In the initial experiment, C_3H/Hen mice were injected subcutaneously with *E. coli* endotoxin in doses of 0.5 and 0.005 mg daily, five times per week. Animals were killed after 15 and 30 injections, and histologic sections of the spleen, liver, and kidney were examined. Amyloid was present in the spleen in 2 of 17 mice examined after 30 injections (Table 1).

White Swiss mice (G.P.) were selected for the next experiment, because they develop amyloidosis more readily and in greater amounts after casein than the C_3H mice do (*3*). These G.P. mice were similarly treated with daily doses (0.25 ml) containing 0.5 or 0.005 mg of lot 1 endotoxin administered subcutaneously five times a week. Control mice were injected with phosphate-buffered saline (0.25 ml) on an identical schedule.

Every G.P. mouse examined after 25 injections of the high dose of endotoxin had moderate to extensive deposits of amyloid in the spleen (Fig. 1). Four of 11 mice receiving the lower dose also had splenic amyloid. In three of these mice, deposits were focal and limited, but in the fourth there was perifollicular deposition around almost every follicle. The amyloid in all cases had the characteristic green birefringence after polarization microscopy of sections stained with Congo red. None of the animals receiving buffered saline developed amyloidosis.

Using lot 2 *E. coli* endotoxin, we found splenic amyloid in every one of the 22 G.P. mice examined after 15 or 20 injections (Table 1). In the mice with extensive splenic amyloid, hepatic and renal amyloid was also detected.

Although relatively small numbers of animals have been used, the findings reported here are with two separate lots of endotoxin and in two strains of mice. In studies to be reported (*4*), we have seen splenic amyloid in G.P. mice as early as 1 week (after five injections) and in most animals within 2 weeks of starting administration of endotoxin.

Many of the features of amyloidosis induced by endotoxin are similar to those seen following repeated casein administration (*3*); histologic involvement of the spleen, liver, and kidney are comparable. The higher incidence of amyloid in the White Swiss (G.P.) mouse after injection of endotoxin as compared to the C_3H/Hen mouse parallels the different susceptibility of the two strains to amyloid induced by administration of casein (*3*). In preliminary studies we found that repeated injections of endotoxin reproduce the abnormality in ribosome function (*5*) found after casein administration.

Endotoxin is a lipopolysaccharide derived from the cell walls of Gram-negative, as well as of some other, bacteria (*6*). This material is potent at very low doses and has many biological effects, including metabolic, circulatory, and immunological (*6*). The ability of endotoxin to induce amyloidosis can now be added to this list. Because endotoxin is a ubiquitous substance, inactivated only after autoclaving at a high temperature for several hours and because as little as 100 μg was able to induce amyloid, it is possible that endotoxin may also be involved in other types of experimental amyloidosis. Furthermore, endotoxin may play a role in human secondary amyloidosis, particularly those forms associated with chronic infection.

Endotoxin is rapidly localized in the reticuloendothelial system after parenteral administration, as has been demonstrated with labeled endotoxin (*7*) and with fluorescent antibody studies (*8*). Cohen's concept of the pathogenesis of amyloid suggests that chronic stimulation of the reticuloendothelial cell leads to amyloid production (*1*). The ability of endotoxin to induce amyloid lends support to this hypothesis.

WERNER F. BARTH*
JOYCE K. GORDON
JAMES T. WILLERSON
Arthritis and Rheumatism Branch, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

References and Notes

1. A. S. Cohen, in *International Review of Experimental Pathology*, G. W. Richter and M. A. Epstein, Eds. (Academic Press, New York, 1965), vol. 4, pp. 159–243.
2. Difco Laboratories, Inc., Detroit, Mich.; lot 1, 510667 and lot 2, 513390.
3. J. T. Willerson and W. F. Barth, in preparation.
4. W. F. Barth, J. T. Willerson, S. M. Wolff, in preparation.
5. N. Talal and W. F. Barth, *Clin. Res.* **16**, 325 (1968).
6. I. L. Bennett and L. E. Cluff, *Pharm. Rev.* **9**, 427 (1957); H. Raskova and J. Vanecek, *ibid.* **16**, 1 (1964).
7. D. Rowley, J. G. Howard, C. R. Jenkin, *Lancet* **1956-I**, 366 (1956); H. E. Noyes, C. R. McInturf, G. J. Blahuta, *Proc. Soc. Exp. Biol. Med.* **100**, 65 (1959).
8. N. Cremer and D. W. Watson, *Proc. Soc. Exp. Biol. Med.* **95**, 510 (1957).
9. We thank Dr. S. M. Wolff, NIH, for his helpful suggestions in this study.
* Present address: University of Maryland School of Medicine, Baltimore, Maryland 21201.

