THE ONTOGENY OF ISOZYMES OF LACTIC DEHYDROGENASE IN TWO AMPHIBIAN SPECIES

by

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ABSTRACT

The ontogeny of the enzyme LDH has been studied in two species of amphibians (*Amblystoma gracile* and *Rana aurora*) as it provides a sensitive gauge of the state of differentiation of the organism, since the number and proportions of LDH isozymes present exhibit temporal and species specificity, thereby reflecting the degree of activity of the controlling genes. The presence of LDH in all stages of both species examined was established by assaying embryo homogenates for LDH activity, and the LDH was resolved into isozymic patterns by the methods of starch gel and disc electrophoresis. Specific enzyme activity for each developmental stage was correlated with the morphological events then occurring and the isozyme patterns obtained were discussed in terms of showing an increase in complexity during ontogeny and in terms of the current LDH isozyme hypothesis. A modified hypothesis was advanced to account for some of the experimental findings.

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INTRODUCTION

The process of differentiation (i.e., the temporal appearance of specific protein species in cells) depends, at least in part, upon the differential activity of genes controlling the synthesis of particular macromolecules at specific times and loci in a developing organism and thus investigators have been led to the study of cellular products as a measure of differentiation (Davidson et al, 1963). The use of an enzyme as a protein which reflects in a sensitive manner the state of differentiation of a tissue is advantageous because its presence can be tested for in a meaningful way by assaying for its functional activity. An even more sensitive gauge of tissue differentiation may be obtained if the enzyme studied is one which occurs in a number of molecularly distinct but functionally similar isozymic forms, as is the case for lactic dehydrogenase (LDH)* (Markert and Møller, 1959). Studies on the ontogeny of LDH in a variety of mammals (Markert and Møller, 1959; Flexner et al, 1960; Markert and Ursprung, 1962; Fine et al, 1963; Wiggert and Villee, 1964) and in the chick (Lindsay, 1963; Ebert, 1964) have shown that the pattern of isozymes found exhibits temporal, tissue, and species specificity and it seems reasonable to assume that changes observed in proportions of isozymes at different stages of development would reflect the degree of activity of the controlling genes.

There is as yet little information on the ontogeny of LDH isozymes in amphibians. Accordingly, this study has concerned itself with an investigation into the LDH content of two species of amphibians, Amblystoma gracile and Rana aurora, during early developmental stages as part of a larger project on the problem of differentiation.

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* other abbreviations are: NAD+, NADH — nicotinamide adenine dinucleotide, oxidized and reduced forms; FAD, $FADH_2$ — flavine adenine dinucleotide, oxidized and reduced forms.

MATERIALS AND METHODS

I Preparation of Embryonic Material:

Amblystoma gracile eggs collected in the Vancouver area were reared in the laboratory in dechlorinated water until the appropriate developmental stages were reached. Assignment of developmental stages was by comparison with the Harrison schema for *Amblystoma punctatum*, as in Rugh (1962). The stages selected for study were: 7 (late cleavage), 10 (early gastrula, with dorsal blastopore lip visible), 12 (mid gastrula, with yolk plug visible), 16 (open neural folds), 20+ (closed neural folds), 25 (approximately 10 somites visible), 30 (tail bud definite), 33-34 (muscular response to mechanical stimulation -- C-flexure), 36-37 (autonomous movement -- S-flexures), and 40 (free swimming). Groups of 30 embryos, at each of the selected stages, were freed of jelly and, where possible, also of fertilization membranes; washed in ice-cold 0.1 M phosphate buffer (pH = 7.0); and then ground by hand in an all glass Potter-Elvehjem homogenizer with the minimum possible volume of the same buffer. An additional small volume of buffer used to rinse out the homogenizer was pooled with the first homogenate. The brei was centrifuged at 10,000 x g for 10 min. in a Servall model refrigerated centrifuge in order to precipitate any remaining whole cells, yolk platelets, cell debris, nuclei, and mitochondria. The assumption was made that the same relative constituents occurred in the remaining fraction in all of the stages studied. The relatively clear supernatant located between the light fatty layer and the dark precipitate in the bottom of the centrifuge tube was withdrawn by pipette, thoroughly mixed, and divided into two fractions: one to be used for spectrophotometric analysis, and the other for electrophoresis. Both fractions were stored in small, capped plastic test tubes in a GE deep-freeze at -20°C until used. Since it has been suggested by Zondag (1963) that freezing does cause some loss of the enzymatic activity of LDH unless certain precautions are taken, an attempt was made to standardize the length of time each sample was frozen before being tested for activity, and repeated freezing and thawing of the samples was avoided as much as possible. Accordingly, enzyme activity was measured after the samples had been frozen for 30 days.

