THE EFFECT OF DIVALENT CATIONS ON THE RECOMBINATION OF LACTIC DEHYDROGENASE SUBUNITS*

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The in vitro formation of lactate dehydrogenase (LDH) isozymes 2, 3 and 4 from mixtures of isozymes 1 and 5 frozen in sodium chloride solution (Markert, 1963) has been used as evidence for the subunit structure of the LDH isozymes. Recombination experiments have also been used to characterize a third type of interacting LDH subunit in testicular tissue (Zinkham, Blanco, and Kupchyk, 1963 and 1964). Although no quantitative data has been available, the electrophoretic patterns obtained following recombination have been qualitatively consistent with tetramers formed randomly from the available types of subunits. An attempt to elucidate the mechanism of the hybridization has been made by Chilson and Costello (1964) who studied other monovalent cationic halogen salts, variation in freezing and thawing rates, and the influence of DPNH and acetyl pyridine DPNH. It is the purpose of the present communication to provide quantitative data demonstrating the multiple phenomena occurring during the process of hybridization in vitro and to report the efficacy of calcium and magnesium chloride in promoting it. A recent report by Kirshner and Tanford (1964) has also shown the relative efficiency of magnesium chloride in promoting the dissociation of hemoglobin although recombinants are not demonstrable due to the presumed symmetrical nature of the process.

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Methods and Materials. Homogenates of human psoas muscle, collected at autopsy and stored for several months at -20°, were prepared with equal volumes of cold distilled water using a Teflon pestle homogenizer of the Potter-Elvejhem type. The clear supernate was diluted 1-2 with either phosphate buffer pH 6.9 containing sodium chloride or with tris* buffer pH 6.9 containing calcium or magnesium chloride in appropriate concentrations to give the indicated molarities of the salts in 0.1 M buffer in the final solution. Each solution was divided and one aliquot was placed at -20° for 16-18 hours and then permitted to warm up to room temperature. The other aliquot of each solution was maintained the entire period at room temperature as a control.

LDH assays were performed on each aliquot using a 3.0 ml assay mixture composed of 1×10^{-3} M pyruvate, 1.73×10^{-4} M DPNH, and 0.05 M phosphate buffer pH 7.4. The reaction was started by the addition of 25 microliters of a 1-20 dilution of homogenate (0.1 M phosphate buffer containing 0.06% bovine albumin) and was followed over the initial 1-2 minutes at 38° in a thermostatted Zeiss PMQ II Spectrophotometer equipped with a Photovolt recorder.

Agar gel electrophoresis of the frozen and control solutions was carried out on microscopic slides for 1 1/2 hours in 1.2% agar in 0.05 M veronal buffer pH 8.6 with a field strength of approximately 7 v/cm. Following electrophoresis, the slides were stained 30 minutes in 0.035 M tris buffer pH 8.3 containing 0.14 M lactate, DPN, 1.4 mg/ml, phenozine methosulfate 0.034 mg/ml, and Nitroblue tetrazolium 0.35 mg/ml.

Densitometry was performed using either the Spinco Analytrol with micro scanning attachment or the Joyce Chromoscan both of which gave comparable results.

Results and Discussion. Preliminary experiments with sodium chloride in the range of 0.1 to 2.0 M in 0.1 M phosphate buffer in the range of pH from 6.5 to 7.5 indicated that 0.5-1.0 M NaCl in 0.1 M phosphate buffer pH 6.5-7.0 would provide favorable conditions for the recombination of LDH subunits after freezing. Under these conditions, however, there was a 16 to 27% loss of activity after

* tris(hydroxymethyl)amino methane

freezing. The control solutions maintained at room temperature were stabile. In the same concentration range KCl and phosphate buffer alone were not effective as replacements for NaCl, i.e., neither change in isozyme pattern nor loss of activity occurred. The losses thus appeared related to the process of recombination. Concentrations of ammonium sulfate higher than 0.4 M protected the enzyme from the sodium chloride dissociation. A concentration of 0.5 M NaCl in 0.1 M phosphate buffer pH 6.9 has been adopted as the standard reference concentration for subsequent experiments since it is consistent with that of other investigators (Zinkham, Blanco, and Kupchyk, 1963 and 1964).

Because both calcium and magnesium chlorides precipitate as phosphate salts it was necessary to utilize a tris-HCl buffer with these divalent cationic chlorides. Table I compares the percent distribution found in an experiment with magnesium chloride and the recombination obtained with sodium chloride. The human psoas muscle isozyme pattern does not fit a random binomial distribution; presumably because the muscle is composed of a mixture of muscle cell types, i.e., red and white muscle fibers rather than a single cell type (Blanchaer and VanWijhe, 1962; Fine, Kaplan and Kuftinec, 1963). Following the freezing in salt treatment the distribution should approximate that predicted from a random binomial distribution. The predicted distributions have been calculated based on the fractional contribution of each type of subunit found in the room temperature control. While the recombinants appear to approximate the predicted distribution, the quantitative data reveals several interesting deviations from the predicted distribution. In sodium chloride a higher percentage of isozyme 3 than expected is found with less than predicted values for isozymes 1 and 2. Such a distribution can only be partially explained on the basis of a selective loss in isozyme 1 which would increase the percentage of M subunits from 52 to 61 percent. The expected random distribution, however, would still be different from that actually found (i.e., 2.6, 15.4, 34.6, 34.6 and 13.0 percent for isozymes 1 through 5 respectively).

