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Lactate Dehydrogenase Isozymes. Turnover in Rat Heart, Skeletal Muscle, and Liver†

Paul J. Fritz,* E. Lucile White, Kenneth M. Pruitt,‡ and Elliot S. Vesell

ABSTRACT: Turnover values for 12 lactate dehydrogenase isozymes from three rat tissues were estimated by ^{14}C-labeled amino acid incorporation during the adult, steady state condition. The continuous dietary administration method, accompanied by specific immunoprecipitation, was used; the data were analyzed by the zero-order synthesis, first-order degradation model. The values for heart lactate dehydrogenases 5, 4, 3, 2, and 1 were 3.2, 1.7, 11.8, 39.6, and 53.2 pmol/g per day, respectively. For skeletal muscle lactate dehydrogenases 5, 4, 3, 2, and 1, the rates of synthesis and degradation were 13.5, 0.6, 0.8, 1.3, and 2.4 pmol/g per day, respectively. According to this method of analysis, rat liver isozyme 5 turns over at a rate of 79.2 pmol/g per day, whereas the value for rat liver isozyme 4 is 0.8 pmol/g per day. No correlation between the turnover and the molecular weight of the lactate dehydrogenase isozymes was observed in skeletal or cardiac muscle. In liver there was a correlation between the relative, but not the absolute, rate of isozyme-5 and -4 degradation.

Proteins within animal cells are in a state of dynamic flux, being continuously synthesized and broken down, a phenomenon designated turnover.[1] In recent years intensive investigations of the turnover of various proteins have made it clear that theories dealing with the regulation of intracellular protein levels must take into account both synthesis and degradation (Schimke and Doyle, 1970).

Of the many enzymes that occur in animal tissues in multiple molecular forms (isozymes), rates of biosynthesis and degradation, which may control the tissue distribution of these isozymes and thereby provide an insight into their functional significance (Fritz *et al.*, 1969), have been measured only for the lactate dehydrogenases (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) (Fritz *et al.*, 1969, 1970b). We previously measured rates of synthesis and degradation of lactate dehydro-

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[1] If turnover is defined as the rate of replacement of a protein in a particular tissue (Reiner, 1953), then at steady state turnover is equal to the rate of synthesis or the rate of degradation. For the model in which synthesis is zero order and degradation is first order: turnover = $k_d[P]_{ss}$. Since half-life = $(\ln 2)/k_d$, turnover = $([P]_{ss} \ln 2)/\text{half-life}$. The symbols have the following meanings: k_d is the rate constant for degradation; k_s is the rate constant for synthesis; and $[P]_{ss}$ is the steady state concentration of the protein under investigation. Thus, it is misleading to use the terms "turnover" and "half-life" synonymously. Half-life estimates the rate of removal of a protein relative to the amount present and $k_d \times 100$ can be considered to be the per cent of the protein replaced in a given time. On the other hand, k_s is a measure of the absolute amount of the protein made (or degraded) per unit of tissue per unit of time. Proteins with short half-lives do not necessarily have a fast turnover. If the steady state protein concentration is small, the turnover will be slow. For example, rat skeletal muscle lactate dehydrogenase 5 has a half-life of 138 days, as calculated from the first-order rate constant for degradation (k_d), whereas rat skeletal muscle lactate dehydrogenase 1 has a half-life of 13 days. However, the turnover of these isozymes is 13.5 and 2.4 pmol/g per day, respectively (Table I).

