of continental drift. A relative motion of the continents must involve the mantle to depths of several hundred kilometers; it is no longer possible to imagine thin continental blocks sailing over a fluid mantle.

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Lactic Dehydrogenases: Functions of the Two Types

Rates of synthesis of the two major forms can be correlated with metabolic differentiation.

David M. Dawson, Theodore L. Goodfriend, Nathan O. Kaplan

The molecular heterogeneity of many proteins, particularly of individual enzymes, has recently come to be clearly recognized. The various forms of these proteins are usually separable by physical means, but the physiological significance of the separate molecular species has frequently been obscure. Regarding the enzyme lactic dehydrogenase (LDH), however, the different molecular forms appear to have different functions and it has become clear that they are associated with different modes of metabolism and are quite characteristic of specific tissues.

The molecular forms of LDH were first separated by electrophoresis; by this means up to five separate forms can be distinguished. Recent studies have demonstrated that in fact only two principal or parent forms of LDH exist. The present view, now supported by evidence from many sources, is that LDH is a tetrameric molecule made up of four subunits of the two parent molecules. We have termed the parent forms H (heart) and M (muscle), for the organs from which they are readily obtained; "pure" H type is thus composed of four H subunits (H_4), "pure" M type is composed of four M subunits (M_4), while the other three forms of LDH are molecular hybrids consisting of mixtures of subunits: M_3H, M_2H_2, and MH_3. The evidence that we and others have adduced in support of this concept has been presented elsewhere in detail (*1–4*). It rests on the orderly differences among these molecular hybrids in terms of their catalytic behavior, heat lability, and amino acid composition, and the ability of pure parent forms to yield the intermediate forms under certain in vitro conditions (*5*). A comparison of some of the physical, chemical, and catalytic properties of the H_4 and M_4 forms, as compared to the hybrid H_2M_2, are given in Table 1. The H and M types are also immunologically distinct. Antibodies prepared against the H type do not cross-react with the M form. The converse is also true: antibodies against the M LDH do not react with H protein. Hybrids of LDH will react with both types of antibody (*1*). The two types are synthesized within the same cell, as shown by tissue cultures of pure cell strains and by histochemical demonstrations (*6*).

We have previously proposed that the two types of LDH have significantly different functional roles (*1*). This concept is based on the demonstration that there is a marked difference in the degree to which the activity of the two types is inhibited by pyruvate. The LDH found in the heart (predominantly H_4 in most species) is maximally active at low concentrations of pyruvate and is strongly inhibited by excess pyruvate (approximately 10^{-2} *M* in vitro). The M form of LDH, on the other hand, maintains activity at relatively high pyruvate concentrations. These facts may be related to function in the following way. In the heart, a steady supply of energy is required, and this is maintained by the complete oxidation of pyruvate and lactate in mitochondria. The inhibition of heart LDH by pyruvate favors this oxidative pathway. In skeletal muscle there is a requirement for sporadic, sudden releases of energy in the relative absence of oxygen. This energy is supplied by glycolysis, which produces large amounts of pyruvate and requires its reduction to lactate. Muscle LDH allows this reaction to take place despite temporarily high levels of pyruvate. The lactate formed enters the blood stream and joins metabolic pathways elsewhere.

We have used the catalytic differences between muscle and heart enzymes to demonstrate the proportions of M and H subunits in tissue extracts. In this method we have measured the rates of reaction at high and low concentrations of pyruvate and have compared these rates with those for pure M_4 and H_4.

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Since both kinds of subunits occur in the cytoplasmic fraction and frequently in the same molecular hybrid, they serve as convenient controls for each other, making it possible to avoid artifacts of the extraction process. In practice (*1, 3, 4*) we have found that the differences between the two enzymes can be magnified by use of the hypoxanthine analog of nicotinamide adenine dinucleotide. Most of the assays reported here were performed in two ways: (i) with hypoxanthine analog of reduced nicotinamide adenine dinucleotide (NHXDH) and $3.0 \times 10^{-4}M$ pyruvate, and (ii) with reduced nicotinamide adenine dinucleotide (NADH) and $1.0 \times 10^{-2}M$ pyruvate. The ratio of these two rates was obtained. It gives an accurate representation of the percentage of H and M enzymes, whether they are present in crude extracts as hybrids, as separate hybrids isolated by electrophoresis, or as artificial mixtures of the two parent enzymes (Fig. 1). The percentage of H can then be read directly from a standard curve prepared for each animal species.