Rana aurora eggs were collected near Haney, B.C., and reared in the laboratory. The same procedure for preparing extracts of the various developmental stages was followed as for *Amblystoma*, with the following exceptions. Developmental stages were designated by comparison with the Shumway schema for *Rana pipiens*, as in Rugh (1962); a total of 9 stages was studied, of which

eight stages (viz., 12+, 14, 15-16, 17, 18, 19, 20, and 21) corresponded to stages 12-40 studied in Amblystoma. The ninth stage of Rana studied consisted of over-ripe, unfertilized eggs. Enzyme activities were assayed immediately on the fresh supernatants, before they were frozen and stored for other studies.

II Enzyme Activity Assays:

The activity assay used as the criterion for determining the presence or absence of LDH in the samples was based on that given by Nielands (1955) and Amador et al (1963) (see Appendix A). Activities were measured on a Unicam Sp. 500 spectrophotometer. The following reagents were pipetted into a 1 cm. fused silica cell:

2.70 ml. 0.1 M glycine-NaOH buffer, pH = 10.0
0.15 ml. 0.5 M Na-lactate solution
0.15 ml. 2×10^{-2} M NAD^+

The mixture was stirred with a smooth glass rod and the cell was placed in the holder in the instrument and positioned so that the light beam from the tungsten lamp passed through the solution. This served as the blank with which to zero the instrument. At zero time, 0.03 ml. of the embryo sample was pipetted into the cell, the mixture was stirred rapidly, and the optical density readings at 340 mμ were recorded at 15 sec. intervals for the first minute of reaction, and at 30 sec. intervals for the next two minutes. Enzyme activity was assayed five times for each embryo sample and also for seven serial dilutions of a commercial preparation of rabbit muscle LDH, ranging in protein concentration from about 1 mg./ml. to 0.0125 mg./ml.

Controls: For each new batch of reagents used, a no-enzyme control was assayed, i.e., in place of the enzyme test solution, the cell contained an equivalent volume of water. For each sample tested, a no-substrate and a no-NAD^+ control were each assayed, in which appropriate volumes of water were substituted for Na-lactate and NAD^+, respectively.

III Protein Determinations:

The protein content of each of the samples studied was determined after Lowry et al (1951), using the Folin-Ciocalteu phenol reagent (see Appendix B). 0.40 ml. of the embryo sample was added to 2.00 ml. of a fresh solution containing:

50.00 ml. 2% Na_2CO_3 in 0.10 N NaOH
0.50 ml. 1% $CuSO_4.5H_2O$
0.50 ml. 2% Na-K tartrate

The contents were mixed well and allowed to stand for at least 10 min. at

room temperature. Then 0.20 ml. of commercial Folin reagent, diluted immediately prior to use with an equal volume of water was added, while the contents of the test tube were mixed, and the solution was allowed to stand for 30 min. at room temperature. The optical density of the solution was read at 500 mμ in a Beckman Model B spectrophotometer. A reagent blank which contained an equivalent volume of water in place of the protein sample was used to zero the spectrophotometer. Since, according to Lowry et al (1951), the method gives most accurate readings for samples containing 5-100 μg. protein/ml., i.e., showing an optical density between 0.05 and 0.30, appropriate dilutions were made on the samples where necessary. For each assay performed a standard curve was prepared by plotting the optical density against the known protein concentration for each of 5 dilutions of commercial egg albumin ranging in concentration between 50 and 400 μg. protein/ml. Tests on samples and standards were performed in duplicate.

IV Starch Gel Electrophoresis:

1. Electrophoresis:

The LDH of the samples was resolved into isozyme patterns by starch gel electrophoresis after the method of Smithies (1955 and 1959), modified by Tsuyuki et al (1962) and Tsuyuki (1963), in 0.3 M borate-NaOH buffer, pH = 8.5, for 18 hours at 0°C and 250 volts (see Appendix C). Preliminary runs showed that the ionic strength of the phosphate buffer in which the samples were dissolved (viz., 0.1 M) was too high and caused some drying out of the gel during the run and slight distortion of the migration fronts of the bands. Repeated runs on samples which had been dialyzed for several hours against distilled water or against 0.023 M borate-NaOH buffer, pH = 8.5, gave poor results in that much of the protein seemd to have been lost from the dialyzed samples as compared to the undialyzed ones. Thenceforth samples were run without prior dialysis. In preliminary testing, some of the samples were appropriately diluted with the same phosphate buffer in which they had been prepared so that the same specific enzyme activity was inoculated into each slot in the starch gel. However, the patterns obtained were, in most cases, too pale for analysis and therefore in subsequent runs all samples were inoculated at full strength.

