throughout the rat brain. Folch-Pi (9) has shown the period of myelination in brain parallels formation of proteolipids and cerebrosides. It is interesting then to speculate whether isozyme activity may be related to the metabolism of these classes of compounds (10).

Major changes in LDH isozymes were also noted during the period of development at the time of onset of carbohydrate and lipid metabolism in hepatic cells of the chick (11) and suggested a possible correlation between these events. The distribution of LDH activity was somewhat correlative with the glycogen and lipid content of the cells (12).

Conklin, Dewey and May (13) found seven LDH isozymes in untreated rat kidney homogenate. Only a single band could be observed in the sample after butanol treatment. The explanation proposed by these authors is that the enzyme is associated with various lipoproteins or lipids which alter the electrophoretic mobility. If the isozymes were due to binding of a single enzyme component to various lipoprotein fractions, multiple dehydrogenase activities could be easily explained by assuming that each lipoprotein band bound a number of different dehydrogenases.

The above was criticized by Ressler (14). Butanol treatment provided no reason to question the concept that LDH exists in different molecular forms. Attempts were also made to detect specific lipid associated with the isozymes by staining technique but none could be demonstrated. Negative results by these staining techniques, however, have no equivocal meaning.

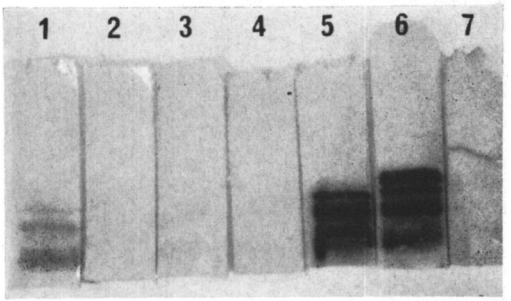

FIG. 9. Brain lactic dehydrogenase: 24 hr. Extracting solvents: 1. 1% $CaCl_2$ + 1% butanol + 0.5 g PL (Liver); 2. 1% $CaCl_2$ + 1% butanol + 1.5 g PL; 3. 1% $CaCl_2$ + 1% butanol + 2.0 g PL; 4. 1% $CaCl_2$ + 1% butanol + 2.0 g PL + NL; 5. 1% $CaCl_2$ + 1% butanol + 2.0 g crude lipids; 6. 1% butanol; 7. 1% $CaCl_2$ + 1% butanol.

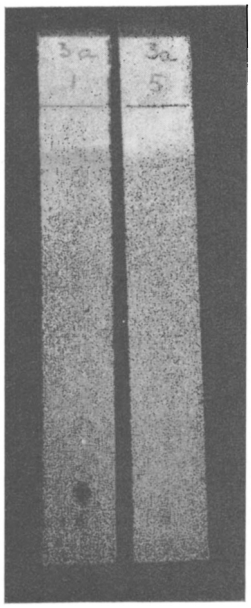

FIG. 10. Neutral lipids extracted from LDH_1 and LDH_5: Lipid extract of LDH_1 (left) and LDH_5 were compared in a neutral lipid solvent system, hexane:diethyl ether:acetic acid (80:20:15).

The results of Moss and Kind, 1962, may have a bearing on the subject. They found that a number of active alkaline phosphatase fractions could be extracted from segments of the gel after electrophoresis of concentrated butan-1-ol extracts of human bone, liver, kidney and small intestine (15). Each of the fractions from a given organ was said to have the same K_m value but there have been differences between fractions from different tissues. It was suggested that the different bands were possibly complexes with different proteins but actually fractions of the same enzyme. Recovery of activity from the β-lipoprotein region by freezing and thawing followed by a second electrophoresis fractionated the band into a portion moving with the original mobility together with a faster moving component. It was suggested that the latter could have resulted from the dissociation or degradation of a complex between the enzyme and the lipoprotein.

Addition of acetone to serum inactivates all isozymes except LDH-1 and this effect has been used in a simple test for this isozyme in serum (Latner and Turner, 1963).

Chloroform has also been shown to inhibit all except the fastest moving serum LDH isozyme (Warburton *et al.*, 1963). If diluted serum is shaken with choloroform, the enzyme activity remaining is a good measure of the amount of the serum enzyme which is of cardiac origin alone.

Our own results with brain LDH using organic solvents as part of the extracting solution also tended to affect the slowest moving band first (LDH-5).

The protection offered by lipids to the slower moving bands in our experiments is not explainable at this time. Also, the recombination of fractionated neutral and phospholipid fractions was not as effective as the crude lipid extracts. One possibility raised was that some protective peptide or lipid was left on the column during fractionation.

The experiments indicating a higher concentration of unknown lipids in LDH-1 vs. LDH-5 may explain their differential response to the chemical solvents and storage. Tentative identification of the lipid suggests it may be a glycolipid.

While the initiation of these experiments

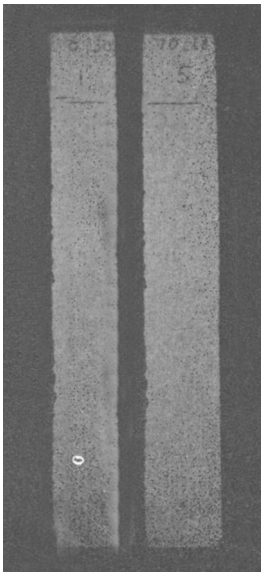

FIG. 11. Phospholipids extracted from LDH_1 and LDH_5: Lipid extract of LDH_1 (left) and LDH_5 were compared in a phospholipid solvent system *n*-propanol:ammonium hydroxide (70:30).

was begun in the hope of finding a solvent system which would best extract brain protein, some interesting results were generated. The results of the various salt solutions and alcohol solutions all seem to indicate, or at least be explained, by the fact that we are dealing with a lipoprotein. The differential effect of sodium and calcium on enzyme activity certainly fits this pattern. The addition of crude lipids showing some protective action is more circumstantial evidence that lipids are involved in certain brain isozymes. The fact that more lipids could be extracted from LDH-1 than LDH-5 plus the greater stability of LDH-1 adds credence to this kind of interpretation. Obviously, many more experiments must be done before the real role of lipids can be implicated in isozyme activity. However, we feel that the experiments are sufficiently broad based and sound to give direction for other experiments in this field.

Summary. Mouse brains were extracted with a variety of solutions composed of inorganic salts, organic solvents, or mixtures of each. During these experiments, it was noted that a differential effect of certain salts and solvents on isozyme patterns could be demonstrated. These effects of isozyme systems [lactic dehydrogenase (LDH) and nonspecific esterase] could best be explained on the basis of lipid–enzyme complexes. Circumstantial evidence is provided for viewing LDH as a lipoprotein catalyst.

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