Since the two kinds of subunits have different amino acid compositions, it is reasonable to suppose that their synthesis is determined by separate genes. Indirect evidence for the existence of separate genes is provided by examples in which there appears to have been mutation of one but not of the other. Electrophoretic patterns of LDH from some mice (*7*) and some humans (*8*) show splitting of the bands containing M subunits but not of the H_4 band. This splitting is probably caused by two kinds of M subunits in the presence of one kind of H subunit. The two M subunits propably differ in amino acid composition, reflecting mutation of the M gene independently of the H gene.

Since the two kinds of subunits have different physiological roles, it is likely that the two genes controlling their synthesis are regulated independently. We have observed independent variations of M and H synthesis in many situations. We discuss these under five headings: normal differentiation, hormonal responses, removal of nerve supply, disease states, and change in oxygen tension.

Subunit Synthesis during Development

Changes in the molecular forms of LDH during development have been observed in many species and many tissues. For example, embryonic chicken

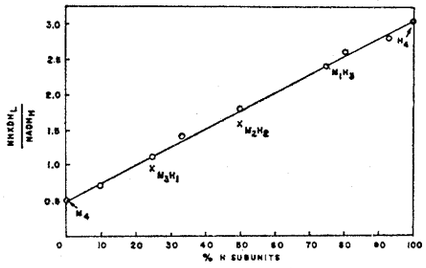

Fig. 1. Relationship of the ratio $NHXDH_L/NADH_H$, for chicken LDH, to the percentage of H subunits. (Circles) Values obtained when different proportions of crystalline H_4 and M_4 are added; (crosses) hybrid molecules isolated by electrophoresis from crude tissue extracts (from Kaplan and Cahn, *4*). The data show that the subunits have the same catalytic action whether they exist separated in pure tetramers or together as hybrid tetramers. In the other figures and in the tables, therefore, the concentrations of M and H refer to total M and H subunits, whether found in hybrid molecules or in pure tetramers.

skeletal muscles contain a relatively high proportion of H subunits, and the LDH from this tissue has catalytic properties resembling those of mature heart (*1, 4*). After hatching, the concentration of M subunits increases at a striking rate, while the concentration of H remains fairly constant. As a result, the percentage of M and the catalytic properties of the enzyme achieve the characteristics of mature skeletal muscle. (It should be emphasized that, although the percentage of H subunits decreases, the absolute concentration of H subunits remains nearly constant, and the changes in the character of the enzyme are caused by the accelerated production of M protein.)

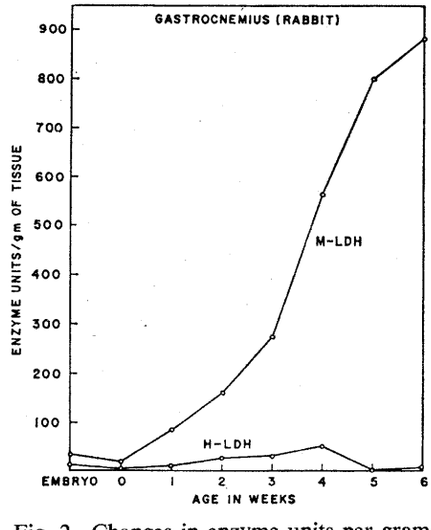

Fig. 2. Changes in enzyme units per gram of tissue (fresh weight) with age in rabbit gastrocnemius. The tissue was excised, homogenized in $0.1M$ tris buffer, *p*H 7.4, or in $0.25M$ sucrose, centrifuged, and diluted appropriately. An enzyme unit is defined as that amount which will produce an absorbancy change, at 340 mμ, of 1.000 in 90 seconds with NADH and $3.0 \times 10^{-4}M$ pyruvate. The total M and H subunits were calculated from the ratio $NHXDH_L/NADH_H$.

