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ONTOGENIC CHARACTERISTICS OF LACTATE DEHYDROGENASE TURNOVER IN THE MOUSE

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Summary

1. In order to investigate the ontogenic and turnover characteristics of lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) in the foetal mouse, activities of this enzyme and isoenzyme distributions have been measured during development, and the time sequence of incorporation of radioactive amino acids have been determined during the last weeks of gestation.

2. In the early foetal stages, the enzyme was present with low specific activity and in the form of lactate dehydrogenase-5 in all regions, but a marked increase in specific activity became evident about the seventeenth day of gestation, and was accompanied by the formation of tissue specific patterns of isoenzyme distribution.

3. Measurement of turnover parameters was undertaken by both double-label ([^{3}H] and [^{14}C]leucine) and pulse-chase ([^{3}H]leucine) techniques. Both procedures provided indications that appreciable degradation may occur during embryogenesis, with general proteolysis proceeding at a faster rate than with lactate dehydrogenase, and with higher rates of degradation occurring in foetuses which were smaller than average.

4. This data has been discussed in relation to the regional variation in these characteristics and the significance to studies of growth and differentiation.

Introduction

Lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) is both the archetypal example of an isoenzyme system, and the enzyme which has been most widely utilized in studies of tissue differentiation [1—3]. There have been a number of studies on the ontogenetic progressions of lactate dehydrogenase during the early and later stages of gestation, and many valuable contributions by this means towards a molecular definition of the central bio-

logical problem of cell and tissue differentiation [4—6]. As yet, however, our understanding of the factors which act to specify the isoenzyme complement of tissues remains incomplete; and one area, in particular, in which our knowledge is deficient is related to the turnover characteristics of this enzyme at particular stages of development. A significant question that needs answering, for example is whether protein degradation is occurring during foetal development and to what extent compared with protein synthesis.

The purpose of this study was to shed light on these questions by the use of two separate radioisotopic methods. In the first experiment, a double-label experiment (^3H and ^{14}C) was performed on pregnant mice in order to provide information of the relative turnover rates for general and specific proteins in separate foetal regions; and in the second experiment, a pulse of [^3H]leucine was administered to a group of pregnant mice, and the amount of radioactivity in soluble protein and lactate dehydrogenase was determined sequentially in order to provide information on the retention of label in the foetus in the later stages of pregnancy.

Materials and Methods

1,6-diaminohexane was supplied by B.D.H. Chemicals Ltd., Poole, England, and the potassium oxalate and cyanogen bromide by Ajax Chemicals Ltd., Sydney, Australia. Carbodiimide used in the substitution of the Sepharose was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A., as were the $NADH_2$, NAD, pyruvate and heparin.

Starch for gel electrophoresis came from Electrostarch Co., Madison, Wisconsin, U.S.A., and the Packard Instrument Company, Inc., Downers Grove, Illinois, U.S.A. supplied the 2-5-diphenyloxazole (POP) and the 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) used in the scintillation fluid.

All other chemicals and solvents employed were of analytical reagent grade.

Protein determinations were made by the method of Lowry, Rosebrough, Farr and Randall [7] and lactate dehydrogenase activity was measured by the method of Wroblewski and La Due [8] with the use of a Unicam SP800 recording spectrophotometer.

In the double labelling experiment 6 mice, pregnant for 12 days, each received 25 μCi of [^{14}C]leucine by an intraperitoneal injection followed 3 days later by an injection of 75 μCi of [^3H]leucine. 20 h after the final isotope injection, the animals were killed by decapitation, the foetuses were removed, blotted dry and weighed. The foetuses were separated into groups of eight, according to average weights, which were 0.74, 0.64 and 0.31 g respectively. Each foetus was dissected into four regions: head, limbs, chest and abdomen, and the dissected regions were pooled and homogenized in 2 vols. of cold 0.02 M sodium phosphate buffer, pH 6.8, centrifuged in a refrigerated L265-B ultracentrifuge at 165 000 $\times g$ for 60 min and the clear supernatants were dialysed overnight against 0.02 M sodium phosphate buffer, pH 6.8, 0.5 M sodium choride. $NADH_2$ was added to give a final concentration of approximately 100 μM immediately before chromatography.