2. Staining:

In some of the preliminary runs, gels were stained with the general protein stain Amido Black 10 B for about 3 minutes and then rinsed several times with water-methanol-acetic acid (50:50:10 v/v) to remove background, unspeci-

fic stain. However, Amido Black 10 B is not a very sensitive stain and a protein concentration of about 8 mg./ml. in the sample is required in order to obtain a clear pattern. The samples studied generally contained much less protein (viz., 2.7-9.8 mg./ml.) and the patterns obtained were faint. For this reason, staining for total protein was not carried out routinely on all of the samples. Instead, only the much more sensitive stain based on the activity assay for LDH was used, with some preliminary runs and all the subsequent ones discussed in Results. The staining solution used was slightly modified after Dewey and Conklin (1960) and Kaplan and Cahn (1962) to contain:

80.0 ml. 0.1 M tris-HCl buffer, pH = 8.0
20.0 ml. 0.5 M Na-lactate solution
2.5 mg. phenazine methosulfate
50.0 mg. nitro blue tetrazolium
60.0 mg. NAD^+.

Each gel was stained with 100 ml. of this solution at room temperature for 1 hour in the dark. They were then rinsed in several changes of distilled water under the same conditions. All staining and rinsing was done in stainless steel pans on a shaker. The stained gels were wrapped in polyethylene film and stored in the dark in a refrigerator until photographed.

V Disc Electrophoresis:

1. Electrophoresis:

The LDH in the embryo samples was also resolved into isozymes by the method of disc electrophoresis, Ornstein (1962), and Reisfeld et al (1962), in 0.05 M tris-0.38 M glycine buffer, pH = 9.4, for 1½-2½ hours at room temperature and a current of 2.5 ma./gel (see Appendix D). Runs were made on all of the embryonic stages studied and also on rabbit muscle LDH, which served as a standard. Amblystoma gracile samples were run at several different concentrations to obtain good resolution of the isozymes for all stages examined; the concentration used and the length of duration of the run for each sample are given in the legend to Fig. 5. Rana aurora samples were run at full strength. Since the sample is applied to the top of the gel and migration takes place in only one direction (viz., toward the cathode) duplicate samples of certain stages and of the standard were run in reverse in order to ascertain whether or not any migration of protein occurred toward the anode.

2. Staining:

The conditions and reagents used for staining the LDH isozyme discs on the gel columns were identical to those used in the starch gel studies though a much smaller volume (viz., 2.0 ml./gel) of stain was required. The rinsed

gels were stored in corked, water-filled tubes in the dark in a refrigerator until photographed.

VI Calculation of Results:

1. Enzyme activities:

For each assay performed, the largest change in optical density per minute was obtained by finding by inspection the largest change in optical density per half minute and multiplying this value by 2. The change in optical density per minute per ml. of sample was calculated by multiplying the above figure by 100/3.

2. Protein concentration:

The optical density values for duplicate tests on each sample were averaged. Protein concentration for each sample was then read directly from the egg albumin standard curve prepared for each series of assays, and corrected for any dilution made on the original sample.

3. Specific enzyme activity:

The specific enzyme activities, i.e., changes in optical density per minute per mg. of protein, were calculated for each of the 5 assays performed on each sample. Then for each sample the arithmetic mean of its specific enzyme activities was calculated to be used in tests for significance of differences of the means, for which a modified Tukey's test (Snedecor, 1957) was applied. For the set of data for each species a D value was obtained which allows for comparisons between any two points (i.e., LDH specific activity values of any two developmental stages) on each graph (see Table I and Fig. 1). The LDH specific activity means for each sample were also used to construct the graphs in Fig. 1 and to make comparisons of relative enzyme content between samples, as they represent measures independent of the original volumes of the embryo homogenates. These values were used again to standardize the amount of sample used (i.e., its degree of dilution) in some of the preliminary electrophoretic runs so that in each case the same amount of activity was inoculated into the gel slot since this would make relative comparisons of staining intensity in the isozyme bands more meaningful.