TABLE 1: Rate Constants for Synthesis and Degradation of Lactate Dehydrogenase Isozymes in Rat Tissues as Determined from Isotope Data at Steady State.[a]

Tissue and Isozyme	k_d (day^{-1})	[P] (pmol/g)	k_s (pmol/g per day)
Heart			
5	0.077	42 ± 3 (15)	3.2
4	0.015	116 ± 11 (14)	1.7
3	0.021	560 ± 24 (14)	11.8
2	0.022	1801 ± 88 (13)	39.6
1	0.028	1902 ± 78 (12)	53.2
Skeletal muscle			
5	0.005	2692 ± 76 (15)	13.5
4	0.009	68 ± 7 (5)	0.6
3	0.018	42 ± 4 (5)	0.8
2	0.031	41 ± 4 (4)	1.3
1	0.052	47 ± 7 (5)	2.4
Liver			
5	0.036	2199 ± 146 (17)	79.2
4	0.028	27 ± 6 (5)	0.8

[a] Steady state levels are expressed as the mean ± the standard error. The numbers in parentheses beside the steady state levels indicate the number of animals used in the determinations. The lactate dehydrogenase levels in the animals had reached a steady state concentration as judged by measurement at various times during postnatal development.

genase 5 from rat liver, skeletal muscle, and heart (Fritz *et al.*, 1969) and in a preliminary communication reported values of the rate constants for synthesis and degradation of the five lactate dehydrogenase isozymes in rat heart and skeletal muscle and for two isozymes in rat liver (Fritz *et al.*, 1970b). These reports were based on data obtained from adult rats using the method of continuous administration of ^{14}C-labeled amino acids in the diet (Schimke, 1964). In the present investigation, more extensive documentation of the radioisotope studies on lactate dehydrogenase isozymes in these three rat tissues is provided.

Experimental Procedure

General. Methods for lactate dehydrogenase assay, protein determination, electrophoresis, and column chromatography were previously described (Fritz *et al.*, 1969, 1970a).

We expressed the level of isozymes in picomoles rather than in international enzyme units because we were more concerned with the actual amount of protein present than with the catalytic potential. Methods for converting enzyme activity to picomoles were previously described (Fritz *et al.*, 1970a). Since the five isozymes differ in molecular activity (Pesce *et al.*, 1967), a different view is gained depending upon how the results are expressed. For example, if we compare rat liver isozyme 5 to rat heart isozyme 1 (Table I), we note that in terms of picomoles per gram of tissue they are similar (mean value 2199 *vs.* 1902). However, there are more than twice as many isozyme 5 units per gram of liver than isozyme 1 units per gram of heart (176 *vs.* 76).

Purification of Enzymes and Preparation of Antibodies. LACTATE DEHYDROGENASE 5 (A_4). The enzyme was purified from rat liver by the method of Hsieh and Vestling (1966). The final preparation showed a single band on polyacrylamide disc electrophoresis when stained either for enzyme activity or for protein (Figure 1). For antibody production we adhered to the following schedule: (i) 1 ml of sample containing 3 mg of protein was emulsified thoroughly with an equal volume of Freund's complete adjuvant (Difco) and injected into the toe pads of New Zealand white rabbits. (ii) The procedure was repeated 1 week later. (iii) One week later the sample without the adjuvant was injected subcutaneously in the back of the rabbits in four equal amounts. (iv) One week later, 50 ml of blood was removed from each rabbit by cardiac puncture. The blood was allowed to remain in glass at room temperature for 1 hr while the clot formed. Then the clot was freed from the glass by rimming with a stainless steel spatula and the sample was placed in a refrigerator at 4° and allowed to remain overnight. In the morning the serum was decanted and the clot was centrifuged at 3000*g* for 15 min in order to recover more serum. About 30 ml of serum could usually be obtained from 50 ml of blood.

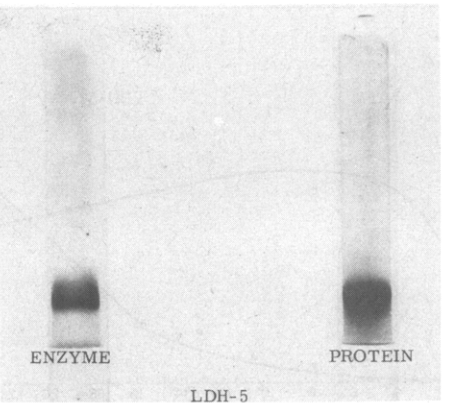

FIGURE 1: Acrylamide gel electrophoresis of purified rat lactate dehydrogenase (LDH) 5. The enzyme was purified by the method of Hsieh and Vestling (1966). A total of 16 μg of protein representing 8 international units of enzyme activity were applied to the acrylamide gel columns and electrophoresis was performed as previously described. After electrophoresis, the enzyme was visualized with a tetrazolium stain as described (Fritz *et al.*, 1970a) and the protein was visualized by staining with 1% Amido Schwarz in 7.5% acetic acid.