Columns of the substituted sepharose used for affinity chromatography [9] were equilibrated with 0.02 M sodium phosphate buffer, pH 6.8, 0.5 M sodium

chloride at 4°C, the temperature at which all chromatography was performed. Just before sample application, buffer containing 100 μM $NADH_2$ was substituted for the eluting buffer. After application of the sample, washing was continued with $NADH_2$ until no further protein, as indicated by the $A_{280\ nm}$ values, could be detected in the eluant. All fractions containing activity were pooled and concentrated.

Starch gels were prepared with 48 g of electrostarch in 440 ml of 0.03 M Tris/glycine, pH 9.0, buffer; 9 mg of heparin (sodium salt, 179 USP units/mg) were dissolved in the gel buffer as directed by the suppliers. Vertical starch gels, with 20 sample slots, were run for 3.25 h at 4°C at a constant 600 V, which a discontinuous electrode buffer system of 0.12 M Tris/glycine, pH 9.0 for the cathode, and a 0.08 M solution of the same buffer for the anode. The concentrated samples of lactate dehydrogenase from each tissue were run in triplicate in adjacent slots in the gel, after which the gel was cut into longitudinal strips of equal width. One of the strips for each tissue was then sliced and stained for lactate dehydrogenase activity. The regions of isoenzyme activity were excised from the corresponding positions of the remaining two strips of gel, and each of these starch blocks was finely chopped and placed in a 20 ml screw-top counting vial. Up to 0.8 ml of hydrogen peroxide (30% w/v) was added to each vial depending on the quantity of starch to be digested. The vials were securely capped and placed in an overn at 70°C for 12 h, after which they were allowed to cool before 15 ml scintillator (xylene/Triton X-100/POP, POPOP) were added, mixed thoroughly, and allowed to equilibrate in the dark for several hours to ensure that fluorescence had been eliminated before counting.

The concentrated lactate dehydrogenase samples obtained from each tissue after affinity chromatography, were counted by dissolving either 0.1- or 0.2-ml aliquots directly in 3 ml of xylene/Triton scintillator. 0.1 ml aliquots of the clear supernatants obtained after centrifugation of the tissue homogenates were placed directly in the counting vials. Protein was precipitated by the addition of 0.2 ml of 10% trichloroacetic acid, and the vials were centrifuged for 5 min at 3000 × *g*. The supernatant was removed and the precipitates washed once with diethyl ether, then dissolved in 0.1 ml of 0.2 M sodium hydroxide. Finally, 3 ml of xylene/Triton scintillation fluid was added, and the samples were counted. All samples were counted in a Beckman LS-250 liquid scintillation system, with the ^{14}C-isoset provided by the manufacturers.

In a separate, pulse-chase experiment, 21 mice, pregnannt for 12 days, were each injected with 25 μCi of ^{3}H-leucine, and were killed (groups of three) at daily intervals. The foetuses were removed, blotted dry, weighed and dissected into heads, limbs and bodies. The foetal regions from the three mice killed on the same day were pooled, homogenized and the LDH and protein contents were measured, after which the lactate dehydrogenase was purified and the radioactivity was measured as in the previous description. Foetuses weighing less than 0.40 g each were not dissected, but processed in toto and are henceforth referred to as "small foetuses".

Results

The lactate dehydrogenase activities per foetus in each of the pooled groups which were used in these experiments are presented graphically in Fig. 1, and it