A striking synthesis of M subunits also occurs in the gastrocnemius muscle of the developing rabbit, as shown in Fig. 2. In the heart, this rapid synthesis of M does not occur, and there is a more moderate increase in H units (Fig. 3). Not all striated muscles show the pattern of the developing gastrocnemius. Soleus, a red muscle, demonstrates a developmental pattern which resembles that of the heart, as illustrated in Fig. 4. The diaphragm also develops into a muscle with predominantly H subunits. The LDH present in these muscles is correlated with their physiological function. Muscles which contract tonically or rhythmically have an LDH composition like the heart, while muscles which contract more abruptly have M LDH. Physiological considerations have long made it clear that red muscle such as the soleus is concerned with posture and support and hence is more continuously contracted.

The accumulation of large amounts of M enzyme in muscle proceeds to a varying degree in different muscles during development. Data for the adult rabbit, chicken, and human are given in Table 2. In the rabbit, the distinction between red and white muscle tends to be sharper, but in all species a spectrum is observed. Correlation with the physiological function of individual muscles, where this is known, is excellent. It is interesting that these alterations in the synthesis of LDH subunits in muscle occur well after birth. The factors that allow the synthesis of M enzyme in muscle appear to come into play with physiological demand.

Subunit Synthesis in Response to Hormones

When estradiol was administered to immature rats and rabbits, the synthesis of M subunits by the uterus was stimulated much more than the synthesis of H subunits. When the administration of estrogen was discontinued, the concentration of M subunits fell, and the proportion of M to H subunits returned to the control value (Fig. 5). Puromycin, administered after hormone treatment, resulted in a proportionate decline in the concentrations of both M and H

subunits, indicating that the alteration in relative proportions of M and H was caused by differences in rates of synthesis rather than by differences in rates of degradation. Like other effects of estradiol on uterus, the preferential stimulation of the synthesis of M subunits was blocked by prior administration of actinomycin D. This antibiotic inhibits the DNA-dependent synthesis of RNA and, therefore, blocks those effects that are mediated by gene action. Thus, the effect of estradiol provides an example of independent regulation of the synthesis of M and H proteins and probably represents differential action of the hormones on separate genes (*9*).

The LDH of the mature uterus contains a greater proportion of M subunits than LDH of the immature uterus and thus resembles the enzyme in skeletal muscle. It appears that this transformation is probably physiologically significant, in view of the demands for anaerobic metabolism of the uterus during parturition. The mature uterus, in other words, functions more like a skeletal muscle than does the immature uterus (this is emphasized by Richterich *et al.*, *10*). The effect of estradiol "anticipates" the demands to be placed on the uterus and transforms the LDH in such a way that it is ready for mature function. This is unlike the development of skeletal muscle itself, in which LDH composition changes coincidently with physiological demand.

In contrast to the effects of estradiol on rat uterus, progesterone and testosterone, acting on uterus, stimulated a parallel increase in both M and H subunits (*9*). Several other examples of hormone effects were characterized by synthesis of both kinds of subunits. In all these instances the composition of LDH was the same in the stimulated organ as in the immature organ. These other target organs, such as chick oviduct, rat mammary gland, and pigeon crop sac, serve biosynthetic functions. Unlike uterus, they probably do not rely to an increasing degree on anaerobic metabolism, and therefore they would not be expected to show changes in LDH composition during development.

Effect of Denervation

It is well known that, after the division of a motor nerve, the muscles it supplies will gradually atrophy. In two rabbits the sciatic nerve of one leg was divided; one animal was killed 10 days later and one 30 days later. After 30 days the total weight of the denervated muscles had fallen to 70 percent of the weight of muscles of the normal side. Histological sections of the muscle showed neural atrophy, mild in the tibialis anterior, and slightly more advanced in the gastrocnemius.

The LDH present in the denervated muscle was markedly different from normal (Table 3). Ten days after denervation no change could be found except for a possible slight decrease in total amount of enzyme. This result parallels many histological studies (*11*) which

Table 1. Properties of crystalline chicken lactic dehydrogenases. [Data from Pesce *et al.* and Fondy *et al.*, 22]

Form	K_m, pyruvate	Turnover No., pyruvate at V_{max}	$NHXDH_L/NADH_H$*	Molecular weight†	Histidine/mole	Threonine/mole	Inhibition by 3×10^{-4} oxalate (%)
H_4	$8.9 \times 10^{-5} M$	45,500	3.2	151,000	30	75	84
H_2M_2	$5.2 \times 10^{-4} M$		1.97	154,000	45	63	61
M_4	$3.2 \times 10^{-3} M$	93,400	0.55	140,000	63	51	33

* NHXDH, hypoxanthine analog of reduced nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide. Subscript L indicates that $3.3 \times 10^{-4} M$ pyruvate was used in the assay; subscript H, that $1.0 \times 10^{-2} M$ pyruvate was used. † Differences in the three values do not exceed the error of the method.

