Studies on the Mechanism of Hybridization of Lactic Dehydrogenases *in Vitro**

Oscar P. Chilson,† Louis A. Costello, and Nathan O. Kaplan

ABSTRACT: The nature of subunit-subunit interactions in lactic dehydrogenases has been investigated by studying the conditions which are required for hybridization *in vitro*. The effects of various pyridine nucleotides, temperature, and different ions on hybridization as well as inhibition by certain organic compounds have been examined. Under the conditions used, hybrids were not formed without freezing and the rate of hybrid formation was faster if sodium phosphate plus either thiocyanate or a halide ion were present. The order of effectiveness was as follows: $SCN^- , I^- > Br^- > Cl^- >>> F^-$. Phosphate could not be replaced by arsenate, pyrophosphate, or sulfate. Reduced acetylpyridine analog of DPNH (AcPyDPNH), DPNH, and DPN were found to be good protectors against enzyme inactivation during freezing and thawing but only AcPyDPNH inhibited hybrid formation. In the presence of AcPyDPNH, chicken heart lactic dehydrogenase and beef heart lactic dehydrogenase appeared to dissociate and recombine (or exchange subunits) at unequal rates. Data are presented suggesting that the mechanism of hybridization of lactic dehydrogenases *in vitro* may involve some conformational changes and that part of the inactivation which occurs as a result of freezing and thawing is caused by oxidation of sulfhydryl groups. Hybridization of lactic dehydrogenase subunits may occur as a result of a combination of increased salt and protein concentration and/or decreased *p*H near the eutectic point.

Specific ion effects, weakening of hydrophobic forces, and freezing out of bound water at low temperature may also be rate-limiting factors. Since hybridization occurs between lactic dehydrogenases from widely divergent species, it is suggested that during evolution there has been a considerable degree of conservation of structural features which are required for tetramer formation in lactic dehydrogenases.

There is a considerable amount of catalytic, physical, and immunological evidence that lactic dehydrogenase is composed of four subunits and that various multiple forms which are found in nature are hybrids containing 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 LDH[1] in sodium chloride (1 M) causes the formation of five multiple forms in a binomial pattern. The implication was that *in vitro* hybridization occurred via complete dissociation and random recombination of subunits, but the mechanistic aspects of this novel intermolecular rearrangement remain obscure. We have studied the conditions which are required for hybridization in order to gain an understanding of the nature of subunit-subunit interactions. In the course of the present studies we have examined the effects of various pyridine nucleotides, temperature, and different ions on hybridization as well as the inhibition by certain organic compounds. We have also studied the effect of hybridizing conditions on reactivity of sulfhydryl groups with *p*-mercuribenzoate.

Materials and Methods

Acetylpyridine-DPN (AcPyDPN) was purchased from the Pabst Laboratories and reduced enzymatically according to the general method described previously (Anderson *et al.*, 1959). DPNH and *p*-mercuribenzoate were purchased from the Sigma Chemical Co. Beef H_4 and chicken H_4 were prepared as described by Pesce *et al.* (1964) and dogfish M_4 was prepared by Mr. M. Buchwald and Mr. F. Stolzenbach of this laboratory. All enzymes were crystalline homogeneous preparations unless otherwise indicated. Protein was determined by measuring the extinction at 280 mμ; molecular weights of the LDH's were assumed to be 140,000 and ϵ_{max} at 280 mμ was assumed to be 1.8×10^5 for chicken H_4 and 2.0×10^5 for all other enzymes used. *p*-

* Publication No. 335 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. *Received October 22, 1964.* Supported in part by grants from the American Cancer Society (P-77F) and the National Institutes of Health (CA-03611), and by a contract (PH-43(64)534) from the National Institutes of Health. Part of the data included in this paper has been presented in preliminary form at the 48th Annual Meeting of the Federation of American Societies of Experimental Biology, Chicago, 1964.

† Postdoctoral Fellow of the U.S. Public Health Service (5-F2-GM-13, 398-02) from the National Institute of General Medical Sciences.

[1] Abbreviations used in this work: AcPyDPNH, reduced acetylpyridine analog of DPNH; LDH, lactic dehydrogenase; chicken H_4, chicken heart LDH; beef H_4, beef heart LDH; dogfish M_4, dogfish muscle LDH; rabbit H_4, rabbit heart LDH; ϵ_{max}, molar extinction coefficient; TMV, tobacco mosaic virus.

Mercuribenzoate binding was assayed spectrophotometrically as described by Boyer (1954) and an ϵ_{max} of 7.6×10^3 was applied for increase in optical density at 250 mμ.

Reagent grade ethylene glycol and propylene glycol were purchased from the Fisher Scientific Co. and used without further purification. Starch-gel electrophoresis and enzyme activity measurements were carried out as described previously (Fine *et al.*, 1963). Starch gels were stained for protein with amido black using the method described by Smithies (1959). All *p*H determinations were apparent, uncorrected values obtained at room temperature on a Beckman "Zeromatic" *p*H meter.

Results

Freezing Requirement. In order to determine whether freezing was required for hybridization, equal concentrations of beef H_4 and chicken H_4 were mixed in 0.9 M NaCl + 0.1 M sodium phosphate (*p*H 7.0) to give a final enzyme concentration of 7.6×10^{-6} M. One-ml samples were placed in a dry ice and methanol bath and unfrozen center portions were withdrawn during the freezing process. A control tube was kept on ice and the frozen fractions were allowed to thaw at room temperature before being placed on ice. After all samples were dialyzed overnight at 4° against 0.1 M potassium phosphate (*p*H 7.0), to lower the salt concentration, the various mixtures were resolved by starch-gel electrophoresis. Table I summarizes the results of this experiment. The control and the unfrozen center portions did not contain hybrids and there was only a slight loss of enzyme activity; considerably more activity was lost in the frozen fractions and starch-gel electrophoresis demonstrated a binomial distribution of the LDH forms.

