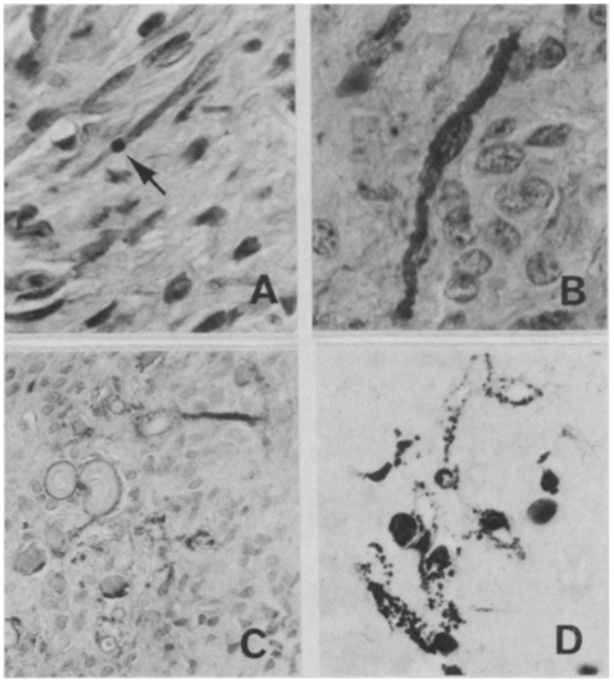
buffer (pH 5.0) and used to stain the hydrolyzed slipes, the granules in the walls of the capillaries were especially apparent. The latter technique was also effective in staining all layers of the multilayered corpuscles, but especially the most superficial layer. The observed Gomori positive material was visualized after treating the tissue for the PAS reaction and with the basic dye procedure of SOLCIA et al.[15] for endocrine cells. These techniques also stained droplets in glial cells and in an occasional pinealocyte. After 1 to 1.5 h hydrolysis with warm HCl, the basic dye procedure yielded well stained droplets on a background of unstained tissue (Figure D). Each of the dyes recommended by SOLCIA et al.[15] stained the above-mentioned material. One of the most important features was that the material also gave a masked metachromatic reaction with toluidine blue in McIlvaine buffer.

Generally, the histochemical observations reported here are consistent with a 'peptidergic' type of neurosecretion. The presence of stainable droplets in the perivascular space and in the endothelium of the capillary may represent hormone being secreted into the blood. The similarly stainable material in the multilayered corpuscles indicates that these structures somehow participate in the secretory process, possibly by acting as a site at which certain by-products are deposited. As is the case with the hypophyseal neurophysin[16–19] the neurosecretory material which stained with the methods reported here probably represents the carrier molecule (as a carrier-hormone complex) for the polypeptidic hormones. This may explain the presence of the stainable material (carrier protein ?) in the multilayered corpuscles; the carrier may normally be a by-product of the secretory processes and possibly deposited on the multilayered corpuscles. A pineal peptide-binding protein has been biochemically identified by KRASS et al.[20]. This protein is similar to neurophysin of the posterior pituitary but is almost devoid of amino acids containing SH groups and is incapable of binding the posterior lobe hormones. The origin of the axons which contain the neurosecretory material is presently unknown. Although there were some neuron cell bodies found in the pineal parenchyma they were never seen to contain neurosecretory material. The findings prompt the conclusion that (at least in the monkey) neurosecretion may be one of the mechanisms responsible for secretion of polypeptide hormones by the pineal gland.



A) A single globule (arrow) of neurosecretory material (stained with Gomori's method) in a nerve fibre in the monkey pineal gland. ×720. B) Multiple globules in a nerve fibre. ×720. C) Neurosecretory fibre entering a perivascular space. Stainable material also present in the vicinity around the blood vessel and in outer layers of multilayered corpuscles. ×290. D) The substance stained by the metachromatic reaction with toluidine blue after hydrolysis with warm HCl. ×290.

Zusammenfassung. In der Epiphyse von Rhesusaffen (*Macaca rhesus*) wurden Neurosekrete histochemisch nachgewiesen und gezeigt, dass die Affenepiphyse Nervenfortsätze mit färberisch und histochemisch mannigfach darstellbarem Neurosekret enthalten, was ebenso für den perivaskulären Raum und die Wände der Blutgefässe gilt.