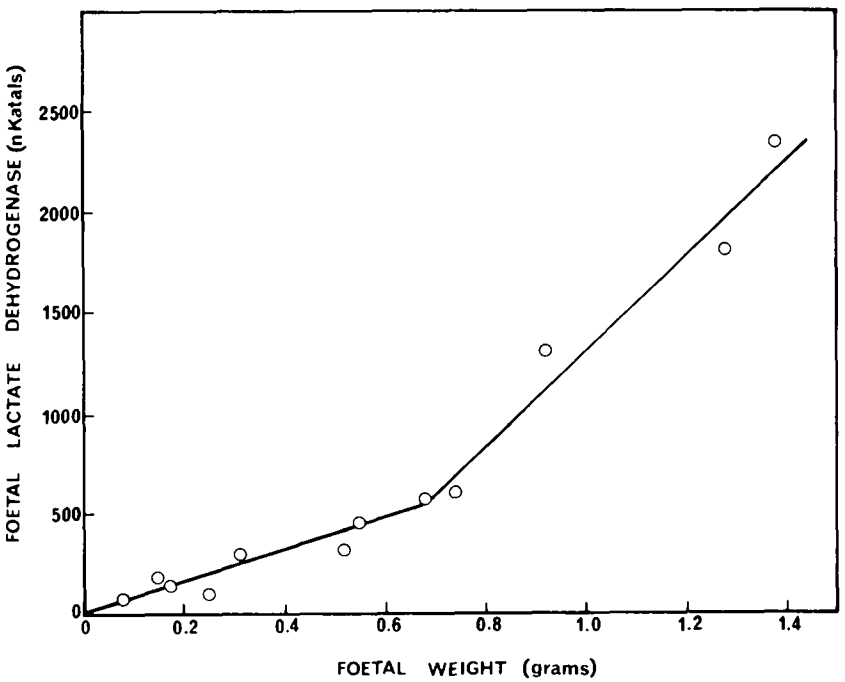

Fig. 1. Diagrammatic representation of the relationship between the total lactate dehydrogenase activity and weight of mouse foetuses. LDH, lactate dehydrogenase.

may be seen from the nature of this progression, that an appreciable augmentation of the rate of increase in specific activity occurs about the 0.75 g stage.

With the larger foetuses, it was practicable to carry out dissection into various regions, and the data for these are shown in Table I. In every case, the major portion of the lactate dehydrogenase activity was located in the foetal mid-regions, with lesser amounts in the heads and limbs; but the highest specific activity of lactate dehydrogenase occurred in the foetal limbs.

Electrophoretograms of the lactate dehydrogenase samples from the double-label and pulse-chase experiments are shown in Figs. 2 and 3. Lactate dehydrogenase-5 is the dominant isoenzyme in all early embryonic tissues, although progressions through lactate dehydrogenase-4, -3, etc. are discernible in the latter stages of embryogenesis.

Table II lists the double isotope ratios for total protein and lactate dehydrogenase in foetuses which were graded according to size. Higher values for these ratios were obtained for the larger foetuses in all cases but for the liver regions with total protein, and these relativities were also reflected in the indicated rate constants for degradation. With lactate dehydrogenase, the small foetuses had the highest ratio, in each of the foetal regions, whilst the large foetuses had the lowest, a difference which was most marked in the case of the liver region. Variations in specific radioactivities followed a similar general pattern to that observed with total protein, but values were significantly higher in all regions but liver. Again, when allowance was made for the influence of growth on these ratios, and the rate constants for degradation calculated [10—12], proteolysis was indicated as being higher in the average total protein than in lactate dehydro-

TABLE I

LACTATE DEHYDROGENASE SPECIFIC ACTIVITY IN MOUSE FOETAL REGIONS

Foetuses were weighed and dissected as described under Methods. The individual regions were homogenized, assayed for their content of lactate dehydrogenase and protein [7,8] and specific activities calculated.

Average foetal weight (g)	Approximate conceptual age (days)	Lactate dehydrogenase activity (nkat per g tissue)		
		Heads	Mid-regions	Limbs
0.31	15	800	972	1567
0.52	15½	480	684	823
0.55	16	633	986	228
0.55	16½	675	994	850
0.64	17	610	1105	672
0.74	17½	577	869	980
0.92	18	569	1560	2717
1.24	18½	901	1684	1504
1.38	19	1100	1853	2174

genase and greater in the smaller foetuses than in the larger ones.

