Lactate Dehydrogenase Isozymes of Chick Embryo: Response to Variations of Ambient Oxygen Tension

Abstract. *Incubation of chick embryos in an hypoxic environment causes an increase in the proportion of tissue lactate dehydrogenase (LDH) made up of subunit M, whereas incubation in aerobic conditions decreases the proportional amount of subunit M. The variation of ambient oxygen tension does not change the total LDH activity. These results support the hypothesis that oxygen or oxidative metabolites have an effect on the synthesis of the subunit peptides.*

Lactate dehydrogenase (LDH) exists in five different molecular forms, or isozymes (*1, 2*), which are tetramers of two subunit peptides, H and M (*2*), or B and A (*3*). The different forms of the enzyme from LDH_1 to LDH_5 can be written HHHH, HHHM, HHMM, HMMM, and MMMM. The synthesis of peptides H and M is controlled by two different genes (*4*), and the activity of these genes determines the amount of each isozyme in the cell. In tissues largely composed of one type of cell, the synthesized peptides aggregate to form tetrameric enzyme molecules according to binomial distribution. Thus the isozymic pattern is derived from the equation

$$(p+q)^4 = p^4 + 4p^3q + 6p^2q^2 + 4pq^3 + q^4$$

where p and q indicate the proportions of peptides H and M. If the amount of both peptides is equal ($p = q$), the proportion of the isozymes is 1:4:6:4:1.

Tissues in which the metabolism is mainly aerobic (for example, heart, kidney cortex, and brain) have isozymes LDH_1 and LDH_2, which are mainly composed of H subunits, while tissues capable of anaerobic metabolism (for example, white skeletal muscle and uterus) contain LDH_4 and LDH_5, which are chiefly composed of M subunits (*5–7*). The isozymes differ from each other in their ability to reduce pyruvate to lactate at 30°C (*6*). Concentrations of pyruvate exceeding $10^{-3}M$ inhibit LDH_1 at *p*H 7.0, whereas the activity of LDH_5 remains maximum in higher substrate concentrations.

A suggestion has been made, based on these observations, that metabolic demands regulate isozymic composition of tissues, providing anaerobic cells with enzymes capable of converting great quantities of pyruvate to lactate. In aerobic cells high concentrations of pyruvate inhibit LDH_1, and pyruvate is metabolized by the enzymes of the Krebs cycle. How this regulation operates is obscure.

We studied the effect of variations of ambient oxygen tension on the proportion of H and M peptides in the tissues of the chick embryo. Chick embryos were used because eggs could easily be subjected to various oxygen tensions. We thought that the tissues would be most sensitive to alterations during embryonic development since, at this time, there are normally changes in the activity of genes controlling production of peptides H and M (*2, 5, 8*). White Leghorn eggs were incubated in wooden, hermetic boxes, which were ventilated with 15 percent O_2 in N_2, air, and 40 percent O_2 in N_2. The developing embryo tolerates an oxygen tension of 15 percent, but considerable hypoxia is produced (*9*). The same authors (*9*) have used 40 percent O_2 as an optimum oxygen concentration. The temperature was kept at 38.5°C in an isothermal room; the relative humidity was 70 percent (*10*).

Heart, liver, and pectoralis superficialis (muscle) were excised from 7- to 21-day-old embryos, weighed, and homogenized with a Potter-Elvehjelm homogenizer. The homogenates were diluted in 0.067*M* Sørensen phosphate buffer (*p*H 7.4) and immediately analyzed for lactate dehydrogenase activity. In the assay mixture, the final concentrations of pyruvate and reduced nicotinamide-adenine dinucleotide (NADH) were $3.3 \times 10^{-4}M$ and $1.3 \times 10^{-4}M$, respectively. The temperature was 25 ± 0.1°C; the *p*H, 7.4. The reaction was started by the addition of tissue homogenate, and the oxidation of NADH was followed at 340 mμ by means of a Beckman DB spectrophotometer using a recorder. We determined the percentage of each subunit in the tissues by making use of the different activities of the isozymes in low and high concentrations of pyruvate (*6*). We found the best concentrations for the analysis of the isozymes of chicken LDH by our method to be $3.3 \times 10^{-4}M$ for a low concentration of pyruvate and $40.0 \times 10^{-4}M$ for a high concentration. The activity ratios for LDH_1 (100 percent subunit H) and LDH_5 (100 percent subunit M) were assayed with chicken heart LDH_1 and chicken m. pectoralis superficialis LDH_5, which were prepared by starch-gel electrophoresis (*11, 6*). The ratio signifies the rate of pyruvate reduction obtained when a high concentration of pyruvate was used divided by the rate obtained when a low concentration was used. Our values are 0.543 for LDH_1 and 1.337 for LDH_5; these values were used in the calculation of the percentages of the subunits.

