CHAPTER IV

QUANTITATIVE AND ELECTROPHORETIC ANALYSIS OF LACTATE DEHYDROGENASE DURING TAIL REGENERATION IN THE SCINCID LIZARD, MABUYA CARINATA

Lactate dehydrogenase (L-Lactate; NAD-Oxidoreductase, EC 1.1.1.27)(LDH) is a tetramer composed of two different types of subunits. LDH which is most predominant in skeletal muscle is named as the Muscle or A type and the other, predominant in heart muscle is named as the Heart or B type (Wieland and Pfleiderer, 1957; Markert and Møller, 1959; Kaplan and Ciotti, 1961 a,b; Cahn et al., 1962; Markert, 1963; Markert and Appella, 1963 and Wilson et al., 1963). Wilson et al. (1963) have equivocally demonstrated the inhibition of B subunits under low concentrations of pyruvate and the relative resistance to inhibition of A subunits to low concentrations of pyruvate. Susceptibility to 'pyruvate inhibition' of an isozyme containing both kinds of subunits is reported to be directly proportional to the relative content of B subunits (Goodfriend and Kaplan, 1964). Therefore, with suitable modifications of pyruvate concentration, assays of tissue LDH can yield measurement of two distinct proteins, the A and B subunits (Wilson et al., 1963 and Goodfriend and Kaplan, 1964) and the ratio of the B type to the A type

expressed as the 'analogue ratio' may reflect the metabolic status of the tissue (Temperley, 1971).

These two LDH subunits are thought to be controlled by separate genes and they combine, at random, to give rise to two pure forms, i.e. A_4 and B_4 and three hybrid forms, A_3B_1, A_2B_2 and A_1B_3. The mechanism controlling the formation of various subunit combinations remains enigmatic todate. These LDH isozymes when separated electrophoretically on polyacrylamide gels, run to different lengths, with the B_4 isozyme (LDH 1) being fast moving and most anodic and the A_4 isozyme (LDH 5) slow moving and least anodic (see the review of Roman, 1969).

Tail regeneration in lizards involves the operation of multifarious factors, both at the site of regeneration as well as in other visceral organs like liver, skeletal muscle, kidney and spleen. Regulation of the processes like wound healing, dedifferentiation, blastema formation, differentiation and growth, characteristic of vertebrate regeneration may possibly involve a shift in the LDH isozyme pattern marked by a change in the subunits. Previous enzymological studies on tail regeneration in Mabuya carinata had indicated a possible metabolic shift from a post-autotomic anaerobiosis to an aerobic one, during the blastemic and early differentiation phases, with a final shift back to the anaerobic pattern characteristic of the normal tail tissues (Shah and

Ramachandran, 1970; 1972; 1973; 1974; 1976). Moreover, the later works of Kinariwala(1977), Kinariwala et al. (1978), Ramachandran et al. (1979) and Shah et al. (1980 a, 1981) have also suggested the prevalence of an aerobic environment in the body as a whole during tail regeneration. Relevance of this concept can easily be confirmed by a study of the LDH subunits as well as its isozymic pattern, as these two aspects together can project a clear picture of the metabolic profile. In this light a quantitative evaluation of the A and B type of LDH subunits as well as the electrophoretic distribution pattern of its isozymic components were undertaken in the regenerating tail, liver and skeletal muscle during various stages of tail regeneration in the Scincid lizard, Mabuya carinata to understand the sub-cellular metabolic and molecular mechanisms underlying the phenomenon of tail regeneration.

MATERIALS AND METHODS

Healthy Mabuyas obtained from Hyderabad, Andhra Pradesh, India, were maintained on a diet of insects. The animals were acclimated to the laboratory conditions, for about 10 days prior to autotomy. Animals in the same weight group (20-24 g) of both sexes were selected for the study. Autotomy was done by pinching off the tails, nearly 2-3 cms from the vent. Quantitative as well as electrophoretic analysis of LDH were carried out during different stages of tail

regeneration viz., 3rd, 5th, 7th, 10th, 12th, 15th, 25th, 40th and 60th days post-autotomy and, in animals with intact tails.