Total radioactivity per foetus for total protein and lactate dehydrogenase in the foetal regions is shown in Table III. It can be seen that in the cases of large foetal mid-regions and heads, there is a very small loss of label from the third to the seventh day after injection in the case of total protein, but the loss is much greater in the case of small foetuses and large foetal limbs. On the other hand,

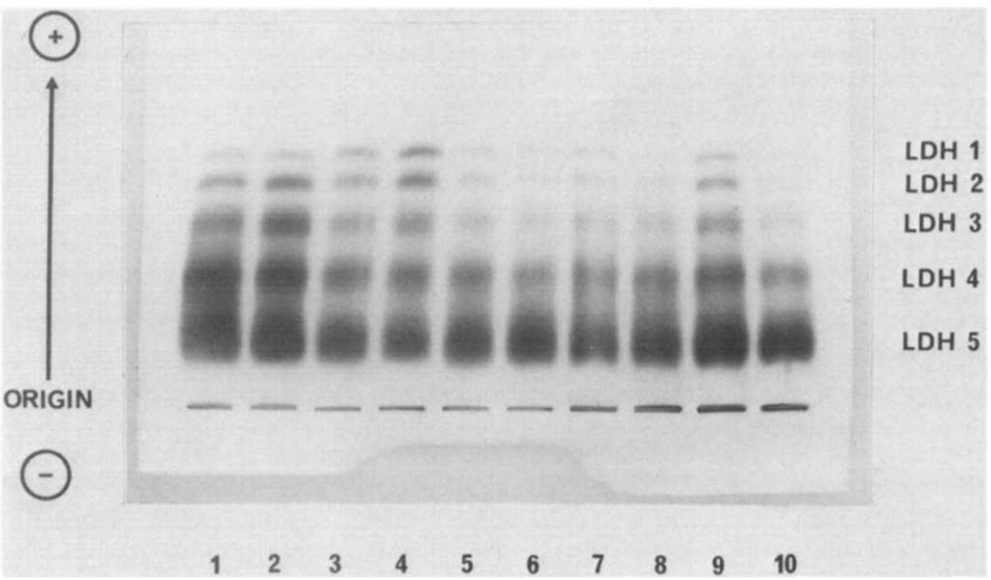

Fig. 2. Starch gel electrophoretogram illustrating the lactate dehydrogenase isoenzyme patterns of maternal uterus and foetal regions at the termination of double label experiment. Each region is represented by two patterns, with the left-hand member representing foetuses or 0.74 g average weight, and the right hand member foetuses of 0.64 g average weight. Slots 1, 2, maternal uterus; 3, 4, foetal heads; 5, 6, foetal limbs; 7, 8, heart region; 9, 10, liver region. LDH, lactate dehydrogenase.

TABLE II

DOUBLE ISOTOPE RATIOS AND DEGRADATION CONSTANTS FOR TOTAL SOLUBLE PROTEIN AND LACTATE DEHYDROGENASE FROM MOUSE FOETAL TISSUES

Six female mice, each twelve days pregnant, were injected with 25 μCi of [^{14}C]leucine followed three days later by 75 μCi of [^{14}C]leucine. 20 h later the animals were killed and the foetuses removed. The foetuses were grouped into "large" (average weight 0.74 g), "medium" (average weight 0.64 g) and "small" (average weight 0.31 g) classifications, with 8 foetuses in each group. The foetuses were dissected and total soluble protein and lactate dehydrogenase fractions separated. Duplication 100-μl samples of these fractions were used for scintillation counting and rate constants for degradation (K_D) calculated, as described in the text.