During the development of the chick embryo, the total LDH activity of each tissue—liver, heart, or m. pectoralis superficialis—grown in 15 percent O_2 equals that of the same tissue grown in 21 percent (air) or 40 percent O_2. The activity is greatest in liver and least in breast muscle. The activity in the heart increases almost fourfold between the ages of 7 and 21 days. In the other tissues the activity is more constant during ontogeny (Fig. 1).

In Fig. 2 are presented the changes in the percentage of subunit M in heart,

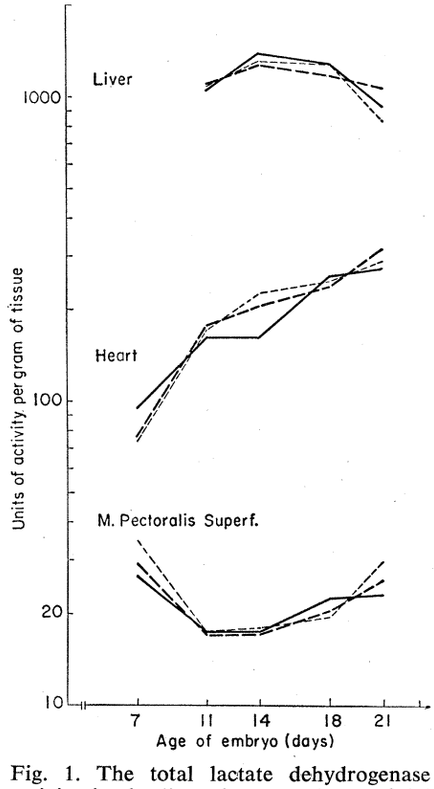

Fig. 1. The total lactate dehydrogenase activity in the liver, heart, and superficial breast muscle of the chick embryo during development. Activity is expressed as change of substrate in micromoles per minute per gram of fresh tissue. The eggs were incubated in 15 percent O_2 (solid line), air (light dashed line), and 40 percent O_2 (heavy dashed line). Each point is a mean of the activities of about ten embryos.

liver, and m. pectoralis superficialis during embryonic development. The chief embryonic form of LDH is composed of subunit H (*5, 8*). In the heart of a 1-week-old chick embryo subunit M constitutes 28 percent of the LDH peptides. When the embryo is incubated in 15 percent O_2 the synthesis of peptide M is proportionally increased in comparison with that of peptide H, and it forms 30 percent of the LDH peptides. If the embryo develops in 40 percent O_2 the percentage of subunit M is lower than that in the hearts of chicks developed in air or in 15 percent O_2, namely 23 percent. The enzymic adaptation to the different oxygen tensions occurs as early as the first week. The pattern of isozymes in the three groups does not change during the rest of ontogeny. The differences between the subunit composition of the hearts of the chick embryos incubated in elevated and diminished oxygen tensions are significant ($P < .05$). The weights of the embryos incubated in 15 percent O_2 were slightly lower than those of other groups. If the percentages of the subunits are plotted against the weights of the embryos the differences between the groups are further accentuated.

The first samples of liver tissue were taken on the 11th day. The percentages of subunit M were 31, 23, and 24 in 15 percent O_2, air, and 40 percent O_2, respectively. The values for embryos developed in hypoxic conditions remain higher than the values for those kept in an hyperoxic atmosphere (Fig. 2). Immediately before hatching, the difference becomes more pronounced. There is more variation in the subunit percentage in the livers of embryos incubated in air. On the 21st day the difference between the chicks incubated in 15 and 40 percent O_2 is significant ($P < .05$).