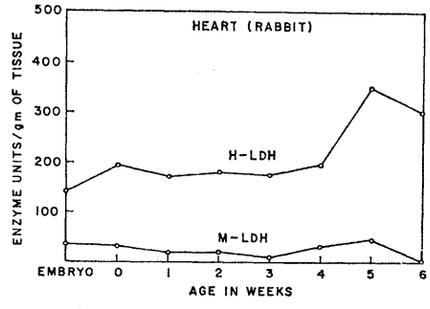

Fig. 3. Changes in enzyme units per gram of tissue (fresh weight) with age in rabbit heart. The methods were those described for Fig. 2.

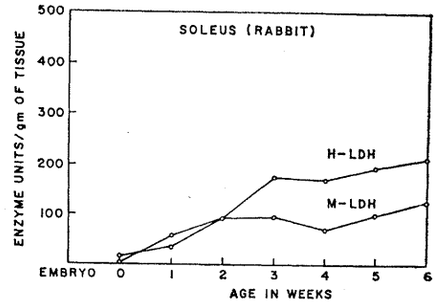

Fig. 4. Changes in enzyme units per gram of tissue (fresh weight) with age in rabbit soleus. The methods were those described for Fig. 2.

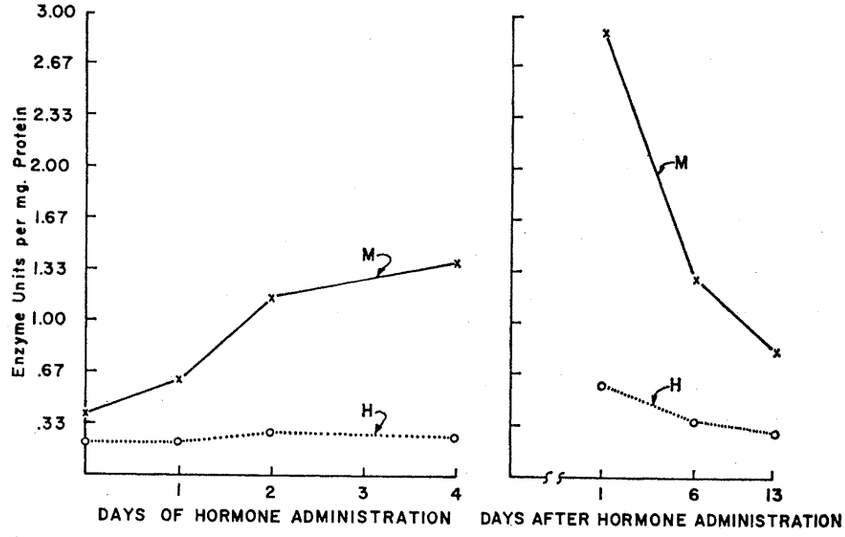

Fig. 5. Effects of the administration of estradiol, and of the termination of such administration, on LDH subunits in rabbit uterus. In experiment 1 (left), immature female rats were injected daily with an oil solution of estradiol (5 μg). The uteri were excised and homogenized, and the activity of total LDH and LDH subunits was assayed, as described for Fig. 2. The data represent means for six or more specimens. In experiment 2 (right), the hormone was administered in ethanol-water for 5 days preceding day 1. The difference between the levels at the end of experiment 1 and the beginning of experiment 2 is probably a result of differences in hormone dosage and duration; the hormone-induced ratio of M to H is the same in these experiments.