Some concentration of protein was found in the center portion; this was particularly marked in the sample removed 20 seconds after placing the tube in the freezing bath. In these and all subsequent experiments hybridization did not occur when the sodium chloride concentration was 1 M or less, unless the samples were frozen—even after storage for several days at temperatures between 0 and 4°.

Effects of Rates of Freezing and Thawing. Having determined that freezing was required for hybrid formation, it was of interest to determine whether hybridization occurred during the freezing or during the thawing step. It was found that this problem could be investigated by varying the rates of freezing and thawing. When 1-ml samples were placed in a deep freeze at −20°, approximately 30–60 minutes were required for complete freezing to occur; this type of freezing will be referred to as "slow freeze." In dry ice and methanol (approximately −70°) samples could be frozen in 30 seconds or less, and in liquid nitrogen (approximately −195°) freezing occurred almost instantly. The dry ice–methanol procedure will be referred to as "quick freeze." The rate of thawing could be appropriately modified by either shaking under running tap water for 10–20 seconds or letting the samples stand at room temperature for approximately 20 minutes. These methods of thawing will be referred to as "quick-thaw" and "slow-thaw," respectively. Table II shows that if mixtures of dogfish M_4 and chicken H_4 were frozen and thawed quickly there was little loss of enzyme activity, and no hybrids were formed. Slow freezing led to hybridization and partial loss of enzyme activity, regardless of the rate of thawing.

TABLE I: Analysis of Frozen Fractions and Unfrozen Fractions Removed during the Freezing Process.[a]

Sample	Fraction	Units/mg Protein	Hybridization
I (Control kept at 0°)		760	−
II	Frozen	410	+
	Unfrozen	650	−
III	Frozen	450	+
	Unfrozen	755	−

[a] All samples were in 0.9 M NaCl + 0.1 M Na_3PO_4 (*p*H 7.0), and freezing was in dry ice + methanol. A unit of enzyme activity is defined as that amount of enzyme causing a change in optical density at 340 mμ of 1.0/min under the assay conditions. Samples II and III were placed in the freezing-bath and the outer portion began to freeze first; unfrozen center portions were removed with a Pasteur pipet at 10 seconds and 20 seconds, respectively, after placing the tubes in the bath. The frozen portion was held in the bath for a total of 1 minute and then thawed at room temperature. The enzyme mixture (final concentration 7.6×10^{-6} M) contained equimolar amounts of chicken H_4 and beef H_4.

TABLE II: Comparison of Rates of Freezing and Thawing on Enzyme Activity and Hybridization of Beef H_4 and Dogfish M_4.[a]

Rate of Freezing	Thawing Rate	Enzymatic Activity Remaining (%)	Hybridization
Fast	Fast	90	−
Fast	Slow	63	+
Slow	Slow	33	+
Slow	Fast	44	+

[a] All samples were in 0.9 M NaCl + 0.1 M Na_3PO_4 (*p*H 7.0). Fast freezes were in dry ice + methanol and slow freezes at −20°. The total enzyme concentration was 7.6×10^{-6} M. (See text for explanation.)

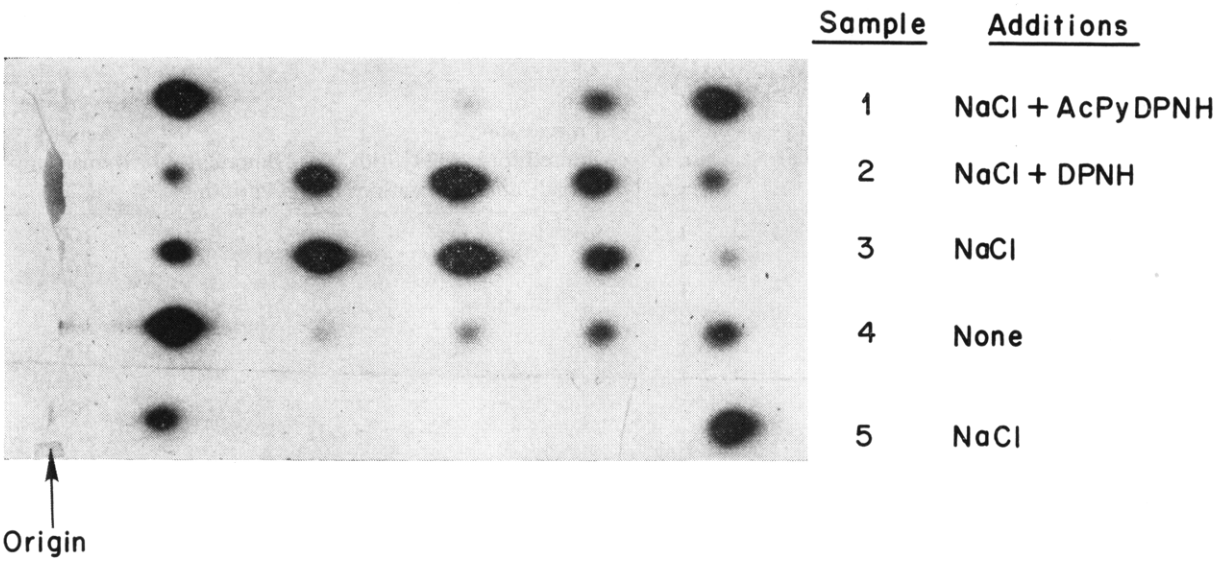

FIGURE 1: Effect of AcPyDPNH and DPNH on hybridization of beef H_4 and chicken H_4. Beef H_4 is the most anodically migrating species and chicken H_4 is the least anodically migrating species. All samples contained equal mixtures of beef H_4 and chicken H_4 at a final concentration of 7.6×10^{-6} M in 0.1 M sodium phosphate (*p*H 7.0). Samples 1, 2, 3, and 5 contained 1 M NaCl and samples 1 and 2 contained AcPyDPNH (6.1×10^{-2} M) and DPNH (6.1×10^{-2} M), respectively. None indicates no NaCl in sample 4. All samples were slow-frozen at $-20°$ and thawed at room temperature, except the control (number 5) which was kept at 0°, and the electrophoretic patterns were obtained by staining for enzyme activity.