After an incubation of 1 week in air 33 percent of the subunits in m. pectoralis superficialis were peptide M; this percentage is slightly higher than that in heart or liver. In the breast muscle of an adult chick over 99 percent of the LDH is composed of subunit M (*5*). The percentage of subunit M in breast muscles of chicks incubated in air during the three embryonic weeks increases and is 54 on the 21st day (Fig. 2). The percentage of subunit M also increases in the other two groups, but, compared with the group incubated in air, it is higher when the oxygen tension is decreased and lower when it is increased. On the 11th and the 21st days, the difference between the chicks incubated in 15 and 40 percent O_2 is significant ($P < .05$). We could not prevent the percentage of subunit M from increasing in embryos raised in 40 percent O_2. This indicates that the oxygen tension was too low or that the oxygen metabolism of tissues depends also on factors other than ambient oxygen, such as blood supply and the biochemical state of the tissue.

Cahn *et al.* have shown that tissue culture of chick embryo breast muscle, leg muscle, and heart in vitro causes a rapid "turning on" of subunit M synthesis (*5, 12*). They noticed that incubation of the tissue culture in 95 percent O_2 causes a delay in this phenomenon, which they interpret as an effect on the gene directing synthesis of subunit M. Other indirect evidence, such as the increase of LDH_5 in neoplasia and hormonal stimulation of the uterus, support our results (*13*). Agostoni *et al.* have reported that electrophoretically fractionated mitochondrial preparations from rat liver always have LDH_1 activity, while other subcellular particles contain isozymes LDH_4 and LDH_5, which are mainly composed of M-subunits (*14*). The fact that mitochondria are the site of aerobic metabolism also supports the view that oxidative metabolism is the factor determining the isozymic pattern of the tissue. Vesell has proposed that the isozymes fulfill distinctive roles by being situated in various regions of the cell (*15*).

As an objection to the theory that aerobic metabolism is an influencing factor in LDH synthesis, it has been stated that red blood cells, platelets, and bovine lens fiber cells contain H-type isozymes (LDH_1 and LDH_2) although they are not capable of aerobic metabolism (*16*). It can, however, be questioned whether these cells can be regarded as ordinary body cells like those of liver and muscle, since they are no longer able to synthesize proteins. Perhaps their pattern of isozymes reflects the more aerobic metabolism of reticulocytes, megacaryocytes, and developing fiber cells.

What is the molecular mechanism in the synthesis of the different subunits? The isozymes are related proteins (*17*), and, therefore, the DNA segments carrying the information for the subunits must also be related. The regulating mechanism, which distinguishes between the two genes, must be highly specific. Perhaps oxygen or intermediates of oxidative metabolism combine with the specific gene repressors and so remove the inhibition of the DNA coding the LDH subunit.

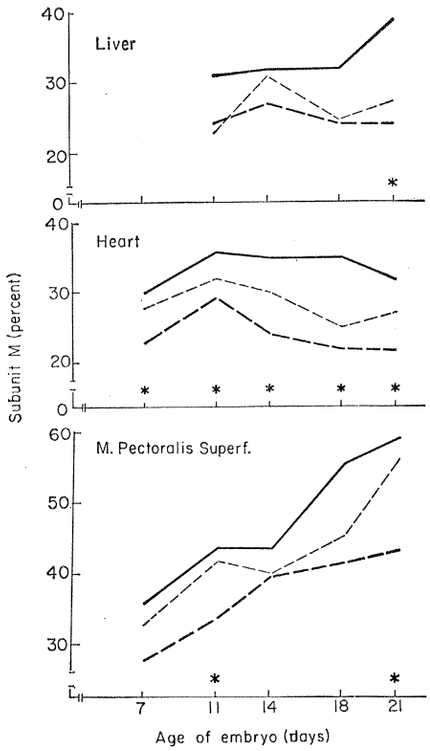

Fig. 2. The percentages of subunit M in lactate dehydrogenase from chick heart, liver, and superficial breast muscle during embryonic development. The eggs were incubated in 15 percent O_2 (solid line), air (light dashed line), and 40 percent O_2 (heavy dashed line). The days when the difference between eggs incubated in 15 percent O_2 and 40 percent O_2 is significant ($P < .05$) are marked with asterisks. Each point is a mean of the percentages in about ten embryos.