Antibody purity was verified by the quantitative precipitin method (Figure 2A) and by double diffusion in agar (Figure 3). The serum of the best antibody producing rabbit had an initial specific antibody titer (milligrams of isozyme 5 precipitated per milliliter of serum) of 0.11 which was increased to 0.42 after subsequent subcutaneous injections.

The antiserum was purified and concentrated in the following way: the serum was diluted 1:1 with 0.02 M Tris (pH 7.4) containing 0.15 M NaCl and after standing for 1 hr in ice was centrifuged at 200*g* for 10 min. An equal volume of ice cold saturated ammonium sulfate was then added slowly with stirring after which the sample was again allowed to stand for 1 hr in ice before centrifuging at 27,000*g* for 15 min. The supernatant was discarded and the precipitate was dissolved in an appropriate volume of 0.02 M Tris (pH 7.4) by allowing the sample to stand overnight in the refrigerator. Insoluble protein was removed by centrifugation at 27,000*g* for 10 min.

LACTATE DEHYDROGENASE 1 (B_4). The enzyme was purified from rat heart by a modification of the method of Hsieh and Vestling (1966). After polyacrylamide disc electrophoresis, the final preparation showed a single band when stained for enzyme activity but two bands when stained for protein

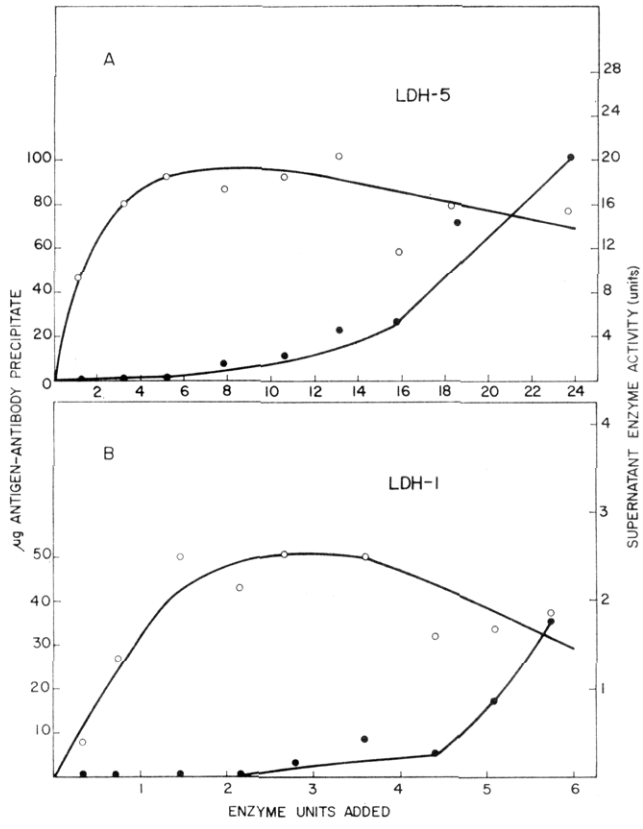

FIGURE 2: Quantitative precipitin reactions of rat lactate dehydrogenase (LDH) 5 (A) and 1 (B). Rabbit anti-rat sera were prepared as described under Experimental Procedure. To 0.05 ml of antiserum or control serum was added a volume of solution containing the enzyme activity indicated. The mixtures were incubated overnight at 4°. The precipitates were collected and washed twice with cold 0.85% NaCl. The supernatant fluids were assayed for isozyme activity, and protein was determined on the washed precipitates.