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COGITATIONES

On the Regulation of Lactate Dehydrogenase Isoenzyme Concentrations in Mammalian Cells

The five lactate dehydrogenase (LDH) isoenzymes (A_4, A_3B, A_2B_2, AB_3 and B_4) found in most mammalian tissues are produced by the association of two different subunit types (A and B) into tetrameric combinations[1]. It was previously assumed that the tissue specific concentrations of the isoenzymes were directly dependent on the synthetic activities of the structural genes for A and B subunits and that the in vivo association of

Table I. Relationship between observed and expected proportions of LDH isoenzymes in rat skeletal muscle

LDH	μg/g[a]	Observed (%)	Expected[b] (%)	% Observed/% expected
A_4	41.2	83.7	79.1	1.06
A_3B	6.46	13.1	18.8	0.70
A_2B_2	0.52	1.06	1.67	0.63
AB_3	0.56	1.14	0.066	17.3
B_4	0.46	0.93	0.00098	949

[a] Taken from FRITZ et al.[10]. [b] The values expected for the random combination of 94.3% A and 5.6% B subunits from a single subunit pool. The relative amounts of each tetramer expected were calculated from the following relationship: $a^4 + 4a^3b + 6a^2b^2 + 4ab^3 + b^4$ where a and b represent the percentages of A and B LDH subunits, respectively, in the subunit pool.

mammalian LDH subunits was a random process[1,2]. More recently, FRITZ et al.[3] have proposed that the overall regulatory process is more complex and involves: 1. differential synthesis of A and B LDH subunits, 2. isoenzyme-isoenzyme interactions involving the exchange of subunits between LDH tetramers, and 3. differential degradation (turnover) of the isoenzymes and subunits. Each cell type would presumably have its own set of rate constants for all of the postulated reactions. According to their model, in vivo subunit associations would not necessarily be random nor would the turnover of A and B subunits within hybrid molecules necessarily be coupled since subunit associations would be changing continuously via subunit exchange reactions. The following points are consistent with their model: 1. synthetic and degradative mechanisms are both involved in the regulation of intracellular protein concentration[4,5], 2. non-random associations of LDH subunits have been observed in tissues of some lower vertebrates[1,6], 3. subunit exchange between LDH isoenzymes has been observed under very mild in vitro conditions suggesting that similar exchange processes may occur in vivo[3,7], 4. heterogeneous rates of in vivo turnover of mammalian ribosomal subunits[8] and of fatty acid synthetase subunits[9] have been observed, demonstrating that the turnover of subunits of these cellular components, at least, is not coupled.

For investigations on the intracellular regulation of multiple enzyme systems, using intact mammalian tissues, it is essential to ensure that the enzymes studied are derived from the same cells and intracellular compartments. For example, non-random distribution of LDH subunits in extracts of heterogeneous tissues may be expected since the intact tissue would contain more than one subunit pool (cell type). Furthermore, differential turnover of isoenzymes within different cell types of the same tissue would not be unexpected in view of the distinct physiological and biochemical characteristics of differentiated mammalian cells. For example, the same protein (LDH A_4) turns over at different rates in rat skeletal muscle, liver, and heart[10]. Although the model proposed by FRITZ et al.[3] is an intriguing possibility, the present study will show that the reported non-random

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Table II. Relationship between observed and expected proportions of LDH isoenzymes in rat skeletal muscle assuming different subunit pools

LDH	μg/g[a]	Observed (%)	Expected[b] (%)	Observed %/% expected
Pool 1: $A_4 + A_3B$				
A_4	41.2	86.5	87.1	1.0
A_3B	6.46	13.5	12.3	1.1
A_2B_2	—	—	0.87	—
AB_3	—	—	0.015	—
B_4	—	—	0.0002	—
Pool 2: $A_2B_2 + AB_3 + B_4$				
A_4	—	—	0.46	—
A_3B	—	—	5.2	—
A_2B_2	0.52	33.9	22.2	1.5
AB_3	0.56	36.4	42.1	0.9
B_4	0.46	29.7	30.0	1.0

[a] Taken from FRITZ et al.[10]. [b] The values expected for the random combination of A and B subunits. Pool 1: 96.6% A and 3.4% B subunits. Pool 2: 26% A and 74% B subunits. The expected values were calculated as described in the legend of Table I.

Table III. Relationship between observed and expected proportions of LDH isoenzymes in rat heart

LDH	μg/g[a]	Observed (%)	Expected[b] (%)	% Observed/% expected
A_4	0.77	0.6	0.1	6.0
A_3B	3.0	2.3	2.0	1.15
A_2B_2	12.8	9.9	13.5	0.73
AB_3	57.2	44.4	39.8	1.12
B_4	55.0	42.7	43.9	0.97

[a] Taken from FRITZ et al.[10]. [b] The values expected for the random combination of 18.4% A and 81.5% B subunits from a single subunit pool. These values were calculated as described in the legend of Table I.

association[11] and differential turnover[3,11] of LDH subunits in rat tissues may simply reflect cellular heterogeneity of the tissues studied.

