

hybrid virus Ad 7 SV40 (Ad 7+) (ref. 25). The synthesis by the hybrid of the SV40 T antigen then becomes as interferon resistant as the synthesis of Ad 7 T antigen, perhaps because the DNAs and messenger RNAs of the two viruses are linked. Moreover, in SV40-transformed cells, some messenger RNA molecules with sequences homologous to viral DNA, are reported to be larger than the genome of the virus²⁶. If cell RNA is transcribed co-linearly with viral RNA, this may modify the sensitivity of the viral messenger RNA to interferon.

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Spontaneous *in vitro* Hybridization of LDH Homopolymers in the Undenatured State

UNTIL now, there has been no totally satisfactory hypothesis proposed for the hybridization of lactate dehydrogenase *in vivo*. This is because hybridization *in vitro*, the only experimental approach, has usually been accomplished by chaotropic agents: freeze-thaw¹, guanidine^{2,3}, urea³, or acid denaturation⁴. Whatever means the cell uses, it follows⁵⁻⁸ that it is susceptible to environmental influences. We now describe a gentle hybridization between heart and muscle types which can occur in more physiological conditions and which mimics many observations *in vivo*.

Crystalline beef heart and rabbit muscle lactate dehydrogenase from Worthington Biochemical were separated into isozymes by DEAE-'Sephadex' chromatography following the procedure of Fritz *et al.*⁹. Pure band 1 and band 5 from Calbiochem were used as such. Hybridizations were done at a total LDH concentration of 0.005 to 0.05 mg/ml. Acrylamide gel electrophoresis was run in a Buchler disk gel

apparatus with ice-water circulating through the cooling chamber. Electrophoresis was allowed to continue 20-30 min after the dye marker migrated off the end of the gels. The isolated gels were stained at 40° C for enzyme activity⁹. Fluorometric measurements were done in an Aminco-Bowman spectrophotofluorometer equipped with a corrected spectra attachment. 1-Anilino-8 naphthalene sulphonate (ANS) from Eastman Kodak was recrystallized from hot distilled water as the magnesium salt. Enzyme activities were determined at 37° C in a Beckman model DB and recorder using 0.1 mM NADH and pyruvate in a 0.02 M phosphate, 0.1 M NaCl (pH 7.2) buffer.

In previous *in vitro* hybridizations, both denaturation and dissociation take place, but it has not been clear whether both of these conditions are necessary for hybridization. Millar first reported that beef heart LDH dissociated with decreasing protein concentration¹⁰. This was confirmed by some¹¹⁻¹⁴, but not by others¹⁵⁻²⁰. Recently Schatz and Segal²¹ and Griffin and Criddle²² have reconfirmed the observation for other tissue LDHs. If dissociation—but not denaturation—is required for hybridization, then mixing of the LDH homopolymers should result in hybridization.

When beef heart LDH and rabbit muscle LDH in 0.02 M phosphate and 0.01 M NaCl were mixed equally, weak hybridization occurred after several hours at 37° C and after 24-48 h at room temperature. These results indicated both time dependence and a large reaction temperature coefficient. Judging from the intensity of the gel bands, some enzymatic activity was lost—possibly by sulphhydryl oxidation. Because sulphhydryls are important to the activity of LDH^{23,24}, we added Cleland's reagent (dithiothreitol) to avoid disulphide formation. Then, from ammonium sulphate suspensions of H and M LDH (10 mg/ml.) separate stock solutions of 1 mg/ml. H and M LDH, 0.02 M phosphate, 0.1 M NaCl, and 0.005 M Cleland's reagent (pH 7.2) were made and kept in an ice bath. Twenty-five µl. portions of H and M LDH stock solutions were added to 1 ml. of this buffer and incubated at 37° C for various times and then placed in an ice bath (Fig. 1). After 10 min of incubation, some M₂H₂* is present. It increases with time and remains the dominant heteropolymeric band throughout the incubation. In several experiments H₃M₁ has appeared at a slower rate than M₃H₁, but this effect has not been highly reproducible. At high M/H, M₂H₂ and M₃H₁ are the predominant heteropolymers, whereas at high H/M, M₂H₂ and H₃M₁ are the favoured isozymes.