TABLE I

Recombination of LDH Subunits by Freezing in $MgCl_2$ in 0.1 Tris pH 6.9

Mg conc.	Isozyme Percentages 1	2	3	4	5	% M Subunits	Assay IU/ml	% of Mean Controls
Room Temperature Controls								
0.0025 M	26.9	31.4	20.8	7.8	13.2	37.2	31.9	
0.025 M	24.8	29.2	19.6	6.8	19.3	41.2	31.2	
0.05 M	23.8	29.4	20.0	6.2	20.6	42.5	31.2	
0.1 M	23.4	28.2	18.6	7.6	22.2	44.2	30.0	
Means:	24.7	29.6	19.8	7.1	18.8	41.3	31.1	
Predicted Binomial Distribution:	12.1	33.6	35.4	16.3	2.8	41		
Frozen Solutions								
0.0025 M	26.0	29.2	18.3	7.2	19.4	41.6	29.6	95.2
0.025 M	22.4	24.7	13.2	21.8	18.1	47	31.9	102.2
0.05 M	31.1	1.6	33.8	23.4	10.3	45.4	30.8	99
0.1 M	17.0	--	55.3	25.4	2.1	48.8	26.8	86.2
0.5 M NaCl in PO_4 Buffer Reference								
Room Temperature Control								
	20.1	24.0	15.2	8.6	32.2	52.3	31.2	
Predicted Binomial Distribution:	5.3	22.9	37.3	26.9	7.3	52		
Frozen Solution								
	--	6	52.8	29.7	11.4	62	26.8	86

The data with magnesium chloride indicates that this slat is effective in producing recombination of the subunits at molar concentrations 1/5 to 1/10 of that required with sodium chloride. Similar results were obtained with calcium chloride in tris buffer. In contrast to the calcium and sodium chloride experiments, however, there is found a selective decrease in isozyme 2 even though essentially no loss in total activity is found on freezing in magnesium chloride solution. In addition, there is evident a large increase in isozyme 4 at only 0.025 M $MgCl_2$ almost approximating the limiting value found at higher salt concentrations. At the highest concentration there is, in the decreased concentration of isozyme 5, a further shift in the values toward those expected in a random recombinants.

A parallel experiment (Table II) was performed with the same homogenate and identical $MgCl_2$ concentrations but with the addition of β-mercaptoethanol and DPNH to a final concentration of 0.006 M and 1 x 10^{-5} M respectively in each solution. The concentrations of β-mercaptoethanol and DPNH used were those used for the reactivation of purified glucose-6-PO_4 dehydrogenase (Kirkman, Schettini, and Pickard, 1964). The β-mercaptoethanol concentration was well below that reported as inhibitory to LDH from rat brain (Bonavita and Guarneri). The DPNH concentration was only 5 or 6 percent of that used in the LDH assay, although it is approximately 1000 times the molar LDH concentration in the magnesium chloride solutions. The changes in the distribution pattern of the isozymes after freezing are similar to those demonstrated in the absence of β-mercaptoethanol and DPNH. In the room temperature controls there was a symmetrical increase in the type 5 and a decrease in the type 1 isozyme that, expressed as the percentage content of M subunits, represents an increase of approximately 14%. A similar but slightly greater increase also occurred in the frozen samples. This does not seem to be due to any effect on the relative activities of the isozyme types in the assay since the calculated H and M content remains essentially the same during the process of recombination.

TABLE II

Effect of β-mercaptoethanol and DPNH on $MgCl_2$ Induced Recombination

Mg conc.	Isozyme Percentages 1	2	3	4	5	% M Subunits	Assay IU/ml	% of Mean Controls
Room Temperature Controls								
0.0025 M	17.3	21.2	16.4	11.2	34.2	56.4	33.0	
0.025 M	19.1	23.4	17.9	12.7	26.9	51.3	31.2	
0.05 M	17.6	21.4	15.9	12.9	31.9	55	33.8	
0.1 M	19.1	22.6	15.8	11.4	31.0	53	32.3	
Means:	18.3	22.2	16.5	12.1	31.0	53.9	32.6	
Predicted Binomial Distribution:	4.5	21.0	37.0	30.0	8.5	54		
Frozen Solutions								
0.0025 M	16.1	19.8	12.9	23.6	27.8	57.2	30.8	94.6
0.025 M	16.4	6.8	23.7	31.3	21.8	59.0	28.8	88.4
0.05 M	17.2	0.6	30.8	31.4	20.2	59.3	29.2	89.5
0.1 M	9.3	2.3	41.5	31.8	15.5	60.5	29.6	90.8

It seems possible then that the effect of β-mercaptoethanol and DPNH on the room temperature controls may represent activation of M type subunits and inactivation of H type subunits. A slightly greater sensitivity to loss of activity on freezing was also noted in this experiment.

The failure to approximate the predicted binomial distribution after recombination might be due to different rates of dissociation and of reassociation among the individual isozymes and to different bond strengths between M-M, M-H and H-H subunits in a system that had not reached equilibrium. The study of the time course of recombination is complicated by the freezing and thawing requirement. Although the experimental conditions used here appear to approximate equilibrium, a more detailed analysis will have to be performed before it can be concluded that these multiple phenomena are not due to a complex set of competing reactions not yet at equilibrium. There might also be present under these conditions a significant amount of monomers, dimers or trimers that have not been identified as present in the equilibrium mixture and yet must be included in the material balance. The presence of a significant concentration of dimers at the time of electrophoresis could explain the inordinately great concentration of isozyme 3 together with the dramatic loss of isozyme 2 since, if randomly associated, half of these dimers should have the same charge as isozyme 3 while none would have the charge of either isozyme 2 or 4. Such dissociated forms are either enzymatically active or reactivated under the conditions of the assay since the stability of the enzyme seems reasonably good.

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