It is known that skeletal muscles are composed of different fibre types. These fibres have been grouped into 3 main classes on the basis of their contraction times, glycolytic capacity, and oxidative capacity[12]. Furthermore, histochemical and electrophoretic studies have demonstrated different relative amounts of the LDH isoenzymes in different fibres of the same skeletal muscle[13,14]. The cellular heterogeneity of rat hind limb musculature with respect to LDH is evident from inspection of Table I. The μg of each LDH form per g of muscle as reported by FRITZ et al.[10] are given in the 2nd column and the percentages of each tetramer in the total LDH population are listed in the 3rd column. From these values, it can be calculated that the total LDH of these muscle preparations is composed of about 94% A and 6% B subunits. The relative amounts of the 5 tetramers expected on the basis of random combination of A and B polypeptides from a single subunit pool were then calculated (Table I, 4th column). The last column lists the observed to expected ratio for each isoenzyme. This ratio should always be 1.0 if all of the subunits are contained in a single subunit pool (cell type) and if they associate in a random fashion. The fact that this ratio is 17.3 for the AB_3 enzyme and nearly 1000 for LDH B_4 while the ratios for the other forms are between 0.63 and 1.06 strongly suggests that AB_3 and B_4 are derived from cells other than those that contain the A_4 homotetramer. The argument may be posed that the variations between observed and expected ratios reflect non-random association of the subunits, although the subunits themselves are all located within the same intracellular pool. This argument seems unlikely in views of the high ratio for the AB_3 asymmetrical hybrid; in those sytems where non-random association of LDH subunits has been demonstrated[1,6] the homotetramers and the symmetrical hybrid A_2B_2 are highly favoured over the asymmetrical forms. Furthermore, good agreement between observed and expected values of isoenzymes is obtained if the total LDH is assumed to be derived from different cell types (subunit pools). As shown in Table II, pool 1 is assumed to contain A_4 and A_3B amounting to about 97% of the total LDH. Pool 2 contains A_2B_2, AB_3 and B_4 amounting to only 3% of the total LDH. The 'observed to expected' ratios for A_4, A_3B, AB_3, and B_4 (0.9–1.1) are now close to the theoretical value of 1.0 (Table II, last column). The somewhat higher ratio for A_2B_2 (1.5) is expected since some contamination of this enzyme by A_2B_2 derived from pool 1 would occur. These results, together with the knowledge that skeletal muscles are composed of different fibre types, strongly suggests that association of LDH subunits within the same rat skeletal muscle cell is a random process.

Similar considerations of the data obtained by FRITZ et al.[10] on rat heart are presented in Table III. As shown, there is a general correspondence between the proportions of the tetramers observed and those expected for the random combination of 18.4% A and 81.5% B subunits from a single subunit pool (Table III, last column). There is one major exception: The proportion of A_4 observed is 6-fold greater than that expected. The amount of A_4 present in the heart preparation is only 0.6% of the total LDH (Table III, 3rd column) and could easily be derived from a small population of cells which are different from a major cell type that contains greater than 99% of the total LDH.

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Table IV. Relationship between observed and expected proportions of LDH isoenzymes during rat heart development[a]

	Birth		+ 10 days		+ 20 days		+ 60 days	
LDH	% obs	O/E	% obs	O/E	% obs	O/E	% obs	O/E
A_4	8.6	(1.1)	4.9	(2.9)	2.3	(3.2)	1.2	(3.7)
A_3B	26.9	(1.0)	14.8	(1.2)	8.7	(1.3)	3.5	(0.9)
A_2B_2	40.3	(1.1)	24.6	(0.8)	21.2	(0.8)	18.6	(1.0)
AB_3	16.1	(0.7)	31.0	(0.8)	38.5	(0.9)	41.9	(1.0)
B_4	8.1	(1.5)	24.6	(1.5)	29.3	(1.1)	34.9	(1.0)

[a] The picomoles of each LDH isoenzyme per g of rat heart were estimated from the data of FRITZ et al[3] and the % of each form in the total LDH popiulaton calculated. The ratio of observed to expected proportions (O/E) of each isoenzyme was calculated as described in the legend of Table I.

Further support of the suggestion that LDH subunit associations in rat heart cells is a random process is obtained from consideration of the developmental data reported by FRITZ et al.[3]. Table IV shows the estimated percentages of each tetramer at different times during development of the rat heart as well as the ratios of these values to those expected for the random combination of A and B subunits from a single subunit pool. For all hybrid tetramers, the 'observed to expected' ratios do not vary by more than 30% from the theoretical value of 1.0 and in many cases are quite close to unity (Table IV). Considering the extensive scatter in the developmental data (ref.[3], Fig. 1) the correlations between observed and expected values are striking. The somewhat higher ratio for B_4 (1.5) at birth and +10 days is partially due to the difficulty in estimating the concentrations of this enzyme at early stages. Finally, the increasing 'observed to expected' ratio of A_4 with development, further supports the suggestion (see above) that most of the A_4 in homogenates of adult rat heart is derived from cells other than those that contain the other 4 isoenzymes: Notice that the proportion of A_4 decreases from about 9% to about 1% of the total LDH between birth and adulthood (Tables II and III), while the 'observed to expected' ratio increases from 1.1 to 6 during the same time. This increase in ratio would be expected since the relative contribution of A_4 derived from the postulated minor cell type would be increasing during heart development.