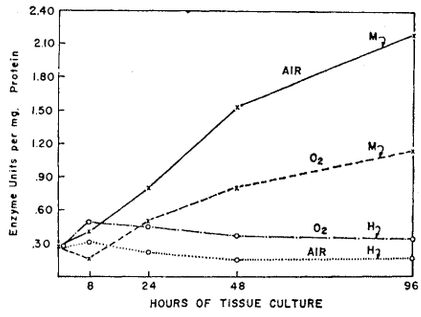

Fig. 6. Effects of tissue culture in air and oxygen on LDH subunits in rabbit uterus. Uteri of immature rabbits were excised, divided into small fragments with a "Mickle" chopper, and grown in tissue-culture medium containing 20 percent horse serum and 5 percent fetal calf serum. The air and oxygen phases each contained 5 percent carbon dioxide. The tissue was harvested and assayed for protein, total LDH, and LDH subunits, as described for Fig. 2.

have shown that, after a week, only the earliest nuclear changes occur. After 1 month, however, the total amount of LDH in the gastrocnemius had been reduced to 19 percent of that in the gastrocnemius of the control, and this loss of enzyme was almost entirely due to loss of M type. In tibialis anterior the change was similar, although less marked, paralleling the histological findings. It is interesting to note that in neither muscle were the histological findings those of advanced disruption of fibers, although shrinkage and nuclear change were evident.

In the soleus, after 1 month, reduction in total amount of enzyme was less (to 30 percent of the amount of enzyme in the soleus of the control), and in this case there was a more striking loss of H type than of M. We have not encountered this lability of H type in any other situation, hence its significance is doubtful, but the implication that for this muscle reversion to an undifferentiated or embryonic pattern would involve loss of H type may be important.

The loss of M enzyme in denervated muscle appears not to be specific for this type of damage, since we have seen the same decrease in M subunits in conditions which affect the health of the whole organism and also in specific muscle diseases. In samples from human muscle we have found that elderly, emaciated people generally have less M enzyme, with a corresponding increase in the percentage of H subunits. Only in healthy adult males was a consistently high concentration of M subunits found (*12*).

Table 2. Lactic dehydrogenase in normal muscle.

Muscle	Percentage of H subunits	LDH (enzyme units/g)	
		Total M	Total H
Rabbit (adult male)			
Psoas	<1	900	<1
Gastrocnemius	<1	800	<1
Semimembranosus	<1	1120	<1
Soleus	65	103	187
Diaphragm	24	280	88
Deep quadriceps	43	165	124
Heart	100	<1	404
Chicken (adult male)			
Pectoralis	<1	4300	<1
Scapulotriceps	4	336	14
Rhomboid	12	487	66
Gastrocnemius	16	668	127
Lateral neck	48	161	156
Human (female, age 58)			
Gastrocnemius	30	55	23
Soleus	70	19	45
Tibialis anterior	27	57	22
Extensor digitorum longus	33	51	25
Extensor digitorum brevis	18	50	11

Composition of Lactic Dehydrogenase in Disease States

In two diseases that we have studied there were deviations from the normal ratio of M to H subunits in tissue LDH. The normal progression of the rates of synthesis of subunits in developing chick muscle is altered in muscular dystrophy of chickens (*4, 13*). In this genetically determined disease the concentration of M subunits is lower than normal throughout the life-span of the affected animals, whereas the concentration of H subunits is approximately normal. Heterozygotes show a pattern intermediate between homozygotes and normal animals (Table 4).

Analysis of several human tumors and the adjacent normal tissue showed a higher concentration of M subunits in the tumor (*14*). There was both an absolute increase and an increase relative to H subunits (Table 5). Lactic dehydrogenase in these tumors was, therefore, more like muscle enzyme, the form present in tissues exhibiting high rates of glycolysis. The high rate of glycolysis manifested by malignant tissue has been emphasized by Warburg. The significance of this coincidence remains uncertain (*15*).

Effects of Oxygen Tension

Another example of independent regulation of M and H genes is provided by alterations in oxygen tension, both in

Table 3. Lactic dehydrogenase after denervation. The analyses were carried out 30 days after denervation. (No change was noted in the muscles 10 days after denervation.)

Muscle	Percentage of H subunits	LDH (enzyme units/g)	
		Total M	Total H
Gastrocnemius			
Normal	5	839	44
Denervated	30	185	79
Tibialis anterior			
Normal	20	1220	310
Denervated	30	165	110
Flexor digitorum longus			
Normal	20	468	118
Denervated	38	240	148
Soleus			
Normal	82	54	246
Denervated	65	33	79

Table 4. Lactic dehydrogenase in muscle of chickens with muscular dystrophy. All samples were of white breast muscle.