RESULTS

I Enzyme Activity and Protein Content Measurements:

LDH activity was found to be present in all of the samples studied and for purposes of inter-stage comparisons the results are presented in terms of specific enzyme activity, i.e., change in optical density per minute per mg. of protein (see Table I and Fig. 1). The graph of specific enzyme activity of A. gracile during development shows a slight initial increase during cleavage, a relatively steady state during gastrulation and neurulation, followed by a large decrease in activity in the post neurulation period. After tail bud development (stage 30) this declining trend is reversed and a sharp increase in specific enzyme activity occurs during the period of initial autonomous muscle movements. Again, this increase is followed by an apparent decrease in activity after stage 35 (during lengthening of the anterior-posterior axis). The results of the statistical tests applied to the data (see Table I) indicate that these differences in LDH specific activity are significant. The graph for R. aurora does not follow the same pattern but certain statistically significant trends are evident in it also. Specific LDH activity remains at a steady state from the initial stage examined (over-ripe, unfertilized eggs) through stage 12+ (mid gastrula; yolk plug visible). Between stages 12+ and 14 (i.e., early neurulation) a decrease in activity occurs, followed by a return to the stage 12+ level by stage 15-16 (i.e., late neurulation). Following stage 15-16 the activity begins to decrease again reaching the stage 14 level by stage 17 (approximately 10 somites visible). The level of LDH activity remains relatively steady throughout the remaining developmental stages of R. aurora studied, though there is significantly more activity at stage $20^{\pm}$ (autonomous movement -- S-flexures) than at stage 17. The results of the enzyme activity studies for the rabbit muscle LDH standard were not clear since the protein determinations on these samples were inconsistent, and thus specific enzymatic activity for rabbit muscle LDH was not calculated.

II Starch Gel Electrophoresis:

Isozyme patterns for the LDH in both amphibian species obtained by starch gel electrophoresis are shown in Fig. 2 and 3. Although a quantitative evaluation of the patterns for the various developmental stages of either species does not appear to be warranted, a qualitative picture is fairly obvious. In stage 7 of A. gracile 10 distinct bands are clearly visible and these 10 bands persist through stage 25. An 11^{th} and a 12^{th} band appear to be present

Table I. Specific LDH Activity in Developmental Stages of *Amblystoma gracile* and *Rana aurora*.

Amblystoma gracile			*Rana aurora*		
Stage number	Mean of specific LDH activities	Increase in activity	Stage number	Mean of specific LDH activities	Increase in activity
			over-ripe eggs	0.363	
7	0.704	0.149*			- 0.013
10	0.853	- 0.018			
12	0.835	- 0.054	12+	0.350	- 0.091*
16	0.781	- 0.108	14	0.259	0.080*
20±	0.673	- 0.265*	15-16	0.339	- 0.088*
25	0.408	- 0.038	17	0.251	0.031
30	0.370	0.170*	18	0.282	0.013
33-34	0.540	- 0.243*	19	0.295	0.067
36-37	0.297	- 0.109*	20	0.362	- 0.039
40	0.188		21	0.323	
	D = 0.109			D = 0.080	

* denotes that the increase in activity (i.e., difference between mean specific activities) is statistically significant (by Tukey's test).

Fig. 1. Graph of Specific LDH Activity vs. Developmental Stage in *Amblystoma gracile* and *Rana aurora*.