In order for hybrids to form in a quick-frozen sample it must be thawed slowly; the hybrid formation is accompanied by some inactivation. There was no distinction between hybrid patterns in samples frozen in dry ice plus methanol and those frozen in liquid nitrogen. Binomial patterns were observed in all cases if slow thawing was carried out.

Effects of Pyridine Nucleotides. Reduced DPN and its analogs have been shown to inhibit the dissociation of LDH into subunits by sodium dodecylsulfate (Di Sabato and Kaplan, 1964) and inactivation by sulfhydryl reagents (Di Sabato and Kaplan, 1963); in addition it has been reported that DPN reduces the extent of inactivation at $-20°$ (Zondag, 1963). Therefore it was of interest to examine the effects of these compounds on hybridization. Figure 1 is a starch-gel pattern showing the effects of various compounds on hybrid formation between chicken H_4 and beef H_4. One-ml samples containing equimolar mixtures of the two enzymes in 0.1 M sodium phosphate (*p*H 7.0) plus the additions indicated were slow-frozen once at $-20°$, thawed at room temperature, dialyzed overnight against 0.1 M sodium phosphate (*p*H 7.0) at 4°, and subjected to starch-gel electrophoresis at *p*H 7.0. The control (sample 5) was not frozen and contained 1 M NaCl; dialysis was carried out as with the experimental samples. Sample 4 contained only 0.1 M sodium phosphate and under these conditions only partial hybridization occurred. The patterns observed after addition of sodium chloride with and without DPNH were essentially the same, but marked inhibition of the hybridization was found in the presence of AcPyDPNH. The pattern observed in the presence of AcPyDPNH suggested that repeated freeze-thawing might lead to a binomial distribution of products. The results of such an experiment are shown in Figure 2, where it is evident that the pattern shifted after multiple freezes and that a nearly binomial pattern was obtained after freezing and thawing the material three times. It is important to note that in Figures 1 and 2 the starch-gel patterns were developed by staining for enzyme activity; identical patterns, however, were also observed when the gels were stained for protein with amido black. The results of these experiments suggest that in the presence of AcPyDPNH dissociation and recombination of the two enzymes does not occur at equal rates. As might be expected, much greater inhibition was observed when similar samples containing AcPyDPNH were quick-frozen in dry ice and methanol. No hybrids were formed unless samples were frozen and thawed many times, and a binomial pattern was never attained.

The effects of varying the ratio of DPN^+, DPNH, and AcPyDPNH to the level of enzyme on hybridization and enzyme activity of beef H_4 and dogfish M_4 are shown in Table III. There was no inhibition of hybrid formation by either DPN^+ or DPNH; however, these nucleotides were very effective in protecting against enzyme inactivation. AcPyDPNH was a very effective inhibitor of hybrid formation and also protected against inactivation. It was necessary to lower the reduced coenzyme analog to enzyme ratio almost 4-fold

TABLE III: Effect of Various Pyridine Nucleotides on Hybridization of Beef H_4 and Dogfish M_4.[a]

Coenzyme	Molar Ratio of Coenzyme/ Enzyme	No. of Freezes	No. of Freezes Required for Hybridization	Hybridization	Binomial Pattern	Enzymatic Activity Remaining (%)
DPNH	800:1	1	1	+	+	100
		4		+	+	100
DPN	800:1	1	1	+	+	100
		4		+	+	100
AcPyDPNH	800:1	1		−	−	100
	230:1	1		−	−	100
		5	5	+	−	100
	140:1	1		−	−	100
		5	5	+	−	100
	100:1	1		−	−	89
		5	5	+	−	83
	80:1	1	1	+	−	67
		5		+	−	65

[a] All samples contained equimolar mixtures of beef H_4 and dogfish M_4 at a total concentration of 7.6×10^{-6} M in 0.1 M potassium phosphate + 1 M sodium chloride + coenzyme or coenzyme analog at the molar ratios indicated (*p*H 7.0). Freezing was performed in dry ice + methanol and samples were thawed at room temperature.

before hybrids appeared during the *fifth* freeze-thaw and 10-fold before hybrids appeared on the first freeze thaw, and even under such conditions a binomial pattern was not observed. The order of effectiveness of AcPyDPNH, DPNH, and DPN as protectors against inactivation at low temperature and as inhibitors of hybridization are the same as was found for protection against sodium dodecylsulfate and *p*-mercuribenzoate (Di Sabato and Kaplan, 1963, 1964). It is of interest to note that at lower ratios of AcPyDPNH/enzyme the drop in enzyme activity which accompanied the first freeze-thaw step was not followed by a proportional loss after several further freeze-thaw steps. At the reduced coenzyme analog to enzyme ratios of 100:1 and 80:1, the per cent remaining activity (relative to an unfrozen control) was essentially the same after the fifth freeze-thaw as after the first freeze-thaw. In the absence of coenzyme, the loss of enzyme activity was 30–40%. This loss of enzyme activity was paralleled by a decrease in protein fluorescence; both fluorescence and catalytic activity appeared to approach constant values after repeated freezing and thawing (Table IV). Similar decreases in enzyme activity and protein fluorescence were observed when the two LDH's were frozen and thawed separately.