(Figure 4). For antibody production, 30 μg of protein per column was subjected to electrophoresis on ten separate polyacrylamide columns in a Canalco Model 12 system. After electrophoresis, the protein bands were located by staining one of the columns for protein. The lactate dehydrogenase zones were then cut from the other columns and the polyacrylamide plugs thus obtained were homogenized in 2 ml of Tris buffer

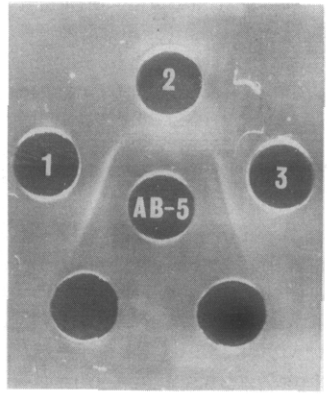

FIGURE 3: Ouchterlony double diffusion analysis of rabbit anti-rat isozyme 5 serum. The center well contained 0.1 ml of antiserum. Well 2 contained 2.0 units of the purified isozyme 5 used as antigen. Wells 1 and 3 contained 2.0 units of isozyme activity in a rat liver homogenate. When the center well contained control serum (not shown) no precipitin lines were observed.

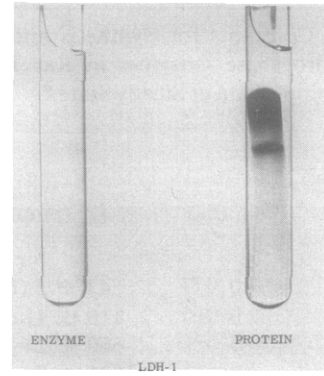

FIGURE 4: Acrylamide gel electrophoresis of purified rat lactate dehydrogenase (LDH) 1. The enzyme was purified by a modification of the method of Hsieh and Vestling (1966). A total of 30 μg of protein representing 1 international unit of enzyme activity was applied to the acrylamide gel columns and electrophoresis was performed as previously described (Fritz *et al.*, 1970a). After electrophoresis the enzyme ws visualized with a tetrazolium stain and the protein was visualized by staining with 1% Amido Schwarz in 7.5% acetic acid.

(pH 7.4), 0.02 M, containing 0.15 M NaCl. This homogenate was then injected into or near the popliteal lymph nodes of the rabbits. The injections were repeated twice at weekly invervals and 1 week after the last injection the rabbits were bled and antiserum collected as described above.

Antibody purity was verified as described for lactate dehydrogenase 5 (Figures 2B and 5). The serum of the best antibody producing rabbit had a specific antibody titer of 0.158 mg/ml. The antibody was purified and concentrated as described in the previous section.

Administration of Isotope. Male Sprague-Dawley rats, weighing 350–450 g, were housed individually. The rats were fed a commercially prepared liquid diet food (Nutrico, Nutritional Biochemicals, Inc.). The food contained 19.6% amino acids by weight. Each day they received 35 ml of the diet food which was supplemented after 5 days with 500,000 dpm per ml of ^{14}C-labeled yeast protein hydrolysate (1000 μCi/mg; Schwarz BioResearch, Inc.). They had water *ad libitum*. At intervals after being placed on the radioactive liquid diet, two rats were decapitated between 6:45 and 7:00 A.M. Hearts, livers, and the skeletal muscles from both hind legs were removed and prepared for counting.

Preparation and Counting of Samples. The preparation and counting of total soluble tissue proteins, free amino acid pools, and isozyme 5 have been described (Fritz *et al.*, 1969). The other isozymes were eluted from DEAE-Sephadex columns as described (Fritz *et al.*, 1970a). Either anti-isozyme 5 or anti-isozyme 1 was used to precipitate isozymes 4, 3, and 2. These isozymes were prepared for counting in the same way as described for lactate dehydrogenase 5 (Fritz *et al.*, 1969).