Experiments were done to test the activity range of dithiothreitol concentration. Hybridization occurred at levels as low as 1×10^{-5} M. Enzyme dialysed at 4° C overnight against 0.01 M phosphate—0.1 M NaCl (pH 7.2) does not hybridize in 1 h at 37° C, but enzyme dialysed in this way and then dialysed against this buffer plus 0.005 M Cleland's does hybridize. The issue is confusing, for Sabato *et al.*²⁸ report no disulphides in beef and muscle LDH. We shall describe any LDH which has either been dialysed or diluted from ammonium sulphate suspension into a buffer with a reducing agent as activated LDH.

Aliquots of ammonium sulphate suspensions of H and M LDH were dissolved in 0.02 M phosphate—0.1 M NaCl—0.005 M Cleland's buffer (pH 7.2), allowed to stand separately for periods up to 41 h, and then combined. After this they were incubated at 37° C for 1 h; incubation was followed by electrophoresis. Good hybridization showed that the activated state of LDH is stable at room temperature for nearly 2 days.

Fig. 2 shows the effect of increasing phosphate concentration. One ml. of reaction mixture contained 0.047 mg H LDH and 0.05 mg of M LDH; the mixtures were incubated for 1 h at 37° C. No. 1 tube is the standard (0.02 M phosphate—0.1 M NaCl—0.005 M Cleland's). No. 2 is the same, with 10^{-3} M oxalate added. Heavy suppression of hybridization

*We shall use customary terminology here because it is thought that a 144,000 molecular weight unit of LDH is a tetramer of four 36,000 subunits rather than eight subunits of 18,000²⁵⁻²⁷.

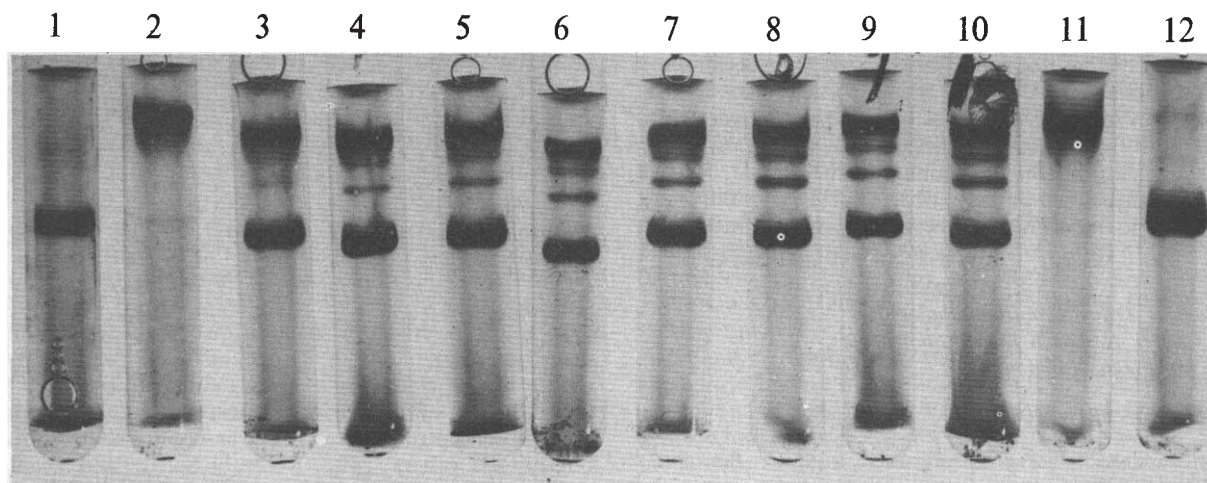


Fig. 1 Hybridization of M and H LDH at 37° C. Tubes 1 and 2 are M and H controls kept in ice throughout the entire incubation. Tubes 3 to 6 represent samples removed at successive 10 min intervals. Tubes 9 and 10 represent samples at plus 97 and 120 min of incubation. Tubes 11 and 12 are unmixed M and H kept at 121 min at 37° C. Commercially purified H and M LDH also forms isozymes in these conditions, but for unknown reasons the reaction frequently takes longer than isozymes purified by the procedures described in the text.

results from the ternary complex. In No. 3, the phosphate concentration is 0.3 M. Here M_2H_2 is reduced in intensity as is M_3H_1 . In 4 and 5, where phosphate concentration is 0.6 M and 1.0 M respectively, suppression of hybridization is evident. In 6 and 7, phosphate concentration is 0.005 M and 0.001 M, and there is considerable hybridization. Finally, 8 contains 0.001 M phosphate, but was incubated at room temperature for 1 h: M_2H_2 is readily observable. When the experiment is done in an ice bath, no hybridization occurs. At pH 6, hybridization did not occur at 0° or 37° C. At pH 8 and 9, it was very rapid at 37° C but nearly suppressed at 0° C (Fig. 2).