TABLE IV: Effect of Freezing and Thawing on Protein Fluorescence and Enzymatic Activity.[a]

Enzyme(s)	Number of Times Frozen and Thawed	Enzymatic Activity (% of control)	Protein Fluorescence
Beef H_4	1	60	76
	2	59	75
	3	50	76
Dogfish M_4	1	70	91
	2	72	86
	3	80	86
Dogfish M_4 + beef H_4	1	69	79
	2	71	81
	3	74	79

[a] Samples contained either beef H_4 and/or dogfish M_4 (alone or in equimolar mixture) at a total concentration of 7.6×10^{-6} M in 0.1 M potassium phosphate + 1 M sodium chloride (*p*H 7.0) and were quick-frozen in dry ice + methanol and thawed at room temperature. Protein fluorescence was determined by excitation at 280 mμ and measurement of emission at 340 mμ in an Aminco-Bowman spectrophotofluorometer. The control was not frozen and was kept at 0°.

Effects of Various Ions. In order to determine whether high ionic strength was sufficient or whether specific ion effects were required, we investigated the effects of various ions on hybrid formations. The data in Table V demonstrate a requirement for sodium chloride when potassium phosphate was used. Chicken H_4 and dogfish M_4 were quick-frozen at a range of phosphate concentrations, with and without sodium chloride. No hybridization occurred in the absence of sodium chloride and there was consistently more inactivation in the presence of sodium chloride than in its absence. It was found that in the presence of sodium chloride hybridization proceeded more readily if phosphate

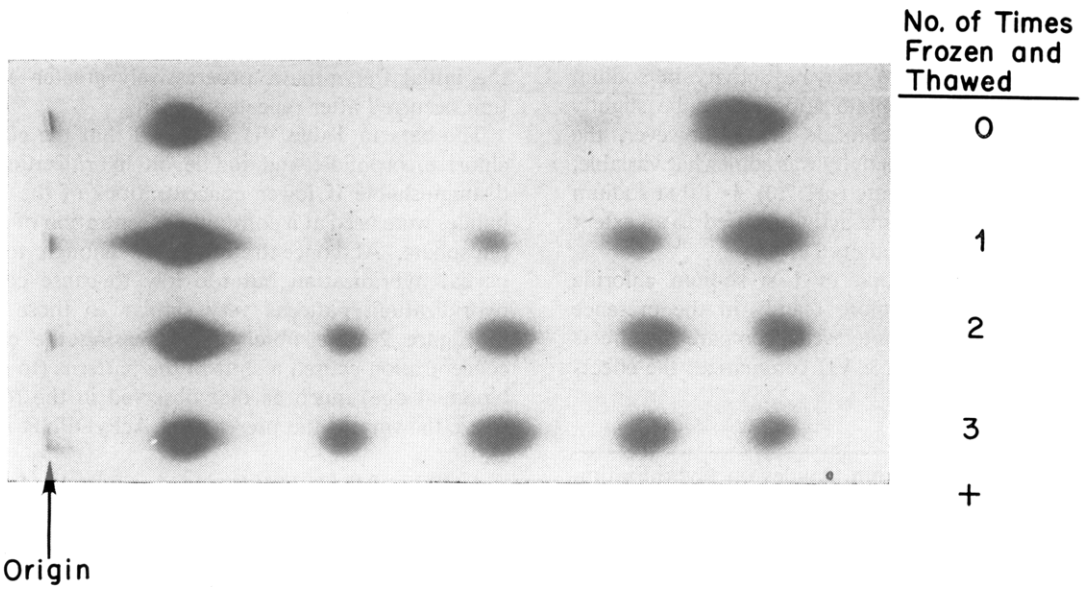

FIGURE 2: Effect of multiple freezes and thaws on hybridization of beef H_4 and chicken H_4 in the presence of AcPyDPNH. All samples contained equimolar mixtures of chicken H_4 and beef H_4 at a total concentration of 7.6×10^{-6} M in 1 M sodium chloride + 0.1 M sodium phosphate + 6.08×10^{-2} M AcPyDPNH (*p*H 7.0). All samples were slow-frozen at −20° and thawed at room temperature for the number of times indicated, except the control, which was kept at 0°, and the electrophoretic patterns were obtained by staining for enzyme activity.

TABLE V: Effect of Varying K_3PO_4 Concentrations with and without NaCl on Hybridization of Chicken H_4 and Dogfish M_4.[a]

NaCl (M)	KPO_4 (M)	Hybrid-ization	Control Activity (%)
1.0	0	−	93
1.0	0.03	+	69
	0.1	−	89
1.0	0.1	+	66
1.0	0.32	+	68
	0.32	−	96
1.0	0.55	+	55
	0.55	−	90
	0.91	−	75

[a] All samples contained equimolar mixtures of chicken H_4 and dogfish M_4 at a total concentration of 7.6×10^{-6} M + the ions indicated (*p*H 7.0), were frozen in dry ice + methanol, and thawed at room temperature (slow thaw). Only one freeze-thaw step was used for all samples in the table.

TABLE VI: Effect of Anions Other than Phosphate in Hybridization of Chicken H_4 and Beef H_4.[a]

Anion	No. of Freezes	Hybrid-ization	Binomial Pattern	Control Activity (%)
Phosphate	1	+	+	80
None (NaCl	1	−		97
alone)	3	+	−	81
	6	+	−	85
Arsenate	1	−		94
Pyrophosphate	1	−		100
Sulfate	1	−		71
	3	+	−	67

[a] All samples contained equimolar mixtures of chicken H_4 and beef H_4 at a total concentration of 7.6×10^{-6} M + 1 M NaCl and the anions indicated (0.1 M, *p*H 7.0). Freezing was performed in dry ice + methanol and the samples were thawed at room temperature. The control was unfrozen and was kept at 0°.