Various tissues viz., liver, skeletal muscle and regenerating tail or normal tail as the case may be, were collected from the animals immediately after decapitation. In case of liver and skeletal muscle a 2% homogenate was prepared in ice-cold glass distilled water, and in the case of tail a 4% homogenate was found satisfactory. The homogenate was centrifuged in Mechanica, Precyzyjna, type 310, high speed centrifuge maintained at 4°C at 30000 X g for 15 mins and enzyme activity was then estimated in the supernatant. The enzyme was assayed by the method described by Kornberg (1955) with 10 mM and 0.33 mM concentrations of sodium pyruvate (obtained from Sigma Chemicals, U.S.A.), in a final volume of 3 ml, to get peak activity of B and A subunits respectively (Ganguly et al., 1978). The rate of fall in the optical density for every 30 seconds was followed at 340 nm in a Carl Zeiss Jena VSU 2-P-Spectrophotometer with cuvetts of 1 cm light path. The ratio of B type subunits to A type was calculated and expressed as the 'analogue ratio' (Wilson et al., 1963 and Goodfriend and Kaplan, 1964). The specific activity of the enzyme was expressed as μ moles of NAD formed/mg protein/min.

The amount of protein in the same supernatant was assayed by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

To every 1 ml of the crude homogenate, 0.1 ml of 1% β-mercaptoethanol was added and centrifuged at 30000 X g for 15 mins at 4°C. The supernatant having approximately 150-200 µg of protein concentration was mixed with a 20% solution of Sucrose (w/v), and was placed on top of the 8.5% polyacrylamide gels. Electrophoresis was performed according to the method of Davis (1964) with a 0.2 M Tris-glycine buffer of pH 8.9 as the reservoir buffer. A constant current of 2.5 mA/gel was passed for 5-6 hrs, as this relatively longer period of run as compared to that for mammalian tissues was found to be satisfactory for the proper resolution of the bands. A similar observation was reported by Lippe et al.,(1977) in the embryonic chicken muscle. After the electrophoretic run, the gels were removed and incubated, in dark, at 37°C, in a freshly prepared medium as described by Dietz and Labrano (1967) for 30-40 mins and were stored in 7.5% acetic acid.

RESULTS

The results of the effect of tail regeneration on LDH subunits in tail regenerate, liver and skeletal muscle are represented in Figs. 1-6, respectively. The relative

concentrations of A and B subunits with the calculated analogue ratios are shown in Figs. 1-3 and the distribution pattern of the isozymes of LDH 1-5 obtained on polyacrylamide gels for all the three tissues mentioned above are given as zymograms in Figs. 4-6.

Figs. 1-3 reveal the preponderance of A subunits in comparison to B subunits in all the three tissues of normal animals with intact tail. Hence, the respective analogue ratios (B/A) are below 1.

The onset of regeneration was clearly marked by a tremendous increase in A subunits and corresponding depletion of B subunits with a resultant fall of analogue ratios to well below the normal levels. Though this trend of increase in A subunits with a decrease in B subunits and resultant fall in analogue ratio in tail regenerate and liver had reached the zenith by 3rd day post-autotomy, in the skeletal muscle such a state was attained only on the 5th day. However, by this time the trend was reversed in both the regenerate and liver and hence there was a slight increase in the analogue ratio in these two cases. On day 5 though in case of liver and muscle the analogue ratios were well below the normal levels, in the case of tail, the analogue ratio was slightly above normal.

Since then, though 7th, 10th and 12th days post-autotomy,

Table 1. Quantitative levels of LDH 'A' and 'B' subunits in regenerate, liver and skeletal muscle during regeneration in M. carinata.

(Values are expressed as mμ Moles of NAD formed/mg protein/min.)

Periods of tail regeneration in days	Tail		Liver		Muscle	
	A	B	A	B	A	B
N	344.66 ±2.83	271.15 ±3.07	267.98 ±5.12	210.20 ±4.47	400.92 ±5.60	281.66 ±6.17
3	482.13*@ ±4.84	253.36 ±4.18	387.72*@ ±5.96	119.40*@ ±4.56	480.79*@ ±4.87	209.43*@ ±4.51
5	421.32*@ ±3.50	341.73*@ ±2.59	335.00*@ ±7.19	144.89*@ ±3.08	501.63*@ ±4.30	200.25*@ ±3.09
7	331.54 ±3.94	331.96*@ ±3.90	245.26*@ ±3.74	256.97*@ ±6.70	450.82*@ ±4.68	200.97*@ ±3.40
10	269.94*@ ±3.56	420.40*@ ±3.43	223.69*@ ±3.72	288.67*@ ±6.38	380.42*@ ±4.20	241.81*@ ±3.55
12	253.86*@ ±4.30	509.87*@ ±4.66	199.79*@ ±4.50	320.05*@ ±4.40	301.34*@ ±5.40	300.25 ±4.56
15	225.32*@ ±3.66	252.08*@ ±6.15	167.88*@ ±3.43	336.96*@ ±4.44	280.23*@ ±3.25	400.43*@ ±3.68
25	375.18*@ ±3.26	421.13*@ ±4.46	232.75*@ ±5.10	303.49*@ ±4.88	310.35*@ ±3.39	340.54*@ ±3.80
40	351.10*@ ±2.32	331.15*@ ±2.30	255.66 ±3.57	258.32*@ ±3.27	361.65*@ ±4.41	310.66*@ ±2.71
60	345.25 ±2.45	282.62* ±3.31	266.47 ±3.72	233.11@ ±3.66	410.77@ ±2.25	272.16@ ±3.21