To make sure our methods caused no denaturation, we heated denatured LDH in stages and compared the properties of the hybridized enzyme with those of the denatured enzyme. In our standard solution, muscle LDH retains 93% activity after 11 min at 37° C and 100% in 0.3 M phosphate. Heart LDH maintains 100% activity in both conditions. There was hybridization at 45° C but it was weaker than at 37° C, possibly because of loss of enzymatic activity. In low phosphate, it seems that initially at 37° C H LDH is not denatured and M

LDH is only slightly denatured; yet hybridization is seen in the first few minutes at 37° C. In 0.3 M phosphate, LDH does not lose activity in the incubation nor does it hybridize as rapidly. Apparently 0.3 M phosphate stabilizes a configuration which is refractory both to hybridization and to heat denaturation. The latter figure may be called a "taut" conformation. That existing in low phosphate may be termed "relaxed" and the site(s) for hybridization are available for reaction. The "relaxed" state is also apparently more susceptible to heat denaturation.

ANS may not react with undenatured LDH, according to Anderson and Weber⁴. To test this, we added aliquots of LDH to 2 ml. of a preheated solution of 0.02 or 0.3 M phosphate, 0.005 M Cleland's reagent and 10^{-4} M ANS. This was kept for 1 min, then quenched with ice. The fluorescence was observed at 20° C. From 20°–50° C, there was little or no reaction; at higher temperatures, reaction began rapidly for M and at slightly higher temperatures (60°–80° C) for H LDH. At 80° C, the amount of ANS reacting at high phosphate with LDH was less than half that reacting at low phosphate concentration. Clearly, the "taut" conformation survives long enough to restrict combination with ANS. These results suggest that activated LDH is not denatured.

Confirming this, there is essentially no difference in the activity of either H or M LDH in 0.02 M phosphate regardless of the presence (or absence) of sulphhydryl reagent. In 0.6 M phosphate, the activity of both LDHs is decreased by about 25%, and this is not relieved by Cleland's reagent. Apparently, the phosphate induced "taut" form is of lower activity than the "relaxed" form.

There are no disulphides in LDH, so there are two possible functions for Cleland's reagent in this hybridization. There may be displacement of a group which masks the hybridization site; or there may be displacement of a group which restrains the enzyme into a configuration which cannot hybridize. For example, in muscle LDH (but not heart), mercaptoethanol treatment removes NADH-X with an accompanying increase in activity³¹. This has not been found here. Or a sulphhydryl may be involved in some way, perhaps as a hydrogen bond donor, in maintaining a non-hybridizing configuration and that the reducing agent competes with the protein sulphhydryl for the acceptor site.

Assuming that hybrid reaction between beef and rabbit LDHs can be projected to homologous LDHs, we suggest that the reaction reported here produces LDH hybrids in the body, where it may be subject to other influences, for example, hormonal and metabolic.

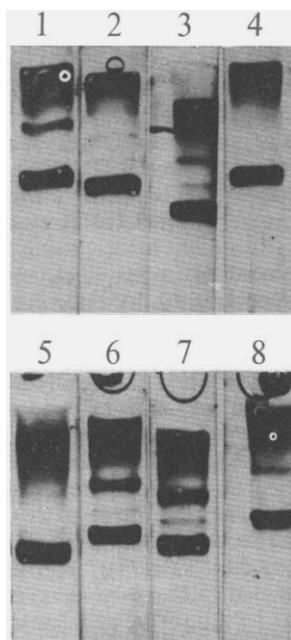


Fig. 2 Phosphate ion as regulator of the hybridization reaction.