Analysis of Data to Obtain Rate Constants for Synthesis and Degradation. The data were analyzed as previously described (Fritz *et al.*, 1969) except that instead of obtaining a value for the degradation rate constant from the slope of the straight line resulting from a plot of ln ($P^*_{max} - P^*$) *vs.* time, we used the integrated form of the rate equation directly. The equation used was

$$P^* = P^*_{max}(1 - e^{-k_d t}) \quad (1)$$

where P^* = disintegrations per minute per picomole of protein at any time t, P^*_{max} = disintegrations per minute per

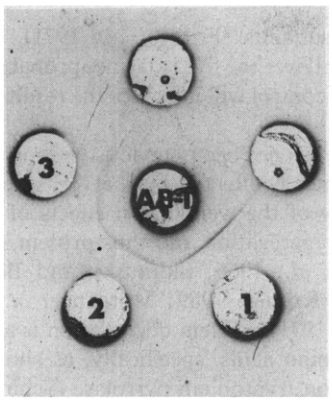

FIGURE 5: Ouchterlony double diffusion analysis of rabbit anti-rat isozyme 1 serum. The center well contained 0.1 ml of antiserum. Well 2 contained 2.0 units of the purified isozyme 1 used as antigen. Wells 1 and 3 contained 2.0 units of isozyme activity in a rat heart homogenate. When the center well contained control serum (not shown) no precipitin lines were shown.

picomole of protein at maximum labeling, and k_d = first-order rate constant for degradation. Based on our measured values for heart lactate dehydrogenase 5 and skeletal muscle lactate dehydrogenase 1, we used a value for P^*_{max} of 1.4 dpm/pmol for all the isozymes. This measured value agrees well with the value of 1.34 dpm/pmol calculated from the measured maximum specific radioactivity of the amino acid pool.

Equation 1 was used to fit the data and thus obtain values for k_d by means of a computer program designated BMDX 85 nonlinear least squares. The program was developed at the Health Science Computing Facility of the University of California at Los Angeles. Knowledge of the steady state protein concentration and of k_d enabled us to calculate k_s, the rate constant for synthesis, since, at steady state, k_s is the product of k_d and the steady state protein concentration (Price *et al.*, 1962; Berlin and Schimke, 1965).

Results

The rate constants for synthesis and degradation of the lactate dehydrogenase isozymes as determined by ^{14}C-labeled amino acid incorporation studies during the adult steady state condition are given in Table I. The time courses of ^{14}C-labeled amino acid incorporation from which the rate constants were calculated are shown in Figures 6, 7, and 8 for rat liver, skeletal muscle, and cardiac muscle, respectively. The rate constants for synthesis and degradation of lactate dehydrogenase 5 from the three tissues differ from the values previously reported (Fritz *et al.*, 1969). Previous studies used a solid diet containing 6% amino acids, whereas in the present work a liquid diet containing 19.6% amino acids by weight was employed. As shown in Figure 7, incorporation of radioisotope into skeletal muscle isozyme 5 was slower in the rats fed the high amino acid diet. Calculation of rate constants from these data yielded the lower values reported in Table I. The incorporation of radioisotopes into isozyme 5 from rat heart and liver was also slower in animals on the higher amino acid diet, resulting in lower rate constants for degradation.

Discussion

Three different methods are available for using radioisotopes to estimate rates of biosynthesis and biodegradation of proteins. Use of the pulse labeling and continuous administration methods have been reviewed by Schimke and Doyle (1970). The third method, involving a double isotope technique, was originally introduced by Arias *et al.* (1969) to examine relative rates of degradation of proteins in liver microsomal membranes. Recently, this method was modified to yield values for first-order rate constants for protein degradation (Glass and Doyle, 1972). In most cases, the data collected by the three methods have been analyzed by the zero-order synthesis–first-order degradation model (Price *et al.*, 1962), which is based on the assumption that each protein is made and broken down independently.