N : Normal (Pre-autotomy state)

* $P < 0.01$; * $P < 0.05$; @ $P < 0.0025$; @ $P < 0.001$; *@ $P < 0.005$

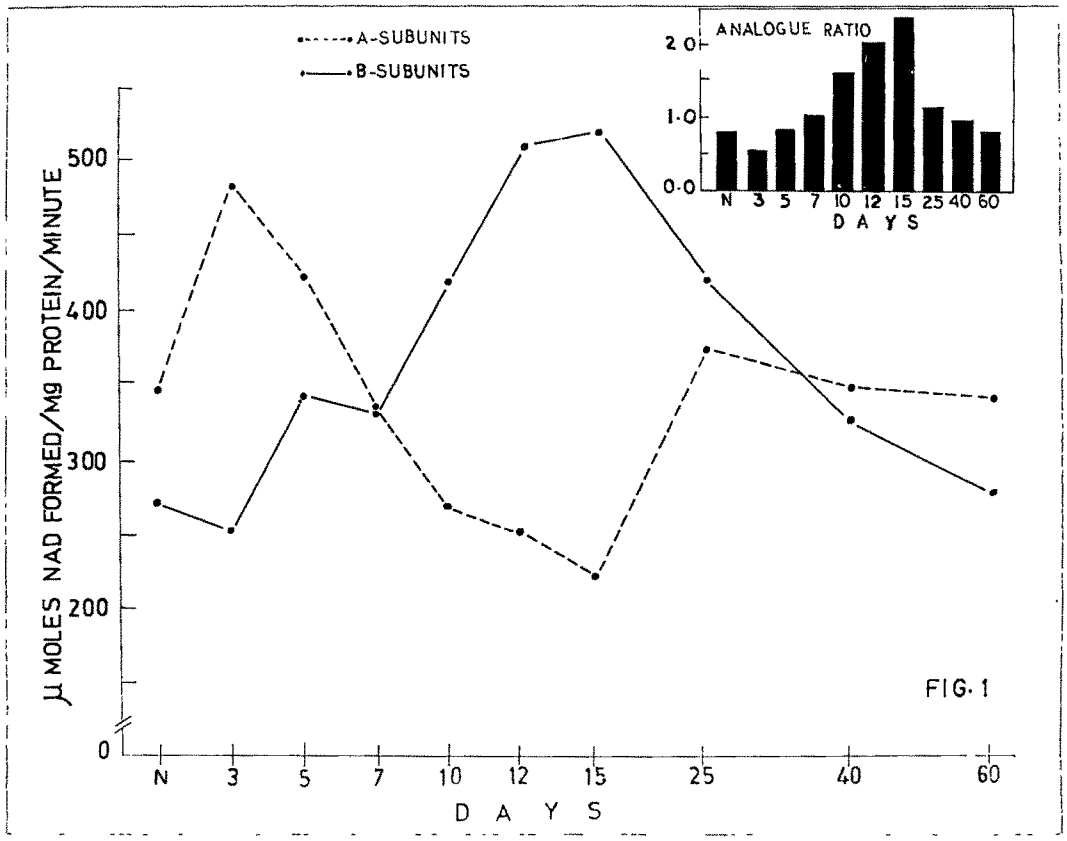

Fig. 1. Changes in the 'A' and 'B' LDH subunits and analogue ratio (B/A) in the regenerate during tail regeneration in M. carinata.