9 September 1968

Lactate Dehydrogenase Isozymes: Dissociation and Denaturation by Dilution

Abstract. *The tetrameric enzyme lactate dehydrogenase dissociates into its constituent monomeric subunits in a high ion to protein concentration ratio, in certain ionic environments when frozen, and at extreme dilution. Dissociation by dilution involves changes in tertiary conformation which inactivate the enzyme. The dissociation is strongly inhibited by homologous native proteins, but only slightly by denatured or unrelated proteins.*

The enzyme lactate dehydrogenase (LDH) exists in many organisms in isozymic (multiple molecular) forms (*1, 2*). It is a tetramer with a molecular weight of approximately 140,000 and a sedimentation coefficient, at infinite dilution, of approximately 7.50*S* (*3*). This enzyme can be induced in vitro to undergo subunit reassociation, that is, molecular hybridization (*4*). Two different subunits, A and B, associate to yield five tetramers, conveniently designated LDH-1 (B_4), LDH-2 (A_1B_3), LDH-3 (A_2B_2), LDH-4 (A_3B_1), and LDH-5 (A_4). Dissociation and recombination of these subunits can be accomplished via reversible denaturation of the LDH tetramer at low *p*H, or by the use of urea, guanidine-hydrochloride, or LiCl at supraeutectic temperatures (*5, 6*), or by freezing and thawing isozymes in the presence of specific hybridization promoting ions (*4, 5, 7, 8, 9*). In addition, prolonged dialysis of certain LDH isozyme combinations against saturated NaCl solutions produces molecular hybridization (*2, 10*). These results suggest that a high ratio of ion to protein is a fundamental requirement for reversible subunit dissociation.

It has also been reported that, at protein concentrations less than 0.5 mg/ml in NaCl solutions, the enzyme dissociates into a dimer with a sedimentation coefficient of 5.5*S* and a molecular weight of about 72,000 (*11*). These results suggest that the enzyme

Table 1. Effect of proteins on freeze-thaw hybridization of LDH isozymes. In the enzyme mixture, the total enzyme protein concentration was approximately 0.5 mg per milliliter of neutral 0.1*M* sodium phosphate buffer (except as indicated below). The mixtures of beef LDH gave the same results as the horse LDH. In assessing the hybridization of the mixtures, the following form is used: three pluses indicate complete random reassociation of subunits as evidenced by a binomial distribution of isozymes; two pluses indicate an equal distribution of staining intensity among the five isozymic forms; one plus denotes the formation of small quantities of the heteropolymeric isozymes; a minus sign indicates no effect. Abbreviations are: h, horse; b, beef; s, shark; y, yeast; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; BSA, bovine serum albumin.

Isozyme mixture	Additive		Hybridization
	Substrate	Amount (mg/ml)	
hLDH-1 + hLDH-5			+++
hLDH-1 + hLDH-5*			−
hLDH-1 + hLDH-5	BSA	300	++
bLDH-1 + hLDH-5	BSA	300	+
hLDH-1 + hLDH-5	sLDH-3	20	−
hLDH-1 + hLDH-5	bLDH-1	25†	+++
hLDH-1 + hLDH-5	hLDH-1	25†	+++
hLDH-1 + hLDH-5	yADH	25	+++

* Total enzyme protein concentration approximately 2.0 mg/ml. † Heat inactivated LDH.

must be active in the dimeric state since enzymatic assays are usually carried out at enzyme concentrations of less than 1.0 μg/ml. If the dimeric enzyme is the catalytically active form, then the equilibrium between dimer and tetramer might be significant in regulating the function of the enzyme in the living cell. We performed the following experiments to examine the molecular state of LDH (*12*) at various concentrations and in the presence of several different ionic environments. By manipulating ion to protein ratios from the standpoint of concentration and molecular species we hoped to disclose the mechanism of in vitro subunit reassociation. In the initial experiments a mixture of LDH-1 and LDH-5 from horse muscle was dialyzed against neutral 0.1*M* sodium phosphate buffer, at a total enzyme protein concentration of 1.0 mg/ml (*7*). This mixture of isozymes was serially diluted 1:2 to a concentration of approximately 0.25 μg/ml, and all 13 dilutions of the series were incubated either at 4° or 23°C from 0 to 48 hours. Molecular hybridization was assessed by tetrazolium staining after starch-gel electrophoresis (*7*). As little as 0.1 unit (*12*) of enzyme activity is detectable by the tetrazolium method, and the enzymatic activity of our preparations was in excess of 500 unit/mg. When necessary, an entire dilution was concentrated by vacuum dialysis and the entire concentrate was applied to the gel.