Schimke studied the synthesis and degradation of arginase and obtained similar values for the degradation rate constant with either the continuous or single isotope administration techniques (Schimke, 1964). Subsequently, most workers have used the single administration technique because of its relative convenience. This method appears to give reasonable estimates for enzyme turnover when the turnover of the protein being studied is slow compared to the turnover of the amino acid pools. Kuehl and Sumsion (1970), working with adult (280–370 g) male Holtzman rats, used the pulse labeling

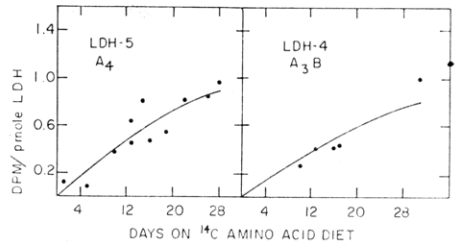

FIGURE 6: ^{14}C-Labeled amino acid incorporation into lactate dehydrogenase (LDH) isozymes of adult rat liver. See Experimental Procedure for details.

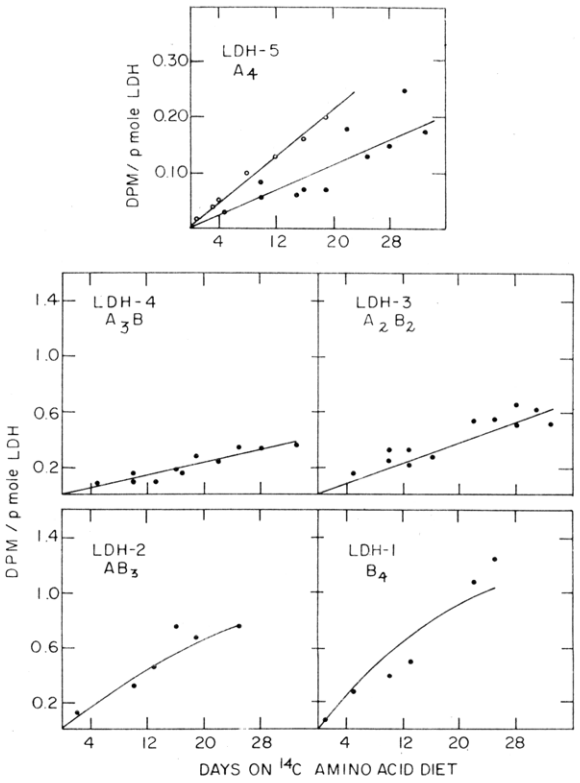

FIGURE 7: ^{14}C-Labeled amino acid incorporation into lactate dehydrogenase (LDH) isozymes of adult rat skeletal muscle. See Experimental Procedure for details: (●) 19.6% amino acid diet; (○) 6% amino acid diet.

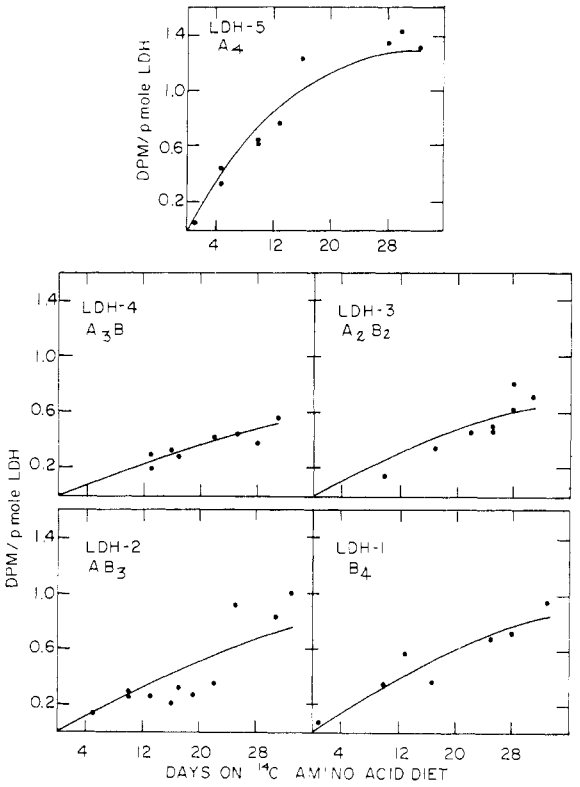

FIGURE 8: ^{14}C-Labeled amino acid incorporation into lactate dehydrogenase (LDH) isozymes of adult rat cardiac muscle. See Experimental Procedure for details.