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References and Notes

1. Th. Wieland and G. Pfleiderer, *Biochem. Z.* 329, 112 (1957); B. R. Hill, *Ann. N.Y. Acad. Sci.* **75**, 304 (1958); E. S. Vesell and A. G. Bearn, *J. Clin. Invest.* **37**, 672 (1958).
2. C. L. Markert and F. Møller, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).
3. E. Appella and C. L. Markert, *Biochem. Biophys. Res. Commun.* **6**, 171 (1961); C. L. Markert, in *Hereditary, Developmental and Immunologic Aspects of Kidney Disease*, J. Metcoff, Ed. (Northwestern Univ. Press, Evanston, Ill., 1962), p. 54.
4. C. R. Shaw and E. Barto, *Proc. Nat. Acad. Sci. U.S.* **50**, 211 (1963).
5. R. D. Cahn, N. O. Kaplan, L. Levine, E. Zwilling, *Science* **136**, 962 (1962).
6. P. W. G. Plagemann, K. F. Gregory, F. Wróblewski, *J. Biol. Chem.* **235**, 2282, 2288 (1960).
7. G. Pfleiderer and E. D. Wachsmuth, *Klin. Wochschr.* **39**, 352 (1961).
8. D. T. Lindsay, *J. Exp. Zool.* **136**, 287 (1963).
9. E. Jalavisto, I. Kuorinka, M. Kyllästinen, *Acta Physiol. Scand.* **63**, 479 (1965).
10. The incubations were performed at the Institute of Physiology, University of Helsinki. We thank Prof. E. Jalavisto for her advice during the work.

11. I. H. Fine and L. A. Costello, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1963), vol. 6, p. 958.
12. R. D. Cahn, *Develop. Biol.* **9**, 327 (1964).
13. R. D. Goldman, N. O. Kaplan, T. C. Hall, *Cancer Res.* **24**, 389 (1964); T. L. Goodfriend and N. O. Kaplan, *J. Biol. Chem.* **239**, 130 (1964).
14. A. Agostoni, C. Vergani, L. Villa, *Nature* **209**, 1024 (1966).
15. E. S. Vesell and A. G. Bearn, *J. Clin. Invest.* **40**, 586 (1961).
16. E. S. Vesell, *Science* **150**, 1735 (1965).
17. C. L. Markert, in *Cytodifferentiation and Macromolecular Synthesis*, M. Locke, Ed. (Academic Press, New York and London, 1963), p. 65.

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Coding Properties of 7-Methylguanine

Abstract. *The abnormal base, 7-methylguanine, has been introduced into copolymers of uridylic and guanylic acids by methylation under controlled conditions. The resulting methylated copolymers have a markedly decreased template activity for polypeptide synthesis in vitro due to steric effects of the 7-methyl groups. Contrary to expectation, these polymers do not permit incorporation of amino acids whose codons contain the bases uracil and adenine.*

The mutagenic effects of alkylating agents have been reviewed recently (*1*, *2*); the methylating agents, methyl methanesulfonate and dimethyl sulfate, have caused mutations (*2*) in several organisms and in TMV RNA (*3*). Since these agents methylate guanine in both RNA and DNA, it has been proposed (*4*) that the mutagenic effects in DNA may result from the mispairing of 7-methylguanine with thymine during replication. This might be anticipated since the methylated nucleoside, with a *pK* of 7.2, as compared with a *pK* of 9.2 for guanosine, is partially ionized in neutral solution. Mispairing of the ionized base could occur through hydrogen bonding between the negatively charged N-1 of 7-methylguanine and N-3 of thymine, and between the amino group of 7-methylguanine and the C-2 keto group of thymine.

If similar mispairing occurred during translation, messenger RNA containing 7-methylguanine would code as if it contained adenine in place of the methylated base. This possibility can be tested by methylating synthetic polynucleotides which contain only uracil and guanine. In these polymers, uracil is alkylated with considerable difficulty (*5*) so that the effects of guanine alkylation can be studied specifically. Protein synthesis in vitro with such polymers as messenger should permit incorporation of amino acids that are normally represented by combinations of the bases uracil and adenine if 7-methylguanine mispairs as adenine.

To test this possibility, we synthesized a UG copolymer (*6*) with polynucleotide phosphorylase from *Micrococcus lysodeikticus*. Polymer was isolated after repeated extraction with phenol in the presence of Macaloid (*7*) by precipitation with three volumes of ethanol. It was then redissolved, dialyzed exhaustively in the cold against a mixture of 0.15*M* NaCl, 0.015*M* sodium citrate, 0.1*M* NaCl, and distilled water, and dried by lyophilization. Chromatography in a mixture of 95 percent ethanol and 1*M* ammonium acetate (60:40) showed only high molecular weight material.