Horse LDH-1 and LDH-5 (both homopolymers) readily undergo molecular hybridization in vitro after being frozen and then thawed in an appropriate ionic environment. Thus, if these isozymes dissociate at high dilution, as reported for beef LDH (*11*, *13*, *14*), then heteropolymer formation should occur when the samples are concentrated. Thus, if the A_4 and B_4 homopolymers were to dissociate into dimers, formation of the A_2B_2 heteropolymer (LDH-3) as well as the homopolymers would be expected upon reconcentration. If the initial dissociation proceeded to the monomer level, then the A_1B_3 (LDH-2) and A_3B_1 (LDH-4) heteropolymers should also be formed. However, in none of our dilution experiments with horse LDH was the formation of heteropolymers observed. Repetition of the experiments with beef LDH likewise failed to generate any heteropolymers.

Reassociation of subunits of LDH molecules from most species may be accomplished by subjecting isozyme mixtures to a freeze-thaw cycle in the following solutions: (i) neutral phosphate buffer containing an appropriate concentration of halide ions (excepting F^-), thiocyanate, 1-anilinonapthalene-8-sulfonate, or urea; (ii) neutral solution of sodium arsenate and HCl; and (iii) neutral solution of sodium nitrate and NaOH solutions (*4*, *5*, *7*, *8*, *9*). In most procedures for obtaining molecular hybridization, total enzyme protein concentrations in excess of 1.0 mg/ml (approximately $7.0 \times 10^{-6} M$) are used. At such enzyme concentrations tris(hydroxymethyl)amino methane-hydrochloride (tris-HCl) is a potent inhibitor of hybridization (*7*, *8*, *9*). However, we have now observed that when the total enzyme protein concentration of mammalian isozymes is reduced to less than 0.5 mg/ml, both intra- and interspecific freeze-thaw hybridization readily occurs in neutral sodium phosphate buffer alone or in neutral tris-HCl. Typical results of such experiments are shown in Figs. 1 (intraspecific hybridization) and 2 (interspecific hybridization) along with results obtained in distilled water. Only in distilled water (resistance = 2.0 megohms) did freeze-thaw hybridization not occur.

This relation between protein concentration and hybridization may be related to the function of bovine serum albumin (BSA) in inhibiting denaturation of dilute solutions of various enzymes and other proteins (*13*). However, whereas BSA is effective only at relatively high concentrations, a small change in LDH concentration markedly inhibits hybridizability of the isozymes. In order to examine the effect on molecular hybridization of various proteins in different structural states we added separately BSA, partially purified shark LDH-3, and heat-inactivated beef and horse LDH-1 to mixtures of horse LDH-1 and -5 and to mixtures of beef LDH-1 and horse LDH-5 (Table 1). Heat inactivation changes the conformation of LDH and essentially destroys its capacity to function as an inhibitor of molecular hybridization, even at 50 times the concentration at which native LDH no longer hybridizes. The concentration of shark LDH-3 reported in Table 1 was not its minimal inhibiting concentration. This particular concentration was chosen to contrast the effectiveness of LDH from an unrelated species with that of heat inactivated homologous (horse) LDH. Clearly, protein to protein interactions must occur to prevent molecular hybridization and these interactions are

Table 2. Effect of coenzymes and substrates on freeze-thaw hybridization of LDH isozymes. In the enzyme mixture, the total enzyme protein concentration was 0.5 mg/ml neutral 0.1*M* sodium phosphate buffer. The hybridization is assessed in the same manner as in Table 1. Abbreviations are: NAD, NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; AcPyAD, 3-acetyl pyridine analog of NAD; TNAD, thionicotinamide analog of NAD.