Tissue	Foetal group	Total protein		Lactate dehydrogenase	
		Ratio ^{3}H/^{14}C	(K_D)	Ratio ^{3}H/^{14}C	(K_D)
Head	Large	5.5 ± 0.1	0.18	4.0 ± 0.3	0.11
	Medium	4.6 ± 0.1	0.14	4.2 ± 0.3	0.12
	Small	5.2 ± 0.1	0.16	6.6 ± 0.5	0.21
Limbs	Large	6.6 ± 0.2	0.20	4.1 ± 0.3	0.15
	Medium	4.9 ± 0.1	0.15	5.5 ± 0.4	0.18
	Small	5.5 ± 0.2	0.18	6.6 ± 0.5	0.20
Heart region	Large	7.0 ± 0.2	0.22	4.2 ± 0.3	0.12
	Medium	6.5 ± 0.1	0.20	6.1 ± 0.4	0.19
	Small	6.9 ± 0.2	0.22	7.4 ± 0.6	0.23
Liver region	Large	4.6 ± 0.0	0.18	3.4 ± 0.2	0.12
	Medium	7.3 ± 2.4	0.28	5.6 ± 0.4	0.21
	Small	7.5 ± 0.1	0.30	7.1 ± 0.6	0.27

TABLE III

PULSE-CHASE EXPERIMENT: TOTAL PROTEIN AND LACTATE DEHYDROGENASE

21 mice, each 12 days pregnant, were injected with 25 μCi of [^{3}H]leucine. The mice were killed at daily intervals in groups of three, dissected into regions, and radioactivity determined in total protein and lactate dehydrogenase, as described in the text. The total radioactivity in each of these pools was then calculated, and tabulated.

Tissue	dpm ^{3}H/foetus × 10^{-3}						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
Total protein							
Small foetuses	104	79	174	167	—	66	—
Large foetuses							
Heads	—	610	141	97	116	87	91
Mid-region	—	170	497	315	390	271	269
Limbs	—	18	119	29	22	42	35
Total	—	798	757	541	528	400	395
Tissue	dpm ^{3}H LDH/foetus						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
Lactate dehydrogenase							
Small foetuses	200	390	310	80	—	80	—
Large foetuses							
Heads	—	120	100	55	70	110	90
Mid-region	—	210	320	310	490	360	350
Limbs	—	8	90	13	54	75	72
Total	—	338	510	370	614	545	512