method to estimate the rate constant for degradation of total liver lactate dehydrogenase which includes a mixture of three isozymes. They reported a value of 0.198 days^{-1} from which they calculated a half-life of 6.0 days. Glass and Doyle (1972) used the double isotope technique to estimate the rate constant for degradation of lactate dehydrogenase from the liver of young (120–140 g) female Sprague-Dawley rats and obtained a half-life of 6–7 days. These values are compared to our data for liver lactate dehydrogenase 5 from adult (350–450 g) male Sprague-Dawley rats for which we calculate a half-life of 19 days based on the first-order rate constant for degradation of 0.036 days^{-1} (Table I). This value is apparently dependent on diet since our previous studies using a diet containing only 6% amino acids gave values of 0.041 day^{-1} for k_d and a half-life of 16.0 days (Fritz *et al.*, 1969). Our estimate of the half-life of total soluble liver proteins of 2.2 days (Fritz *et al.*, 1969) agrees with the value of 2.5 days reported by Schimke (1964), who also used the continuous administration method. However, Kuehl and Sumsion (1970) estimated the mean half-life for total liver protein to be 6.6 days using [^{3}H]-leucine and 3.8 days using [^{14}C]guanidinoarginine; Glass and Doyle (1972) found that the total soluble liver protein half-life was 5.0 days when they used [^{3}H]leucine and 3.2 days using [^{14}C]guanidinoarginine. Several explanations for these differences in the half-lives of total liver protein and of lactate dehydrogenase 5 are possible. It may well be that they are a reflection of age, sex, strain, diet, or other environmental differences in the animals used. Also, they could be within the limits of error of the methods employed. Alternatively, the measured differences may result from the fact that the cells are exposed to the isotope from 10 to 100 times longer in the continuous administration method. Whether there exists a pool of free subunits from which the tetramers are assembled (Rosenberg, 1971) or whether the isozymes arise by *in vivo* exchange of subunits (Fritz *et al.*, 1971), some posttranslational control over the five isozymes probably exists; it is not clear how this control will influence the results obtained by the three methods.

Although it has not been previously reported that the turnover of isozymes is affected by diet, it is perhaps not unexpected in view of the well-known effects of amino acids on polyribosome aggregation and thus protein synthesis in general (Fleck *et al.*, 1965; Sidransky and Bongiorno, 1967; Jefferson and Korner, 1969; Morgan *et al.*, 1971; Wannemacher *et al.*, 1971). Protein degradation is also known to be affected by amino acids, specifically, as shown by the tryptophan effect on tryptophan pyrrolase (Schimke *et al.*, 1965) and by the leucine effect on tyrosine transaminase (Lee and Kenney, 1971), and more generally on total protein, as demonstrated by Woodside and Mortimore (1972) in perfused rat liver. At present we do not know if there is a decreased turnover of proteins other than lactate dehydrogenase in rats on higher amino acid diets.

The pattern of rate constants for degradation of the lactate dehydrogenase isozymes changes significantly from one tissue to another (Table I). In skeletal muscle the rate constants roughly double with the addition of each B subunit. By contrast, in heart the homopolymer A_4 has a higher rate constant for degradation than any of the tetramers containing B subunits. A third pattern is seen in liver where both A_4 and A_3B have essentially the same value. These results relate to the hypothesis that a positive correlation exists between subunit size and the rate constant for protein biodegradation (Glass and Doyle, 1972; Dehlinger and Schimke, 1970). Since the lactate dehydrogenase isozymes have identical molecular weights and number of subunits, this generalization appears to be invalid for the turnover of lactate dehydrogenase isozymes in rat skeletal and cardiac muscle, but may be applicable to the turnover of isozymes in rat liver, the tissue from which these authors developed their hypothesis. It might also be pointed out that the correlation in liver is between relative degradation and not absolute degradation.