Additive	Molar ratio (additive /enzyme)	Hybridization
NAD or NADH	$4.3 \times 10^3 : 1$	+++
	$8.6 \times 10^3 : 1$	+++
	$1.3 \times 10^4 : 1$	++
	$1.7 \times 10^4 : 1$	++
AcPyAD	$4.3 \times 10^3 : 1$	−
TNAD	$4.3 \times 10^3 : 1$	−
Li lactate	$2.9 \times 10^4 : 1$	+
Na pyruvate	$2.6–7.8 \times 10^4 : 1$	+++

dependent upon a precise conformation of the interacting proteins. The conformation may be changed by denaturation or may be inherently inactive because of primary structure.

It has been reported that nicotinamide adenine dinucleotide (NAD) and its analogs protect LDH against freeze-thaw denaturation (*15*). Furthermore, the reduced analogs inhibit freeze-thaw hybridization in sodium phosphate-NaCl solutions at enzyme concentrations in excess of 1.0 mg/ml (*8*). Since changes in enzyme conformation and stability do occur in different ionic environments and after combination with substrates, the effects of NAD and some of its analogs on freeze-thaw hybridization at low LDH concentrations (approximately 0.5 mg/ml) in neutral 0.1*M* sodium phosphate buffer were investigated (Table 2). The effects of lactate and pyruvate can all be interpreted in terms of a primary effect on protein conformation.

Evidence from several laboratories indicates that LDH undergoes dissociation and denaturation when sufficiently diluted. We have not been able to separate these two effects of dilution, and our evidence demonstrates, at least, that enzymatically active dimers which are capable of reassociating at random into tetramers are not produced by diluting concentrated enzyme preparations. Although dilution may promote dissociation of the LDH tetramer into dimers and monomers, the tertiary structure of such subunits must be disrupted because they do not readily reform functional tetramers. The concentration of LDH also has important effects on the dissociability of the tetramer, even under those conditions that do result in subunit denaturation. High concentrations of LDH do not dissociate readily even in the most favorable ionic environments. Thus intermolecular interactions must occur to maintain the tetrameric state of the native LDH. This interaction among LDH molecules shows little or no species specificity. Shark LDH was quite effective in preventing the dissociation of horse LDH. However, denatured LDH, even from the same species and in very high concentration, did not prevent dissociation of LDH upon freezing in a favorable ionic environment. Unrelated proteins such as bovine serum albumin or yeast alcohol dehydrogenase were likewise without effect on the dissociation of LDH.

Normal substrates and cofactors have little influence on LDH dissociation but the acetylpyridine and thionicotinamide analogs of NAD strongly inhibit dissociation of LDH. These results emphasize the fact that the dissociation of LDH is affected by many different molecular environments, not only by the ratio of ion to protein but also by molecular interactions mediated through changes in protein conformation. However, simple dilution of the enzyme in phosphate buffer appears not to promote dissociation without denaturation of the subunits. Therefore, in buffer solutions at all concentrations the tetramer appears to be the only enzymatically active unit.

CLEMENT L. MARKERT
EDWARD J. MASSARO
Department of Biology,
Yale University,
New Haven, Connecticut 06520