was added. Table VI shows the effect of quick freezing in sodium chloride alone (1 M) and in the presence of arsenate, sulfate, or pyrophosphate. None of these ions replaced phosphate, demonstrating that there are specific ion effects essential for hybridization and eliminating simple ionic strength effects as factors in hybrid formation. Partial hybridization occurred in sodium chloride alone and repeated freeze-thaw steps caused a shift in pattern similar to the binomial pattern shown in Figure 2. Addition of sodium sulfate had no effect on the pattern produced by NaCl. In a subsequent experiment it was found that, in the presence of sodium

chloride (1 M) and sodium phosphate (0.1 M, *p*H 7.0), sodium sulfate (0.05 M) was without effect on hybrid formation. The losses of enzyme activity in sodium chloride plus sodium sulfate appeared to be slightly lower than in sodium chloride alone; however, the per cent loss of enzyme activity was somewhat variable. In 0.1 M sodium phosphate (*p*H 7.0) + 1.0 M sodium chloride the loss in enzyme activity varied to an extent of 30–40% of the total activity.

Since it was found that in 1 M sodium chloride hybridization occurred more readily in the presence of phosphate, it was of interest to compare the effects of other halide ions. Table VII summarizes the effects of sodium halides (0.25 M) + sodium phosphate (0.1 M) on hybridization and enzyme activity of chicken H_4 and beef H_4. This table also shows the effects of repeated freeze-thawing on enzyme activity. Fluoride ion can be readily distinguished from the other halides since hybridization did not occur in the presence of this ion unless the samples were frozen and thawed six times; even after many freezes the patterns were still nonbinomial. The order of effectiveness in protecting against loss in enzyme activity was $F^- > Cl^- > Br^- >> I^-$; in the presence of iodide, instead of approaching a constant level of enzyme activity after the initial freeze-thaw, progressively greater inactivation occurred after repeated freezing.

TABLE VII: Effect of Sodium Halides on Hybridization of Chicken H_4 and Beef H_4.[a]

No. of Freezes	Halide (0.25 M)	Control Activity (%)	Hybridization
0	F^-	100	–
	Cl^-	100	–
	Br^-	100	–
	I^-[b]	100	–
1	F^-	97	–
	Cl^-	80	+
	Br^-	76	+
	I^-	53	+
4	F^-	87	–
	Cl^-	81	+
	Br^-	71	+
	I^-	20	+
6	F^-	97	+
	Cl^-	86	+
	Br^-	62	+
	I^-	16	+

[a] All samples contained equimolar mixtures of chicken H_4 and beef H_4 at a total concentration of 7.6×10^{-6} M + 0.1 M sodium phosphate (*p*H 7.0) + the ions indicated, were frozen in dry ice + methanol, and thawed at room temperature (slow thaw). [b] In these and all subsequent experiments salt solutions were prepared immediately before use and trace amounts of thiosulfate were added to the iodide solutions to stabilize them.

The data in Table VII suggested that the effects of chlori e, bromide, and iod de on hybridization were distinguishable if lower concentrations of the sodium halides were used at a constant concentration of sodium phosphate. At concentrations high enough to cause partial hybridization but too low to cause complete hybridization, patterns very similar to those shown in Figure 2 were observed. Increasing the chloride concentration caused a shift in the patterns (to a more binomial one) much as that observed in the repeated freeze-thawing in the presence of AcPyDPNH (Figure 2).

A study of the concentration dependence of sodium bromide and sodium iodide established that, at increasing concentrations, shifts in the hybridization patterns exactly like those observed with chloride ion were found. However, the minimal concentrations required for hybridization were lower than for chloride ion and less iodide ion was required than bromide ion. Table VIII summarizes these results. Remarkably low concentrations were effective and the order of effectiveness of the ions on hybridization as well as enzyme activity are the same as has been shown by other workers in studies of the action of various ions on protein denaturation (Simpson and Kauzman, 1953) and enzyme inhibition (Fridovich, 1963). In subsequent experiments it was found that thiocyanate would replace iodide at the same concentrations; however, chlorate, sulfite, and perchlorate (each at

TABLE VIII: Comparison of Effectiveness of Sodium Halides and Thiocyanate on Hybridization of Beef H_4 and Chicken H_4.[a]

Halide	Minimum Concentration Required for: Hybridization (M)	Binomial Pattern (M)
Fluoride	0.25[b]	
Chloride	0.005	0.01
Bromide	0.002	0.005
Iodide	0.0005	0.005
Thiocyanate	0.0005	0.005

[a] All samples were in 0.1 M sodium phosphate + sodium halides (*p*H 7.0) at the concentrations indicated and contained 7.6×10^{-6} M enzyme. [b] No hybridization occurred in 0.25 M NaF unless samples were frozen six times and the pattern obtained was nonbinomial.

TABLE IX: Effect of Organic Compounds on Hybridization of Lactic Dehydrogenases.[a]

Enzymes	Compound	Concentration (M)	Remaining Activity (%)	Hybridization
Chicken H_4 + dogfish M_4	Ethylene glycol[b]	0	63	+
		0.22	62	+
		0.45	62	+
		0.90	74	−
		1.8	89	−
		2.7	99	−
		3.6	84	−
Chicken H_4 + dogfish M_4	Propylene glycol[b]	0.17	64	+
		0.34	49	+
		0.68	61	+
		1.37	69	+
		2.05	58	−
		2.74	72	−
Chicken H_4 + beef H_4	Sucrose[a]	0	71	+
		0.2	80	−
		0.4	96	−
		0.6	100	−
		0.8	99	−
		1.2	90	−
Beef H_4 + dogfish M_4	Ethylene glycol[b]	0	81	+
		0.45	80	+
		0.90	87	+
		1.8	80	−
		2.7	93	−
		3.6	85	−
Chicken H_4 + dogfish M_4	Tris[c]	0.1	90	−
Chicken H_4 + beef H_4	Tris[c]	0.1	93	−

[a] All samples contained equimolar concentrations of the two enzymes at a total concentration of 7.6×10^{-6} M in 0.1 M potassium phosphate (*p*H 7.0); they were frozen in dry ice + methanol and thawed at room temperature. [b] Frozen in 0.8 M NaCl. [c] Frozen in 1 M NaCl.