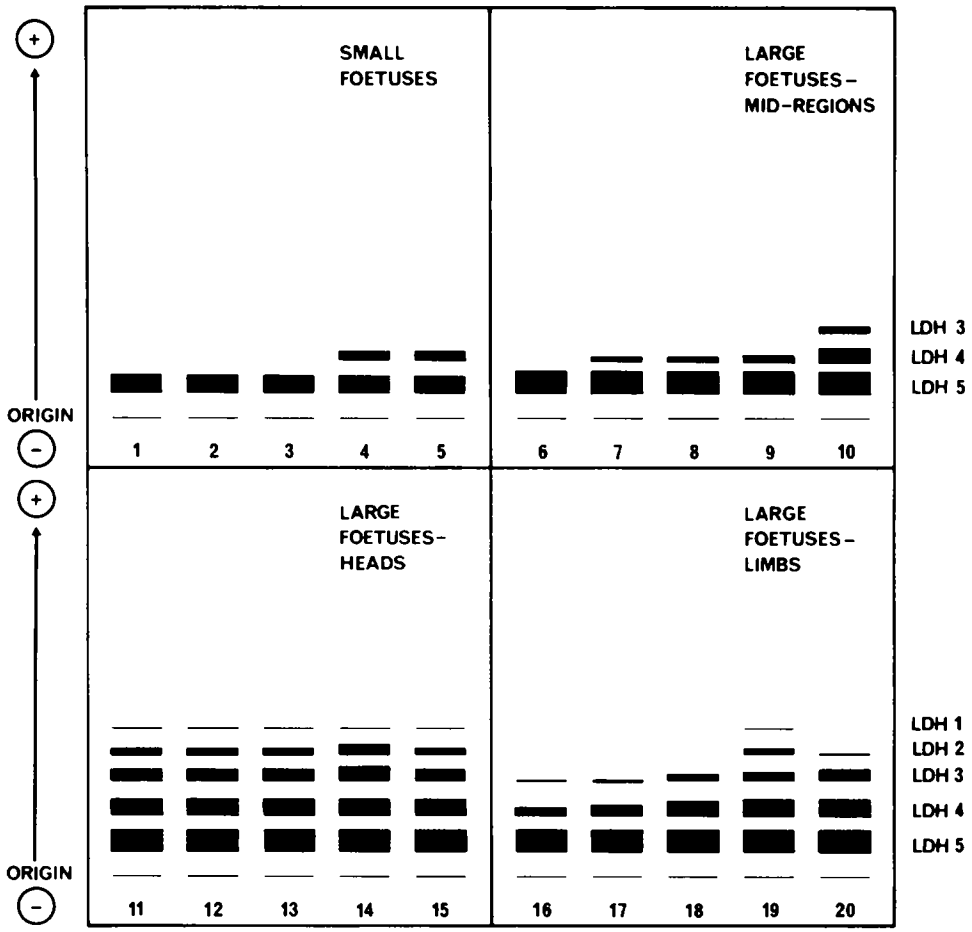

Fig. 3. Diagrammatic representation of the developmental progressions of lactate dehydrogenase isoenzymes during the pulse-chase experiment. The extent of shading is indicative of the relative activity of the individual multiple forms. Time of sampling in each sequence (i.e. 1—5, 6—10, 11—15, 16—20) is 2, 3, 4, 5 and 6 days after injection of tritiated leucine. LDH, lactate dehydrogenase.

the level of counts in lactate dehydrogenase remains relatively constant in heads and mid-regions of larger foetuses, and the rate of loss of radioactivity from small foetal protein is noticeably higher than with the larger foetuses. The radioactivity in foetal limb lactate dehydrogenase presents a complex pattern of incorporation.

Discussion

The present studies allow, firstly, a confirmation and amplification of previous investigations into the ontogenic progressions of lactate dehydrogenase [13—15]. In relation to the significant parameter of specific activity, for example, little information has been available in regard to foetal development in this species previously, but the present results point to the occurrence of a marked upward inflection in the progression of this parameter at about the

0.75-g stage (Fig. 1). In terms of foetal age, this increase corresponds approximately to the seventeenth day of gestation [16], and provides a significant indication of the timing and contribution of changes in lactate dehydrogenase activity to tissue differentiation in this species. When the specific activities of some adult tissues are considered, for example, the direction of this prenatal increase becomes apparent. For adult heart and kidney the lactate dehydrogenase specific activities are approximately 4000 nkat/g tissue; for liver, 3200; and for brain 1600. Hence this increase is the beginning of the transition from the mid-gestational specific activity (about 900 nkat/g) to adult tissue levels.

Furthermore, examination of Figs. 2 and 3 confirms the findings of Markert and Ursprung [15] that lactate dehydrogenase-5 is the chief isoenzyme in early embryonic development of the mouse. The appearance of lactate dehydrogenase-4 in the large foetal bodies on the sixteenth day of gestation is particularly striking, and relates to the changes observed at about that time in foetal heart and kidney by Markert and Ursprung. It may be noted that the foetal head tissues exhibit isoenzymes other than lactate dehydrogenase-5 much earlier than other regions. Furthermore, the general increase in lactate dehydrogenase specific activity occurs one day later with the foetal heads, compared with the other regions. It follows that the transition from the foetal to the adult forms of lactate dehydrogenase involves two processes: an increase in the production of the B-rich isoenzymes (lactate dehydrogenase-1 etc) through activation of the lactate dehydrogenase-B gene by some mechanism, and an increase in the specific activity of all forms of lactate dehydrogenase through an additional activation of the lactate dehydrogenase-A gene, with the relative timing and magnitude of these two processes being very tissue-specific. In the brain, the specific activity increase lags several days being the B-subunit enhancement whilst in the liver, although there is a significant increase in A-gene activity towards the end of gestation, the final lactate dehydrogenase isoenzyme pattern is very similar to the foetal pattern, with only a small increase in B-gene activity.