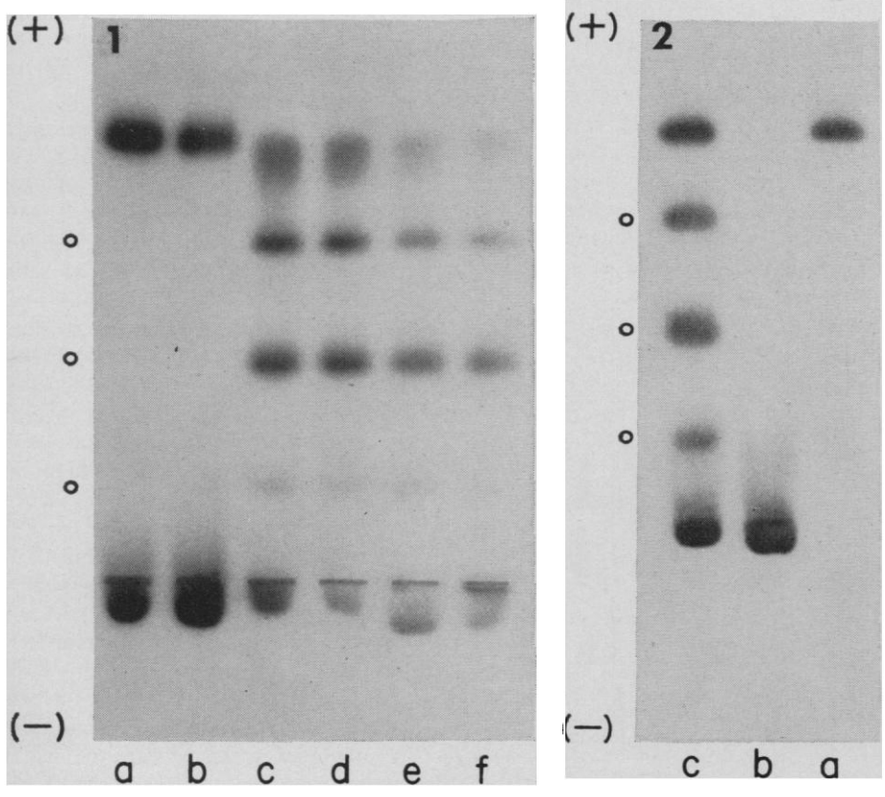

Fig. 1 (left). Intraspecific hybridization of LDH isozymes. Horse LDH-1 plus horse LDH-5. Total enzyme protein concentration was 0.5 mg/ml. The enzyme protein was frozen for 66.5 hours at −20°C, and then thawed at room temperature. Starch-gel electrophoresis was conducted at 23°C for 5.0 hours at 10 volt/cm. (*a*) LDH-1 plus LDH-5 in neutral 0.1*M* sodium phosphate buffer, unfrozen. (*b–f*) Effects of freezing-thawing in: (*b*) distilled water; (*c* and *d*) neutral 0.1*M* sodium phosphate buffer; (*e* and *f*) neutral 0.1*M* tris-HCl.

Fig. 2 (right). Interspecific hybridization of LDH isozymes. Horse LDH-1 plus pig LDH-5. Total enzyme protein concentration was 0.5 mg/ml in 0.1*M* tris-HCl, *p*H 7.0. The sample was frozen for 16 hours at −20°C and then thawed at room temperature. Starch-gel electrophoresis as in Fig. 1. (*a*) LDH-1; (*b*) LDH-5; (*c*) LDH-1 plus LDH-5. Identical results were obtained in neutral 0.1*M* sodium phosphate buffer.

References and Notes

1. C. L. Markert and F. Moller, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).
2. C. L. Markert, *Harvey Lect.* **59**, 187 (1965).
3. E. Appella and C. L. Markert, *Biochem. Res. Commun.* **6**, 171 (1961); R. Jaenicke and S. Knof, *Eur. J. Biochem.* **4**, 157 (1968).
4. C. L. Markert, *Science* **140**, 1329 (1963).
5. S. Anderson and G. Weber, *Arch. Biochem. Biophys.* **116**, 207 (1966).
6. O. P. Chilson, G. B. Kitto, J. Pudles, N. O. Kaplan, *J. Biol. Chem.* **241**, 2431 (1966); G. J. Epstein, M. M. Carter, R. F. Goldberger, *Biochim. Biophys. Acta* **92**, 391 (1964).
7. C. L. Markert and E. J. Massaro, *Arch. Biochem. Biophys.* **115**, 417 (1966).
8. O. P. Chilson, L. A. Costello, N. O. Kaplan, *Biochemistry* **4**, 271 (1965).
9. E. J. Massaro, *Biochim. Biophys. Acta* **147**, 45 (1967).
10. O. P. Chilson, L. A. Costello, N. O. Kaplan, *J. Mol. Biol.* **10**, 349 (1964).
11. D. B. S. Millar, *J. Biol. Chem.* **237**, 2135 (1962).
12. LDH was obtained from horse (*Equus caballus*), beef (*Bos taurus*), pig (*Sus scrofa*), rabbit (*Lepus cunciculus*), and shark (*Carcharrinus platyodon*) tissues (7). Reagents were of the highest quality commercially available and were used without further purification. One unit of LDH activity is the amount required to reduce one micromole of NAD per minute.
13. P. Bernfeld, B. J. Berkeley, R. E. Bieber, *Arch. Biochem. Biophys.* **111**, 31 (1965).
14. G. Hathaway and R. S. Criddle, *Proc. Nat. Acad. Sci. U.S.* **56**, 680 (1966).
15. C. H. Lea and J. C. Hawke, *Biochem. J.* **52**, 105 (1952); H. A. Zondag, *Science* **142**, 965 (1963).
16. Supported by NSF grant GB 5440X.

10 July 1968; revised 23 August 1968