0.001 M) were ineffective. An unexpected cation effect was found when the concentration dependence of sodium chloride in *potassium* phosphate (0.1 M) was compared to that in the presence of *sodium* phosphate (0.1 M). Although 0.01 M sodium chloride was sufficient for complete hybridization in *sodium* phosphate, a 10-fold increase in sodium chloride (to 0.1 M) failed to cause any hybrid formation in the presence of *potassium* phosphate (0.1 M). The initial *p*H and protein concentrations were the same in both buffer systems and identical results were obtained whether chicken H_4 was hybridized with beef H_4 or rabbit H_4. The concentration dependence for sodium phosphate (*p*H 7.0) was also determined and it was found that at a constant concentration of sodium chloride (0.05 M) the minimal concentration for phosphate was 0.03 M. It should be pointed out that the specific ion effects reported here were observed under conditions of quick-freezing and appear to be partially a function of rate. For example, as shown in Figure 1, 0.1 M sodium phosphate without halide caused partial hybridization of chicken H_4 and beef H_4 under conditions of *slow* freezing. Similar results were obtained with these enzymes in 1 M sodium chloride alone after repeating the number of freeze-thaw steps.

Effects of Organic Compounds. As described, conditions which were essential for hybrid formation generally caused partial loss of enzyme activity. In particular, the order of effectiveness of sodium halides as denaturants under hybridizing conditions is the same as shown in studies on denaturation of ovalbumin (Simpson and Kauzmann, 1953). A similar order was obtained by Fridovich (1963) in his studies on anion inhibition of acetoacetic decarboxylase. Simpson and Kauzman found that compounds of the polyhydroxy class reduced the rate of unfolding of ovalbumin in urea, and

Fridovich observed less anion inhibition in the presence of glycerol. These correlations led us to test whether any of these organic compounds could inhibit the hybridization. Table IX summarizes the results of such experiments. Sucrose, ethylene glycol, propylene glycol, and Tris-HCl were all found to inhibit hybrid formation. Sucrose inhibited at concentrations as low as 0.2 M, while for ethylene glycol and propylene glycol concentrations of 0.9 and 2 M, respectively, were required. Tris was tested at only one concentration (0.1 M) but was found to inhibit the hybridization completely. The relation between protective agents and loss of enzyme activity and inhibition of the hybridization phenomenon was not a simple one. Sucrose inhibited hybrid formation at all concentrations tested, but its effects on enzyme activity ranged from no effect at 0.2 M to maximal protection at about 0.6 M. The effect of ethylene glycol varied with the different lactic dehydrogenases. Only slight protection against enzyme inactivation was found when mixtures of dogfish M_4 and beef H_4 were tested, but with combinations of dogfish M_4 and chicken H_4 maximal protection was found at a concentration of 2.7 M; 0.9 M ethylene glycol inhibited hybridization of dogfish M_4 and chicken H_4. Propylene glycol did not significantly reduce the loss of enzyme activity which occurs under freezing but blocked hybrid formation at a concentration of 2 M. The mechanism by which the organic compounds exert their effects is not readily apparent but they are effective at concentrations much below those used for studying solvent effects on protein structure.

Effect of Hybridizing Conditions on Reactivity of Sulfhydryl Groups with p-Mercuribenzoate. There is suggestive evidence that hybridization may involve a conformational change. Gold and Segal (1964) have reported incorporation of *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide into the active site of beef H_4 when the enzyme and reagent were frozen and thawed in 0.25 M sodium chloride + 0.01 M sodium phosphate. Without freezing, even in the presence of salts, the sulfhydryl groups of beef H_4 were not accessible to this reagent. Similar results might be expected with other LDH's and other sulfhydryl reagents. Therefore we tested the effect of hybridizing conditions on the reactivity of the sulfhydryl groups of chicken H_4 with *p*-mercuribenzoate. Table X shows the difference in *p*-mercuribenzoate binding when the sulfhydryl reagent is frozen and thawed with the chicken LDH in the presence (expt 2) and absence (expt 1) of NaCl. In some cases the enzyme was allowed to react overnight at 4° before freezing (expt 2), and in other cases the enzyme was frozen and thawed immediately, with (expt 3) or without (expt 4) *p*-mercuribenzoate, and then allowed to react overnight at 4°. At least eight additional sulfhydryl groups became accessible to the *p*-mercuribenzoate when the enzyme was frozen in the presence of sodium chloride. This was true whether or not the enzyme was allowed to react overnight or frozen immediately (see expts 2 and 3). One to two sulfhydryls were titratable on standing overnight whether or not the enzyme had been frozen previously. It is important to emphasize that, in order to show the increased number of titratable SH groups, freezing with the reagent is essential.

TABLE X: Effect of Hybridizing Conditions on Reactivity of Sulfhydryl Groups of Chicken H_4 with *p*-Mercuribenzoate.[a]

Experiment	Treatment	Time (hr)	Moles *p*-Mercuribenzoate/Mole Enzyme Bound
1[b]	Enzyme + *p*-mercuribenzoate at room temperature	2.5	5.0
	Same incubated at 4°	18	6.8
	Same frozen once after 18-hr incubation at 4°		6.8
2[c]	Enzyme + *p*-mercuribenzoate + NaCl at room temperature	2	6.1
	Same incubated at 4°	18	7.6
	Same frozen once after 18-hr incubation at 4°		15.7
3[c]	Enzyme + *p*-mercuribenzoate + NaCl and frozen immediately		13.4
	Same as above but frozen and then incubated at 4°	18	15
4[c]	Enzyme + NaCl frozen and thawed and then *p*-mercuribenzoate added, incubated at room temperature	2	6.3
	Same as above but incubated at 4°	18	8.4

[a] All samples contained chicken H_4 (7.2×10^{-6} M) + *p*-mercuribenzoate (1.1×10^{-4} M). Freezing was in dry ice + methanol and thawing was at room temperature. [b] Made up in 0.1 M sodium phosphate, *p*H 7.0. [c] Made up in 0.2 M sodium chloride + 0.1 M sodium phosphate, *p*H 7.0.