In an endeavour to shed some light on the turnover characteristics of this enzyme during embryogenesis, the double-labelling experiments were undertaken. Double isotope ratios have previously been shown to provide valuable indications of the relative emphases of synthesis and degradation in animal tissues [8—10], and in the present instance serve to illustrate the existence of a relationship between the size of the foetus and the magnitude of the double isotope ratio (which is especially marked in the case of lactate dehydrogenase), as well as differences between the double isotope values obtained for this enzyme in corresponding regions in the different sized foetuses. The implications of this data is that the turnover of lactate dehydrogenase is greater in the smaller than in the larger foetuses. It is also of interest to note that the double isotope ratios for total protein were significantly higher than the corresponding figures for lactate dehydrogenase; a fact which would indicate that the turnover of total protein is more rapid than that for lactate dehydrogenase.

As to the question of the absolute levels of degradation of these proteins in the developing foetus, it should be realized that although the magnitudes of the double-isotope ratios allow a direct estimation of rate constants for synthesis and degradation in the adult steady state [10—12,17], they do not provide

an immediate answer during embryogenesis because of the variations (e.g. in tissue size) which apply at this time. It is possible, however, to obtain meaningful data on the relative rates of degradation in these foetal tissues by incorporating due allowance for the alterations in the weight of the embryo, and several significant deductions flow from the resultant corrected ratios (Table II). It would appear from these figures, for example, that the rate of degradation in this foetal period is appreciable, and indeed of a comparable order to that in adult tissues [12,17]. Again proteolysis (both general and specific) is higher in the smaller foetuses than the larger, and, in general, higher in regard to total protein than lactate dehydrogenase. There is a further indication in these figures, also, that liver may be the major site of proteolysis in these small foetuses.

With regard to the relative emphasis of degradation between total protein and lactate dehydrogenase, it is worthy of note as well that there is good agreement between the present findings and previous studies on the turnover of this enzyme in maternal tissues during pregnancy [17]. There the conservation of lactate dehydrogenase relative to total protein was noted during this physiological perturbation and led to a conclusion which might well apply to both circumstances; that the favoured response of lactate dehydrogenase may reflect the important role played by this enzyme in cellular function.

In view of the nature of these findings, and the deficiency of comment on this aspect in the available literature, it was considered desirable that confirmation be provided by means of different methodology. To this end, the data from the pulse-chase experiment may be noted; and in particular the fact that the sequential values for the total tritium content per foetus and per region of the foetus provides a useful indication of the degree of protein degradation in this growth situation. This parameter again serves to indicate the presence of appreciable protein degradation in these embryonic stages, points to the higher rate of proteolysis in the smaller foetuses, and illustrates that total protein is degradated more rapidly than lactate dehydrogenase in the larger foetuses.

The overall half-life for total soluble protein in large foetuses was indicated as of the order of six days, for example which is similar to the results obtained in the double-isotope experiment and somewhat greater than average values in adult tissues [12,17].

The indicated half life for small foetuses, however was significantly lower (of the order of two days). With lactate dehydrogenase, overall turnover in large foetuses is indicated by this parameter as relatively minor, but again is apparently considerably accelerated in small embryos. The situation with foetal limbs is complex, exhibiting quite wide fluctuations, and while the reason for this variability is not clear at this state, it may perhaps be related to a connection between the initiation of physiological function and to stimulation of specific protein synthesis in this tissue.

The major deduction from these two different lines of turnover study, then, is that they lead to common conclusions as regards the relative emphasis of proteolysis in embryonic tissues, but the data is also of interest in that it may shed new light on the developmental abnormality known as resorption. Resorption of foetus in utero is known to result in a degree of embryonic loss which averages more than 15% in mice, and is a relatively common occurrence in

other mammals as well [18—20]. Consequently, the occurrence of a relationship between size and degradation rates in the present studies is noteworthy, and may provide an indication of a connection at the level of the relative concentrations of specific inhibitors of protein degradation [21—23].

In summary, then, the present investigation has served to detail the developmental changes in the specific activity and multiplicity of lactate dehydrogenase in mouse tissues, and has demonstrated the presence of appreciable and preferential degradation of proteins during these ontogenic stages, results which bear significantly on previous interpretations of differential gene activation during embryogenesis. Evidence has been provided that foetal proteolysis is of a lower magnitude than in most adult tissues, but sufficient to allow a significant role in the redistribution of protein between foetal regions during growth and differentiation.

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