Very recently, FRITZ et al.[15] have presented further data on the steady-state concentrations of LDH isoenzymes in rat skeletal muscle and heart. These data are considerably different from those published previously[10]. However, calculations of 'observed to expected' ratios using these latter data again suggested the cellular heterogeneity of these tissues with respect to LDH; the percentage of A_4 in heart was 9 times greater than expected, while the 'observed to expected' ratios for the isoenzymes in muscle ranged from 0.16 to 6200.

FRITZ et al.[3] have proposed that differential intracellular turnover of LDH isoenzymes occurs in rat tissues. For example, these workers initially reported that in rat heart the rate constant for degradation of LDH A_4 is about 8 times greater than that for LDH B_4, while in skeletal muscle, the degradation constant for A_4 is about 20 times less than for B_4[11]. Considerably different rate constants appeared in their later paper[15]. It is apparent that, in both tissue preparations, the two homotetramers were derived from different cell types and, consequently, these data cannot be used to describe turnover of isoenzymes within the same cell. Furthermore, although FRITZ et al.[11,15] have reported heterogeneous rate constants for degradation of isoenzymes presumably located within the same cells, their own data on the kinetics of incorporation of ^{14}C-labeled amino acids into the isoenzymes of rat heart[3] is clearly consistent with the suggestion that LDH isoenzymes within the same cell, turn over at the same rate. Although much scatter in the data is apparent from inspection of their figure 2[3], the observed time courses of labeling of LDH A_3B, A_2B_2, AB_3 and B_4 are quite similar. Since the time course of incorporation of radioactivity into protein upon continuous administration of isotopically labeled amino acids is directly related to the rate constant for degradation of the protein (and, therefore, to the rate of turnover), these data suggest that the 4 isoenzymes turn over at the same, or similar, rates. The kinetics of ^{14}C incorporation into A_4 (presumably derived from another cell type) suggests that this enzyme turns over at a considerably higher rate than the other isoenzymes. The simple interpretation that the different kinetics of labeling observed is a reflection of tissue heterogeneity is, perhaps, more consistent with the data than the interpretation offered by FRITZ et al.[3] which is based on computer analyses of complex rate equations for protein synthesis, protein-protein interactions, and protein degradation. The predicted incorporation kinetics for rat heart LDH A_3B[3] (Figure 2), from their computer analysis, are considerably different than those observed and suggest that the rate of turnover of this enzyme is higher than those of A_2B_2 AB_3, and B_4 but is lower than that of A_4. By virtue of the high content of A subunits in this tetramer, the predicted kinetics of ^{14}C incorporation into A_3B would be highly influenced by using erroneous rate constants for synthesis and degradation of A subunits.

In view of the present discussion it is quite possible that: 1. The observed non-random associations of LDH subunits in homogenates of certain rat tissues are due to the existence of different subunit pools in the intact tissue rather than to the non-random association of LDH subunits within the same cell. 2. The observed heterogeneous rates of turnover of the LDH forms may represent different rates of turnover in different cell types rather than differential turnover of the isoenzymes within the same cell. Therefore, it is not yet clear whether or not regulatory mechanisms other than those that control rates of subunit synthesis (gene transcription, message translation, etc.) are involved in controlling the relative levels of LDH isoenzymes in mammalian cells.

The model proposed by FRITZ et al.[3] can surely be tested. Provided subunit exchange between isoenzymes during enzyme isolation can be excluded, then studies on the turnover of A and B subunits derived from the same hybrid tetramers can be used to test the model directly since the two subunit types would necessarily be located within the same cells in the intact tissue. If, as proposed by FRITZ et al.[3], the turnover of subunits within LDH tetramers is not coupled as a result of subunit exchange reactions, and if LDH A and B subunits turn over at different rates within the same cell, then the differential turnover of the two subunits derived from the same hybrid tetramers should be demonstrable, perhaps by using the double isotope method described by ARIAS et al.[16]. Studies using this experimental approach will undoubtedly support or refute the theory of FRITZ and associates on the control of LDH isoenzyme concentrations in mammalian cells.

Zusammenfassung. FRITZ et al.[3] haben in Nature New Biol. 230, 119 ein Modell publiziert, mit dem sie die Regulation der intrazellulären LDH-Isoenzym-Konzentration erklären. Die vorliegende Arbeit zeigt, dass die Resultate von FRITZ et al. einfacher durch Heterogenität der Zellen des untersuchten Gewebes erklärt werden kann als durch ihr Modell mit «non-random association» der LDH-Untereinheiten und verschiedenen «turnover»-Raten der Isoenzyme in derselben Zelle.

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