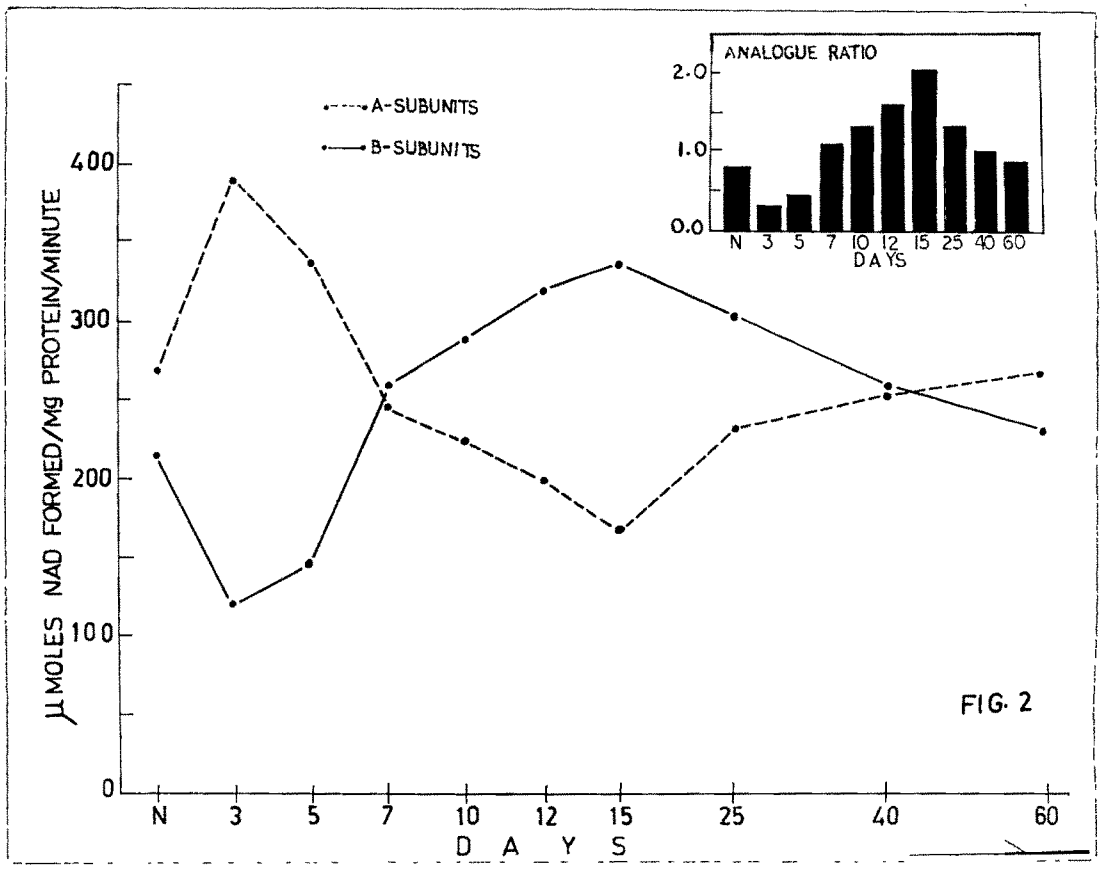

Fig. 2. Changes in the 'A' and 'B' LDH subunits and analogue ratio (B/A) in liver during tail regeneration in M. carinata.