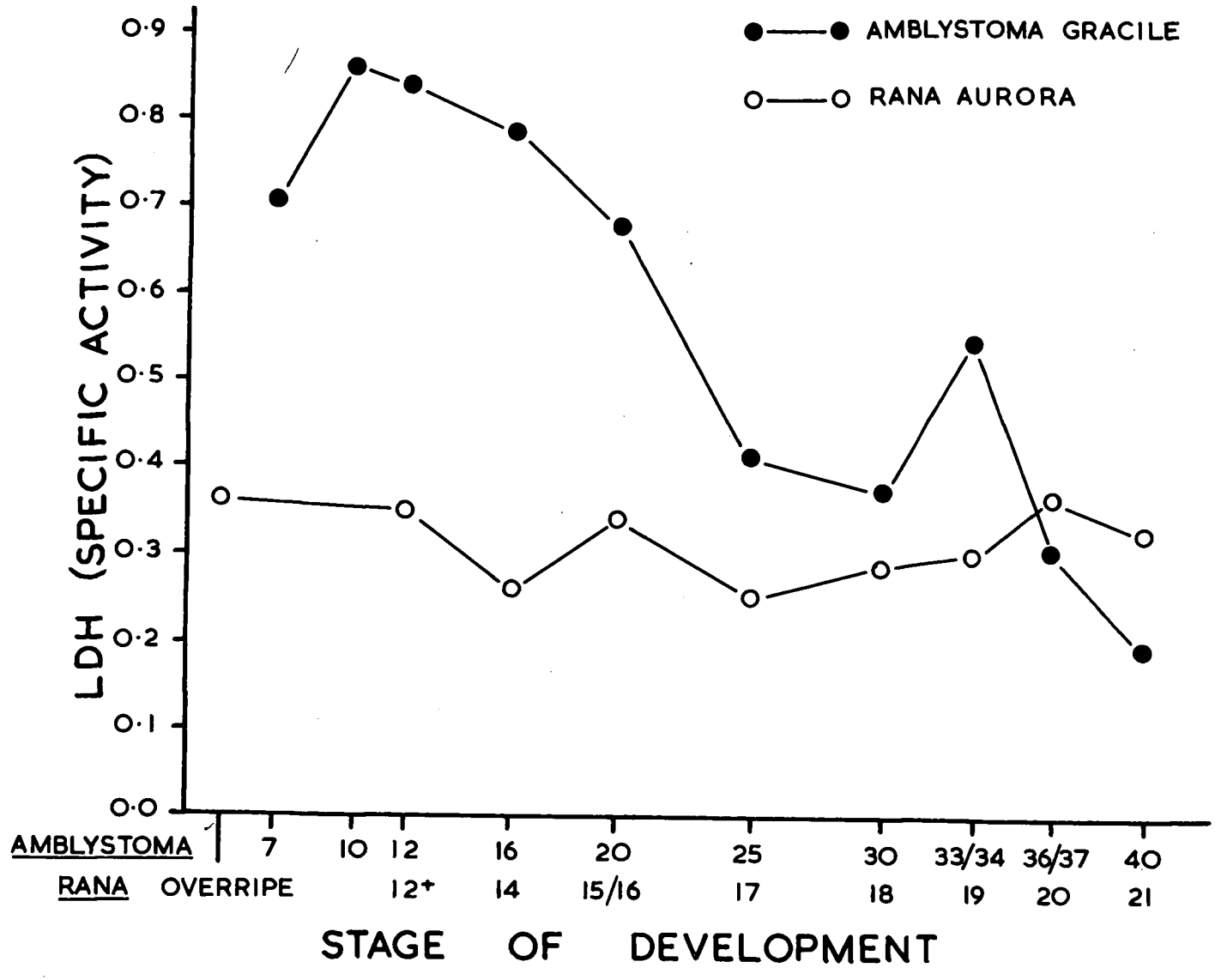

AMBLYSTOMA GRACILE
RANA AURORA
LDH (SPECIFIC ACTIVITY)
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
AMBLYSTOMA 7 10 12 16 20 25 30 33/34 36/37 40
RANA OVERRIPE 12+ 14 15/16 17 18 19 20 21
STAGE OF DEVELOPMENT

Fig. 2. LDH Isozyme Patterns of Amblystoma gracile on Starch Gel. RM: rabbit muscle; 7, 10, ... 40 : consecutive developmental stages. All samples run at full strength for 18 hours.