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Uridylic Acid Synthesis in Ehrlich Ascites Carcinoma. Properties, Subcellular Distribution, and Nature of Enzyme Complexes of the Six Biosynthetic Enzymes†

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ABSTRACT: The six enzymes required for the biosynthesis of uridylic acid have not previously been characterized in a single cell line. To establish the subcellular localization, the general kinetic characteristics, and, more importantly, to be able to consider the possible rate-limiting step, we have studied the six enzymes in the mouse Ehrlich ascites carcinoma. All enzymes except dihydroorotase were studied in the biosynthetic direction. Five of the enzymes (carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, orotidylate phosphoribosyltransferase, and orotidylate decarboxylase) are located predominantly in the soluble (100,000*g* for 60 min) supernatant fraction of the cell as two enzyme complexes, but a significant proportion of these activities can also be localized in the crude nuclear fraction. One complex contains the first three enzymes of the pathway, carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase. This complex, isolated in sucrose gradients containing 30% dimethyl sulfoxide and 5% glycerol, has an apparent molecular weight of 800,000–850,000. The glutamine-dependent carbamyl phosphate synthetase can be dissociated from the complex by reducing the dimethyl sulfoxide concentration to 10% to yield a protein with an apparent molecular weight of 150,000–200,000 whose activity is very labile. When the carbamyl phosphate synthetase is dissociated from the complex, the aspartate transcarbamylase and dihydroorotase activities still cosediment in the sucrose gradient, but both enzyme activities now occur in two new protein peaks with apparent molecular weights of 400,000–450,000 and 650,000–700,000. These latter two peaks are present in a 0.25 M sucrose homogenate. The second enzyme complex contains the fifth and sixth enzymes of the pathway, orotidylate phosphoribosytransferase and orotidylate decarboxylase. This complex sediments in a sucrose gradient containing 30% dimethyl sulfoxide and 5% glycerol with an apparent molecular weight of 105,000–115,000; however, when only sucrose is present, the complex is unstable and a major portion of the activities sediments as a complex of 55,000–60,000 daltons. The fourth enzyme, dihydroorotate dehydrogenase, unlike the other five enzymes, is not found in the soluble supernatant, but sediments from the homogenate at 700*g*. The natural electron acceptor(s) for this enzyme has not been identified; a new assay is described for this enzyme. The dihydroorotate dehydrogenase will utilize dihydroorotate formed by the complex of the first three enzymes and the orotate so produced is converted to UMP by the orotidylate enzymes of the 100,000*g* supernatant. The apparent K_m values and pH optima were determined for four of these enzymes, since they have not been previously determined in extracts of these cells. Although the Ehrlich ascites cell carbamyl phosphate synthetase is subject to product inhibition (by UTP) and precursor activation (by 5′-phosphoribosyl 1′-pyrophosphate), as discovered for this enzyme in extracts of mouse spleen by M. Tatibana and his colleagues, it is not the enzyme with the slowest optimal rate; rather orotidylate phosphoribosyltransferase or perhaps dihydroorotase when it is measured in the biosynthetic direction seem(s) to be the slowest catalyst(s).

The six enzymes required for the biosynthesis of uridylic acid (Figure 1) have not previously been characterized simultaneously in a single cell line under apparently optimal conditions. It is, therefore, difficult from the literature to compare the relative rates of these enzymes for a given cell or to even begin to devise experiments to *properly* assess the factors that may regulate the rate of these enzymes for any given cell *in vivo*. This paper, therefore, represents a start on a study of the

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