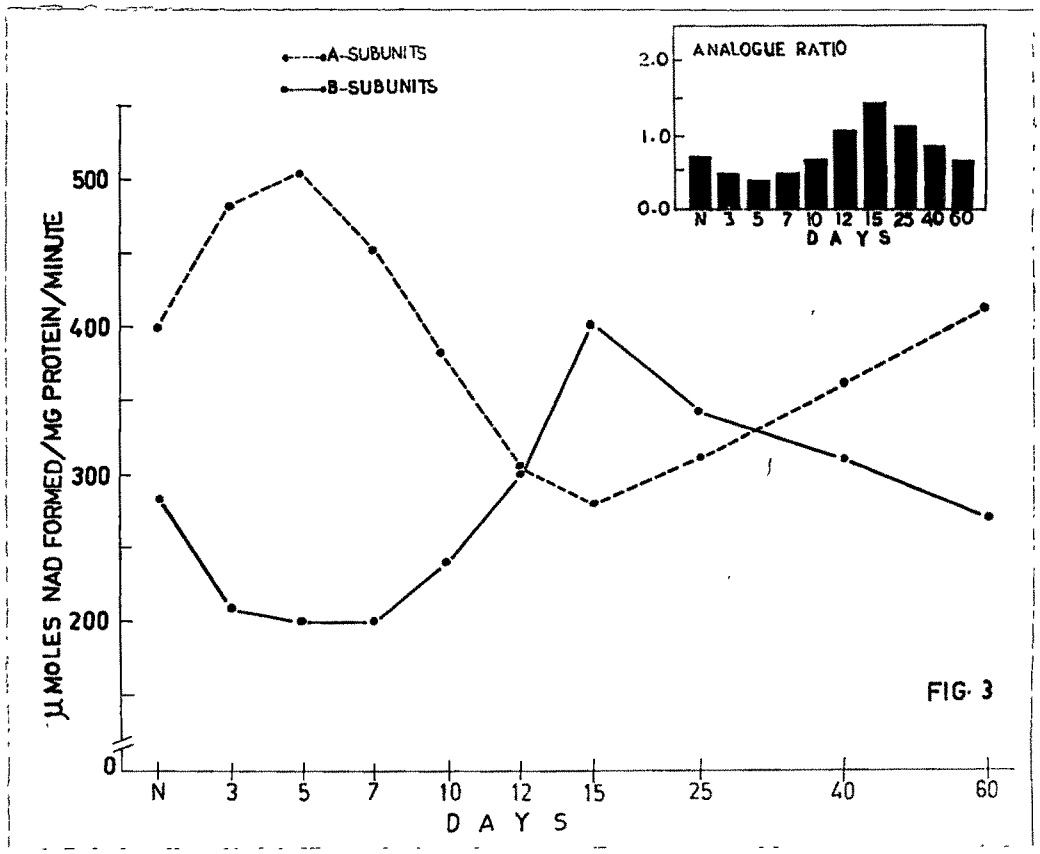

Fig. 3. Changes in the 'A' and 'B' LDH subunits and analogue ratio (B/A) in muscle during tail regeneration in M. carinata.

there was a gradual but definite increase in the B subunits with a concomitant decrease in A subunits and resultant increase in the analogue ratio in all the three tissues, till this trend attained its upper limit on the 15th day of tail regeneration. Thus on the 15th day the B and A subunits registered their highest and lowest levels respectively, with the analogue ratios also attaining their zenith.These reciprocal changes in the two types of subunits as well as the increase in analogue ratio of all the three tissues recorded in the initial stages of regeneration lasting upto 15 days, tend to depict a linear pattern.

The subsequent period of tail regeneration was marked by a decrease in B subunits and an increase in A subunits as seen on the 25th and 40th days post-autotomy. Ultimately both the subunits attained their respective normal levels by about the 60th day of tail regeneration. These changes in LDH subunits and the respective analogue ratios were common to all the three tissues under study.

All the five bands of LDH (1-5) could be discerned in tail, liver and muscle of normal animals with intact tail. However, the anodal bands (LDH 1 and 2) were very weak in all the three cases with 5,4 and 3 in that order (based on staining intensity) being the most prominent. The initial period of regeneration was marked by the disappearance of the anodic bands. The hybrid band (LDH 3) also disappeared leaving behind

Fig. - 4 : Zymograms depicting LDH isozymes in the regenerate during tail regeneration in M. carinata.

Fig. - 5 : Zymograms depicting LDH isozymes in liver during tail regeneration in M. carinata.

Fig. - 6 : Zymograms depicting LDH isozymes in muscle during tail regeneration in M. carinata.