Two separate methylations of a single UG copolymer were performed with methyl methanesulfonate in a light-scattering cell in cacodylate buffer [*p*H 7, ionic strength 0.2 (*8*)]. By following the intensity of light scattered at 90°C, it was demonstrated that there was no change in molecular weight during methylation. The methylated polymers were subsequently freed of buffer by dialysis and lyophilized to dryness.

The compositions of the polymers were determined by paper chromatography and subsequent quantitative elution after hydrolysis with HCl, and by

Table 1. Properties of UG copolymers.

Polymer	Composition (%)				$s_{20,w}$	Mol. wt.*
	U	MeU	G	MeG		
Original UG	69.6		30.4		3.48	3.11×10^5
Methylated UG-1	70.3		23.3	6.4	3.47	
Methylated UG-2	66.2	2.7	15.2	15.9	3.56	

* By light-scattering method.

Table 2. Coding properties of the methylated UG polymers. Each milliliter of reaction mixture contained the following: tris, 0.05 mmole; KCl, 0.03 mmole; magnesium acetate, 0.013 mmole; mercaptoethanol, 0.006 mmole; ATP, 0.003 mmole; GTP, 0.0002 mmole; phosphoenolpyruvate, 0.017 mmole; pyruvate kinase, 50 μg; unlabeled amino acids, 4×10^{-5} mmole; C^{14}-amino acid (specific activity, 40 μc/μmole, New England Nuclear) 2.5×10^{-5} mmole; S-30 fraction, 6 mg of protein per milliliter; and one of the following: polyUG, 90 μg; methylated polyUG-1, 100 μg; methylated polyUG-2, 140 μg. The *p*H was 7.2 at 34°C. For each determination, 0.1 ml of the reaction mixture was used; it was incubated for 7½ minutes at 34°C before the polymer and C^{14}-amino acid were added. The reaction time was 40 minutes at 34°C. Samples were precipitated with 5 percent trichloroacetic acid. The mixture was heated to 90°C for 15 minutes, transferred to glass-fiber filter papers, and washed with 5 percent trichloroacetic acid, ethanol, and acetone. The radioactivity on the filter papers was then counted in a liquid scintillation counter (Nuclear Chicago).

Amino acid	Relevant code words*	Polymer†	Incorporation			
			Estimated from known codons‡			Experimental (%)
			7-MeG = A (%)	7-MeG = G (%)	7-MeG = 0 (%)	
Phenylalanine	UUU	UG	100	100	100	100§
		MeUG-1	100	100	100	100
		MeUG-2	100	100	100	100
Valine	GUU,	UG	62.7	62.7	62.7	40.9 ± 2.3¶
	GUA,	MeUG-1	47.2	57.1	44.2	35.5 ± 4.5
	GUG	MeUG-2	34.1	64.0	28.6	30.6 ± 3.6
Glycine	GGU,	UG	27.3	27.3	27.3	13.5 ± 1.7
	GGA,	MeUG-1	15.6	22.3	14.6	10.9 ± 1.4
	GGG	MeUG-2	7.8	27.0	6.5	6.6 ± 1.8
Tyrosine	UAU	UG				0.93 ± 0.49
		MeUG-1	9.1			.96 ± .45
		MeUG-2	24.1			1.84 ± .69
Isoleucine	AUU	UG				0.02 ± 0.02
		MeUG-1	9.1			.08 ± .08
		MeUG-2	24.1			.07 ± .09
Serine	AGU	UG				2.5 ± .003
		MeUG-1	3.0			2.6 ± .2
		MeUG-2	5.5			1.9 ± 1.3

* Codons containing U, G, and A. See Söll *et al.* (*11*). † See Table 1. ‡ Calculated from experimentally determined base ratios assuming random order of the bases. § Total incorporation of phenylalanine was about 100,000 dpm, 60,000 dpm, and 10,000 dpm for polyUG, methylated polyMeUG-1, and polyMeUG-2, respectively. Controls minus added polymers contained less than 400 dpm for all amino acids except glycine (900 dpm) and have been subtracted. ¶ Average of six independent determinations for phenylalanine, valine, and glycine and four for tyrosine, isoleucine, and serine.