Muscle	LDH (enzyme units/g)	
	Total M	Total H
Two weeks old		
Normal	167	9
Heterozygote	150	9
Homozygote	98	6
Ten weeks old		
Normal	390	1
Heterozygote	101	16
Homozygote	38	7

Table 5. Lactic dehydrogenase in human tumors.

Tissue*	Percentage of H subunits	LDH (enzyme units/g)	
		M	H
Thyroid			
Uninvolved	68	47	100
Tumor	18	492	108
Colon (a)			
Uninvolved	34	88	46
Tumor	15	260	46
Colon (b)			
Uninvolved	49	88	82
Tumor	7	847	65

* "Uninvolved" means that the tissue was not involved in the adjacent tumor.

Table 6. Effects of high and low oxygen tension on subunits of lactic dehydrogenase in cultured heart cells (monkey). Monolayers of a propagated cell line from monkey heart (Salk) were incubated in tissue-culture medium containing 20 percent horse serum and 5 percent fetal calf serum for 46 hours. The cells were subjected to sonic vibrations, and the supernatant was assayed for LDH activity and composition, as described in the legend of Fig. 2 and in (*9*).

Gas phase	Percentage of H subunits	LDH (enzyme units/mg)	
		Total M	Total H
95% air	50	1.26	1.26
95% O_2	66	1.15	1.47
95% N_2	28	2.40	0.95

vivo and in vitro. Incubation of fertile chick eggs in 100 percent oxygen resulted in the suppression of the synthesis of M subunits in the embryonic skeletal muscle. The synthesis of H subunits proceeded steadily in muscle and was increased in the heart relative to synthesis in controls incubated in air.

When explanted into tissue culture, most tissues from chicks and other species began to produce more M than H subunits (*16, 17*). This appeared to be a result of the culture conditions. When uterine fragments were placed in culture, the production of M subunits increased and was unaffected by estradiol in the medium. Thus, removal of the uterus to tissue culture mimicked the effect of estradiol in vivo. Elevation of the oxygen tension of the culture suppressed this overproduction of M subunits by muscle or uterus explants (Fig. 6) and enhanced the production of H subunits by heart explants (*17, 18*). In the presence of actinomycin there was no increase in enzyme concentration under conditions of tissue culture, and no change with alterations in oxygen tension, the proportions of M and H remaining as they were at the time of explantation. Cahn has also observed enhancement of the synthesis of H subunits in chick heart explants incubated in oxygen (*19*).

Conclusions based on the effects of culture conditions on explants are subject to the criticism that in tissue culture there is selection of cell types rather than regulation of the genes within a single cell. However, analogous results were obtained in an established cell strain in tissue culture. When monkey heart cells (Salk) were incubated in an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide, they produced more M subunits than control cells incubated in 20 percent oxygen and 5 percent carbon dioxide (Table 6).

Table 7. Lactic dehydrogenase of various renal zones of the rat.

Zone	Percentage of H subunits
Papilla	9
Medulla	44
Cortex	98

These changes in the synthesis of subunits with changes in oxygen tension are congruent with the proposed physiological role of M and H proteins. The low oxygen tension favors synthesis of M LDH, the form best suited for anaerobic metabolism. High oxygen tension favors synthesis of the H enzyme, the form which predominates in aerobic tissues.

There is one naturally occurring example of graduated oxygen tension and correlation of LDH subunits: the various layers of the kidney. Because of the countercurrent pattern of circulation in this organ, the medullary portions are more anoxic than the cortical portions (*20*). Metabolic studies have shown a greater dependence on glycolysis in the medulla and papilla (*21*). The LDH subunits display a preponderance of H in the cortex and higher levels of M in the interior zones of the kidney (see Table 7).

Conclusion

For the study of differentiation and gene regulation, the synthesis of M and H subunits provides a unique experimental tool. The proteins are similar in many respects—in gross catalytic activity, molecular weight, and ability to hybridize with one another. They are produced in the same cells and are destined for the same general function. Yet they show distinct differences and can be regulated independently. Changes in the ratio of M to H yield direct evidence of the differential synthesis of proteins. It is probable that the effects, on synthesis of the subunits, of embryogenesis, hormones, diseases, innervation, removal to tissue culture, and changes in oxygen tension are expressions of differential regulation of genes. It is hoped that the unraveling of one train of events in regulation will help to elucidate the others as well.

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