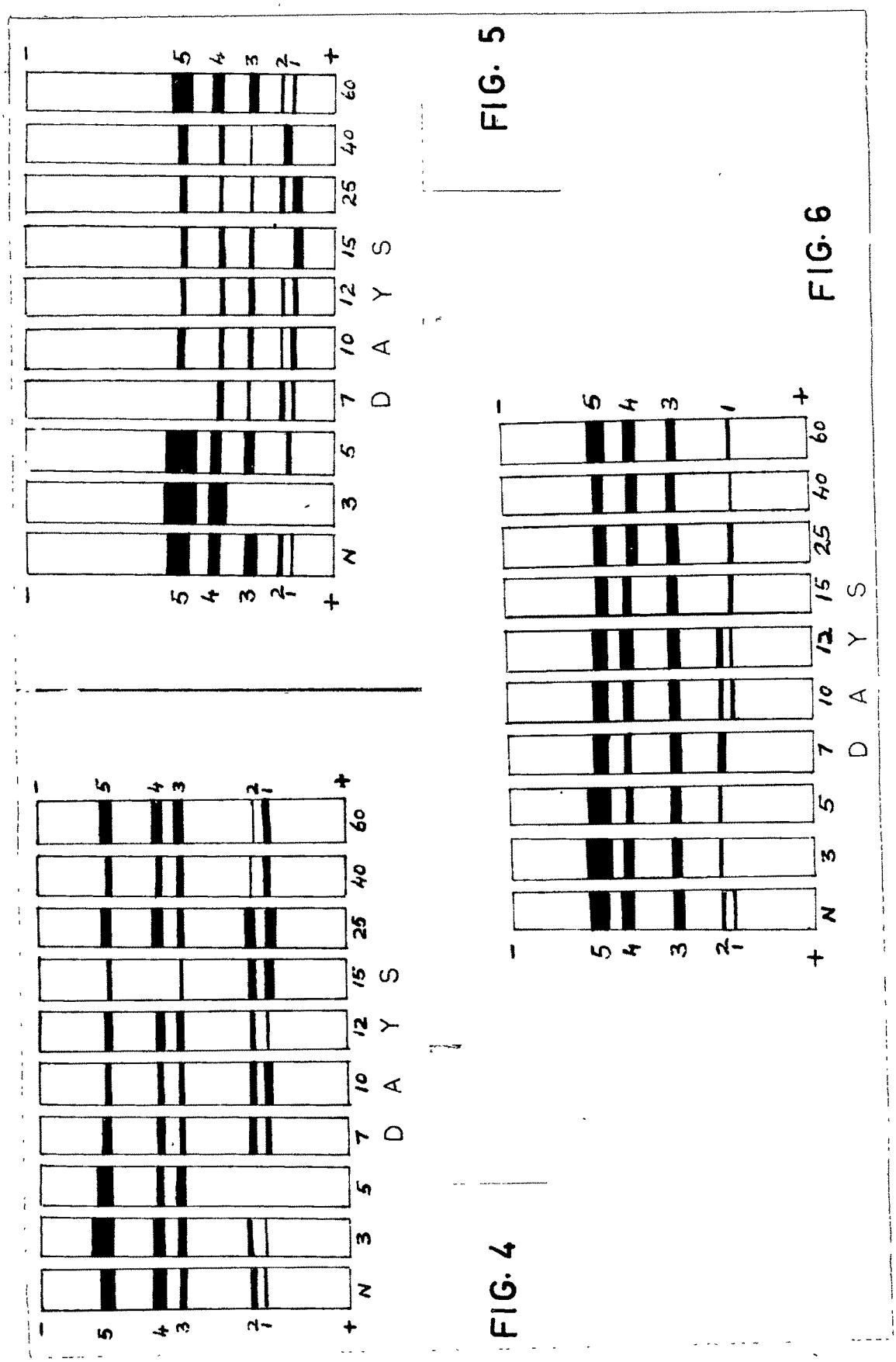
FIG. 5
FIG. 6
FIG. 4
DAYS
DAYS
DAYS
N
3
5
7
10
12
15
25
40
60
1
2
3
4
5
+
-

only bands 4 and 5 on the 3rd post-autotomy, particularly in liver. On day 5th, LDH band 3 along with weak diffused anodic bands reappeared. In muscle on the other hand, 3rd and 5th days post-autotomy were marked by the presence of only bands 2,3,4 and 5 with LDH 1 being completely missing. In the regenerating tail there was a gradual but complete disappearance of both LDH 1 and 2 during 3rd and 5th days post-autotomy. This initial regressive phase of regeneration was marked by increasing intensity of the slow moving muscle type bands (LDH 5 and 4). Though there was no change in the electrophoretic pattern of muscle LDH on the 7th day, all the 5 bands could be clearly noticed in both the tail regenerate and liver at this stage with weaker muscle type of bands and thin but sharp heart type bands. On 15th day, in the regenerate, prominent LDH 1, 2 and 3 bands with a weak 5th band could be discerned. In liver, on 12th day there were both LDH 1 and 2 bands but on 15th day, only strong LDH 1 band could be observed. During both these days bands 3, 4 and 5 were quite weak in comparison to the normal. Muscle also depicted a similar pattern as in liver on 12th and 15th days post-autotomy.