These results suggested that some of the inactivation which occurs during hybrid formation might be owing to oxidation of exposed sulfhydryl groups. This idea is supported by the findings of Zondag (1963); in studies of the effect of low temperature (−20°) on multiple forms of LDH in tissue extracts, addition of reduced glutathione prior to freezing was found to prevent loss in enzyme activity. Therefore we tested the effect of freezing with and without β-mercaptoethanol on enzyme activity and hybridization of

TABLE XI: Effect of Freezing with and without Mercaptoethanol on Enzyme Activity and Hybridization.[a]

Sample	Enzyme Activity Remaining (%)	Hybridization
(1) Chicken H_4	65	−
(2) Chicken H_4 + mercaptoethanol	91	−
(3) Beef H_4	73	−
(4) Beef H_4 + mercaptoethanol	89	−
(5) Chicken H_4 + beef H_4	58	+
(6) Chicken H_4 + beef H_4 + mercaptoethanol	88	+

[a] Samples contained 1 M NaCl + 0.1 M Na_3PO_4 (*p*H 7.0) + 7.6×10^{-6} M enzyme; they were frozen in dry ice + methanol (fast freeze) and thawed at room temperature (slow thaw). Only one freeze-thaw step was used for all the samples in the table. Mercaptoethanol was added at a concentration of 1×10^{-3} M.

chicken H_4 and beef H_4. The data in Table XI show that β-mercaptoethanol (1×10^{-3} M) is a good stabilizing agent and does not inhibit hybrid formation; hence, it appears that at least part of the loss in enzyme activity under hybridizing conditions may be due to oxidation of sulfhydryl groups.

Discussion

The data presented in Tables I and II clearly demonstrate that hybridization did not occur unless the samples were frozen and that one of the steps, either the freezing or the thawing step, must be slow. From the data available it is impossible to say whether at some critical low temperature hybridization would occur without freezing. When approximately 50% ethylene glycol was present hybridization did not occur after storage for several hours at −20°; however, since this compound was found to be a potent inhibitor of hybridization at −70°, interpretation is equivocal. The requirement for freezing is consistent with what would be expected if hybridization required the weakening of hydrophobic forces (Scheraga, 1963; Kauzmann, 1959) and is analogous to the effect of low temperature on dissociation of TMV protein (Lauffer *et al.*, 1958; Fasman, 1964; Caspar, 1963); however, the relatively specific ion requirements are strongly suggestive that ionic interactions between the subunits are also of importance. It seems clear that no single type of force is responsible for subunit-subunit binding in LDH.

Random recombination of subunits implies but does not prove that the mechanism of hybridization involves complete dissociation of subunits. There is some evidence that *in vitro* complementation of the enzyme glutamic dehydrogenase may occur by a mechanism involving exchange of subunits (Fincham and Coddington, 1963). Whatever the pathway, the pattern shown in Figure 2 demonstrates that in the presence of AcPyDPNH dissociation and recombination (or exchange of subunits) by chicken H_4 and beef H_4 does not occur at equal rates. The finding that AcPyDPNH inhibits hybrid formation supports the suggestion (Di Sabato and Kaplan, 1963) that the reduced pyridine nucleotide is a factor in holding the subunits together. This could arise by a combination of conformational changes and/or by bridging two subunits. A closely related possibility must be considered in the light of the specific ion effects reported here. It is possible that there is ion binding at specific sites and that the reduced coenzyme analog and ions compete for the same site. Although the concentration of AcPyDPNH required to inhibit hybrid formation was considerably in excess of that required to inhibit dissociation of LDH by sodium dodecylsulfate, the relative effectiveness was the same, AcPyDPNH >> DPNH or DPN^+.

When examining the effects of various ions, several factors should be considered. One mechanism might involve the effect of ions on the activity coefficients of exposed peptide bonds (D. Robinson and W. Jencks, 1963, unpublished data; Nagy and Jencks, 1964). If, however, the data of Robinson and Jencks concerning the effects of various salts on the activity coefficients of acetyl triglycine ethyl ester are examined, it is found that the solubility of this model compound would hardly be affected at the salt concentrations which were effective in the hybridization system described in our studies (Table VIII). But it must be emphasized that the effective salt concentrations near the eutectic point are very likely to be manyfold greater than the concentrations before cooling. For example, Van den Berg and Rose (1959a) showed that during cooling of 0.02 M NaH_2PO_4 the concentration in the unfrozen portion increased to 3.42 M. Similar increases in solute concentrations (including the proteins) would be expected to occur during freezing of the samples used in our experiments. The effect of phosphate in the presence of halide ions plus the cation effect observed when an attempt was made to replace sodium ion with potassium ion (at limiting halide concentration) should be examined in this context. Van den Berg and Rose have studied the effect of freezing on the *p*H and composition of sodium and potassium phosphate solutions (Van den Berg and Rose, 1959a) and the effect of addition of sodium and potassium chloride on the *p*H and composition of the NaH_2PO_4-Na_2HPO_4-H_2O and KH_2PO_4-K_2HPO_4-H_2O systems during freezing (1959b). Analysis of their phase diagrams shows that sodium phosphate solutions containing the appropriate mole fractions of acidic and basic salts to give an initial *p*H of 7.0 (at 25°) *decrease* in *p*H during cooling (approaching a *p*H of approximately 3.5 near the eutectic point, −9.9°); however, potassium phos-