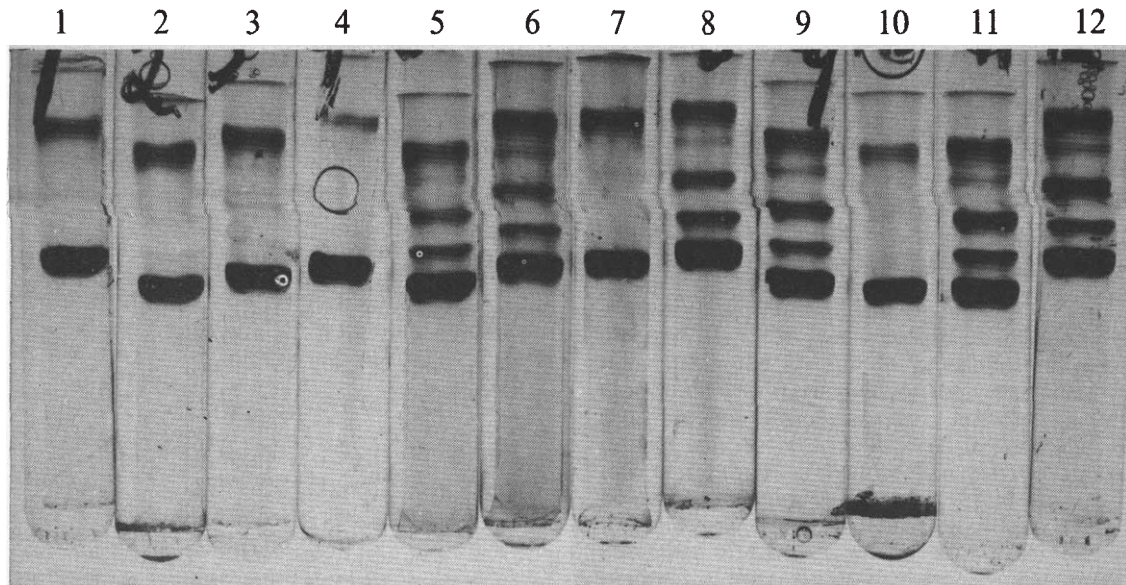


Fig. 3 Effect of temperature and pH on the hybridization reaction. Standard buffer throughout. Tubes 1, 2 and 3 (pH 6, 8 and 9), 2 h at 0° C. Tubes 4, 5 and 6 (pH 6, 8 and 9), 30 min at 37° C. Tubes 7, 8 and 9 (pH 6, 8 and 9), 60 min at 37° C. Tubes 10, 11 and 12 (pH 6, 8 and 9), 2 h at 37° C.

We feel that the most significant feature of these findings is our hypothesis: *in vivo* regulation of LDH isozyme levels could, in part, be by apogenetic parameters. That is, levels of isozymes may not be absolutely controlled by functioning of gene elements or repressors, but may also be sensitive to environmental stresses.

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New Theory of the Control of Protein Concentrations in Animal Cells

THE molecular basis for the regulation of intracellular concentrations of protein has been studied intensively since the discovery of the transfer of genetic information from DNA to RNA to protein^{1,2}. We wish to present experimental evidence in support of a model which considers regulation by post-translational events. This model takes into account the contributions of synthesis, degradation, and protein interactions to the regulation of intracellular protein concentrations. The model explains how protein concentrations could change within a cell while rates of transcription and translation remained constant. Although the experimental evidence in support of the model was collected from studies of the lactate dehydrogenase (LDH) system in the rat, the model may be generally applicable to other subunit proteins and other organisms.

Of the many enzymes known to exist in subunit form, LDH is the most familiar³⁻⁵. In most mammalian tissues the LDH isozymes are tetramers composed of all combinations of two distinct subunits designated A and B. In this notation isozymes labelled A₄, A₃B, A₂B₂, AB₃, and B₄ correspond to LDH-5, LDH-4, LDH-3, LDH-2, and LDH-1, respectively. LDH-1 or B₄ is the most negatively charged of these isozymes. Vertebrate tissues have characteristic distributions of LDH isozyme. For example, in adult mammalian heart muscle, tetramers containing B subunits usually predominate, whereas A subunits are present in the greatest concentrations in mammalian skeletal muscle and liver.