The 25th and 40th days of regeneration were marked by increasing intensity of LDH isozymes, 5, 4 and 3 with gradual weakening of the anodic heart type bands 1 and 2 in all the three tissues under study. A near normal pattern of isozymes

could be discerned in liver, muscle as well as the tail regenerate on 60th day post-autotomy. These changes in the distribution pattern of the various isozymic components are represented as zymograms in Figs. 4-6.

DISCUSSION

Lactate dehydrogenase, the terminal enzyme of anaerobic glycolysis, by its isozymic components controlled by the two subunits, can easily play a pivotal role in bridging the glycolytic process with TCA cycle oxidation. LDH as an index of the changing metabolic profile of a tissue can be gauged not only by the quantitative changes in the two constituent subunits which could be expressed as an analogue ratio, but also by the changes in the electrophoretic pattern of its isozymes. An evaluation of both these parameters in the present study yields a clear manifestation of the occurrence of adaptive metabolic changes not only in the regenerating system but also in the body of the animal as a whole. Increased anaerobiosis associated with the wound healing phases of regeneration reported by many workers, (Okuneff, 1933; Dickens, 1951; Needham, 1952) stands confirmed by the observed increase in the A subunit concentration as well as the LDH 5 band on the immediate post-autotomy period (3rd day). A change in LDH isozymic pattern towards anaerobic type has been shown to occur during early cell proliferation

associated with malignancy (Gerhardt et al., 1963; Richterich and Burger, 1963; Dowson et al., 1964; Goldman et al., 1964; Timperley, 1971; Weinhouse and Ono, 1972; Ananthanarayanan and Ramakrishnan, 1978). Similar changes recorded in liver and skeletal muscle too during this period suggest the prevalence of an anaerobic environment in the body. The absence of the anodic bands in liver and tail during 3rd and 5th days respectively, probably indicates the exclusive assembly of A type subunits.

The periods after wound healing i.e. preblastema, blastema and early differentiation phases are marked by a continuous increase in B subunits paralled by a fall in A subunits and a resultant increase of the analogue ratio in all the three tissues under study. These changes appeared to reach a zenith on the 15th day of tail regeneration. Concomitant changes in the isozyme pattern were also revealed by the appearance of the anodic LDH 1 and 2 bands. These set of changes highlight a shift in the metabolic pattern, from the anaerobic to the aerobic type. Occurrence of such a shift during blastemic and early differentiation phases of tail regeneration was suggested by Ramachandran (1972) and Shah and Ramachandran (1970, 1974, 1976) based on the histochemical localization of dehydrogenases. Prevalence of an aerobic environment in the body as a whole during these periods of tail regeneration was also inferred by Shah et al.

(1980 a), based on their studies on the changes in the total RBC count and haemoglobin content in the blood during tail regeneration in Mabuya carinata. Further evidence available in this context are the shrinkage in the size of visceral fat bodies (Shah et al., 1981), depletion of colloidal content from the thyroid acini (Ramachandran et al., 1981) as well as the increased metabolic activity in various visceral organs (Kinariwala, 1977) during the blastemic and early differentiation periods of tail regeneration in Mabuya carinata.

Appearance of LDH 1 and 2 bands and the attainment of the analogue ratio of 1 by the 7th day post-autotomy itself, in the regenerate and liver are indicative of the definite shift towards aerobic metabolism in both these tissues during the preblastemic phase of regeneration. Peak aerobic activity appears to be established in the tail regenerate as well as the body as a whole by the 15th day post-autotomy as marked by the strong LDH 1 and 2 bands and the maximal analogue ratio obtained at this period which roughly corresponds to early differentiation phase. Strong LDH 1 and 2 fractions which are known to support aerobic oxidation could be purported to play a major role during active cell proliferation and early differentiative events characteristic of blastemic and post-blastemic phases of regeneration. Support to this contention can be drawn from the report of Ananthanarayan and Ramakrishnan