phate solutions *increase* in *p*H during cooling (approaching a *p*H of approximately 7.5 near the eutectic point, −16.7°). The effect of added chloride ion is to alter the *p*H and salt concentrations during cooling. Lovelock (1957) showed that the eutectic temperatures of buffered salt solutions decreased in the order $NaNO_3$ > NaCl > NaBr. He also emphasized (Lovelock, 1953) that the effect of glycerol is to reduce the concentration of salts in equilibrium with ice during cooling. This suggests that the enhancing effects of halide and thiocyanate ions on the rate of hybrid formation in sodium phosphate solutions might be owing to the increased NaH_2PO_4 concentration and consequent decrease in *p*H near the eutectic point, and that the organic compounds (Table IX) inhibit by reducing the degree of increased concentration of salts and protein. This hypothesis would explain the effect of quick thawing on hybrid formation (Table II). If changes in salt concentrations and/or *p*H are brought about too rapidly the protein would not be exposed to critical conditions for time sufficient for hybridization to occur. According to this argument a slow freeze allows enough time for hybrids to form during the freezing process. It may be that the critical difference between sodium and potassium phosphates is that the potassium system maintains a higher *p*H near the eutectic point. It was found that equimolar mixtures of chicken H_4 and beef H_4 do not hybridize in 4.2 M NaH_2PO_4 (*p*H ~ 3.4) unless they are frozen. This demonstrates that additional factors are involved in hybridization of these enzymes. It is possible that increased protein concentration near the eutectic point is a rate-limiting factor. Another physical process which may affect the rate of dissociation and/or exchange of subunits is the freezing out of protein-bound water. For further discussion of the physical processes which occur during cooling of salt solutions and the effects on proteins the book by A. U. Smith (1961) and the recent paper by Leibo and Jones (1964) should be consulted.

Halide and thiocyanate ions may also participate in hybrid formation in a capacity other than by changing the *p*H and salt concentrations during cooling. Many anion effects on proteins have been reported; however, the most instructive parallel to the effects we have observed on hybridization can be found in the data of Fridovich (1963). The order of effectiveness of the anions, SCN^-, $I^- > Br > Cl^- >> F^-$, on hybridization, is in general agreement with Fridovich's data on anion inhibition of acetoacetic decarboxylase. Although perchlorate and chlorate ions were ineffective on hybrid formation, thiocyanate and the halides fell in the same order and were effective at concentrations as low or lower than those required to give 50% inhibition of acetoacetic decarboxylase. Also Fridovich found that the concentration of bromide required to give 50% inhibition was lower at 14° than at 30° and that glycerol reduced the degree of inhibition by anions. Because of the similarity of ion, temperature, and solvent effects on enzyme inhibition and hybrid formation it is tempting to interpret the data reported in this paper in the same context as suggested by Fridovich. Since specific ions are required and are effective at such low concentrations it is reasonable to suggest that phosphate, halide, and sodium ions bind to specific cationic and anionic sites, respectively, on the enzyme. It is possible that these interactions disrupt the ordered structure of water around these sites and when the temperature is lowered the effects are magnified by the freezing out of water. The presence of organic solvents might be expected to modify such interactions and, as shown in Table IX, ethylene glycol, propylene glycol, and sucrose inhibit hybridization and stabilize the enzyme. The fact that the organic solvents are effective at such low concentrations suggests that very specific interactions may be involved.

The observation that additional sulfhydryl groups become available to react with *p*-mercuribenzoate shows that at least some conformational change may occur under hybridizing conditions. This agrees with the results of Gold and Segal (1964) with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide and beef H_4 and is consistent with the observation that additional sulfhydryls are exposed to *p*-mercuribenzoate when LDH is dissociated with sodium dodecylsulfate (Di Sabato and Kaplan, 1964). Preliminary experiments by Mr. David Barker of this laboratory with a tritiated preparation of LDH suggest that the protein does not unfold completely. Details of these experiments will be reported elsewhere.

Since, except for hemoglobin, little is known about the perturbations which result when different kinds of subunits are present in the same molecule (Reithel, 1963), it is interesting to note that hybridization occurs between LDH's from such widely divergent species as the dogfish, chicken, beef, and rabbit, and that even two heart-type LDH's (chicken H_4 and beef H_4) hybridize most readily. These observations suggest that during evolution there has been a considerable degree of conservation of structural features which are required for tetramer formation in lactic dehydrogenase. However, we have also established that, under the conditions described in this paper, H-type LDH's from certain primitive vertebrate species have greatly reduced tendencies to hybridize *in vivo* and *in vitro* with the corresponding muscle type. In addition to the freeze-thaw technique, we have been able to hybridize LDH's by dialysis against saturated sodium chloride (at 4°). The requirements for hybrid formation in concentrated sodium chloride are somewhat different than for the freeze-thaw technique; e.g., phosphate ion had no enhancing effect and a much higher degree of species specificity was found (Chilson *et al.*, 1964). More recently we have also been able to hybridize beef H_4 and chicken H_4 to form a binomial distribution of products after reversible inactivation in urea in the presence of β-mercaptoethanol by using a modification of the technique described by Epstein, *et al.* (1964). Details of these experiments have been reported elsewhere (Chilson *et al.*, 1964).

From analysis of the data reported in this paper and our observations of enzyme specificity as well as reagent specificity on the rate of hybrid formation it

appears that the rate of hybridization of different LDH's is not a simple function of the ease of dissociation and recombination of subunits (or subunit exchange) but also depends on the degree of fit between the subunits.

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