(1978) indicating the elevation in these two LDH isozymic fractions during early carcinomas, multiple myelomas and embryological tumors. An interesting feature is the prolonged anaerobiosis and slow attainment of the oxidative potential shown by the skeletal muscle. Both the electrophoretic pattern and the relative proportion of A and B subunits obtained from skeletal muscle, indicate the persistance of anaerobic glycolysis upto the 7th day post-autotomy as well as the setting in of the oxidative pattern of metabolism only by the 12th day of tail regeneration. Persistance of though declining but higher analogue ratio, and the anodic isozymic bands till about the 40th day of tail regeneration in both liver and the regenerate are clearly suggestive of the operation of the oxidative pattern of metabolism in both the tissues even during the late differentiation period. Pertinent to quote in this context are the reports of Ramachandran (1972) and Shah and Ramachandran (1970, 1974, 1976) wherein they had shown an increased incidence of TCA cycle enzymes in the regeneration blastema and their peak level activity during the late differentiation period, based on which they had suggested the operation of oxidative reactions during the differentiation period. An interesting explanation for the presence of B type of LDH subunits in ruminant liver is the conversion of fatty acids to glucose known to occur in this organ (Fine et al., 1963). Since

gluconeogenesis is suspected to be in operation during blastemic and differentiation periods in the regenerate as well as in liver (Shah and Ramachandran, 1972, 1973, 1976; Shah et al., 1977 b), the currently obtained strong response for B type LDH may assume significance in this context. However, in the muscle, this pattern appears to be operative only for a short period i.e., between the 12th and 25th days of tail regeneration. Since then, whereas the skeletal muscle attained the normal anaerobic pattern (denoted by the isozymic pattern and analogue ratio) by about the 40th day itself, this shift back was evident in the regenerating tail and liver, only by the 60th day post-autotomy, which roughly corresponds to the growth phase.

The present investigation has depicted definite changes in the relative proportion of both A and B subunit proteins during various phases of tail regeneration, not only in the tail but also in the liver and skeletal muscle. A comparatively more pronounced change is indicated in the regenerating system than in liver and muscle. Similar changes in LDH subunits and isozymic pattern are well established in the developing systems (Flexner, et al., 1960; Vessel et al., 1962; Engel and Petzoldt, 1973; Engel et al., 1975; Erickson et al., 1975; Whitt, 1975), and differential gene repression and derepression of the A and B genes are considered to be responsible. Regulation of these changes at the molecular

level might involve control at the transcriptional level, translational level as a well as at the subunit assembly level. Though a differential gene activation and inactivation can be considered to be operative during tail regeneration, as the process of regeneration shows analogy with a developing system, concomitant changes in the adult organs of the body too, tend to denote a different mechanisms of LDH regulation or a multiple factor control. Fine et al. (1963) have opined that the expression of the genetic capacity to produce both types of LDH is controlled by unknown factors which are associated with the underlying mechanisms involved in differentiation and development. Further, Whitt (1975) has cited the suggestion of many workers that though differential gene regulation could probably be the most important component contributing to the developmental changes in isozyme patterns, additional levels of cellular regulation also have to be considered. It has also been suggested that the activity of some isozymes can be regulated not only by their position at specific subcellular locales but also by the presence or absence of various effector molecules within the cell. In the present study on tail regeneration an increase in the B subunits and the concomitant appearance of LDH 1 and 2 isozymes in all the three tissues under investigation tend to favour the activation of B locus by a common factor. Interestingly, Shah et al. (1980 a) have shown the high oxygen carrying capacity of blood by their studies on the changes in

the RBC count and haemoglobin content during the first week post-autotomy in Mabuya carinata. In this light the possible role of the elevated tissue oxygen level in a specific derepression of B locus and a non-specific repression of A locus is worth ascertaining. However, the differential level of elevation of B subunits as marked by the comparatively pronounced one in the regenerate as opposed to the less pronounced but similar one in liver and skeletal muscle during the second week of tail regeneration is probably indicative of the relatively easy transcriptional accessibility of the B locus in the undifferentiated and partly differentiated cells of the regenerate as compared to the fully differentiated cells of liver and muscle. Moreover, alternate regulation by either pituitary or adrenal hormones also cannot be overruled, as some of the unpublished observations from this laboratory indicate these hormones to be playing significant roles during regeneration. Finally, the possible role of thyroxine in elevating the A subunit level and hence LDH 5 and 4 may be inferred by the biphasic increase in these fractions observed during tail regeneration. Both these phases i.e., the initial one, during the 3rd and 5th days post-autotomy and the later one after the 25th day of tail regeneration are incidentally paralleled by the depletion of the colloidal material from the thyroid follicles (unpublished observation). This contention finds support from the observations of Lippe et al. (1977) on the influence of thyroxine in inducing LDH 5 and 4 isozymes in embryonic chick muscle.

* * * * *