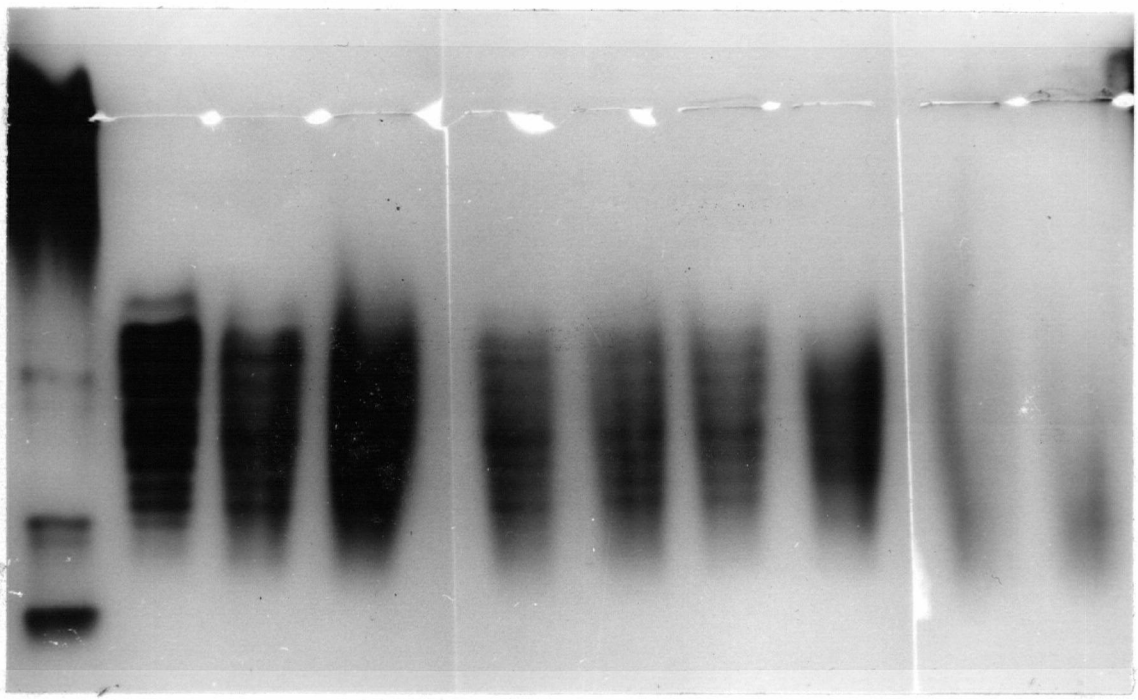
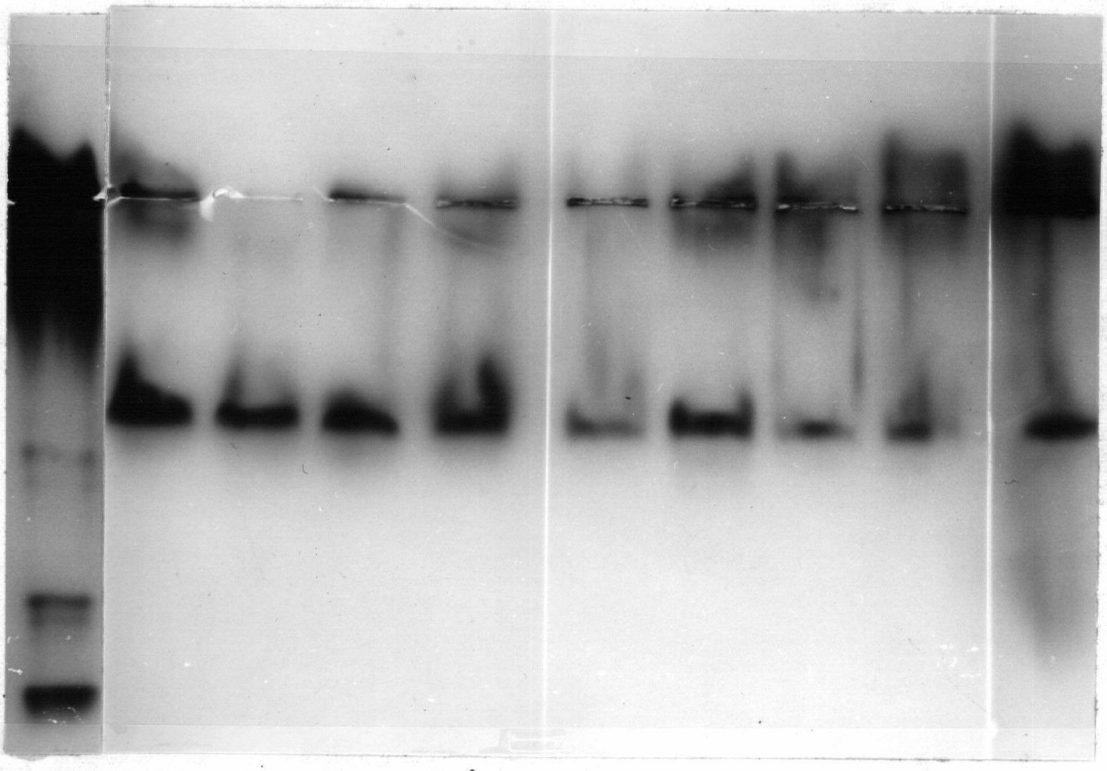

RM
7
10
12
16
20± 25
30
33/34
40

Fig. 3. LDH Isozyme Pattern of Rana aurora on Starch Gel. RM: rabbit muscle; O-R eggs, 12+, ... 21: consecutive developmental stages. All samples run at full strength for 18 hours.


RM OR 12+ 14 15/16 17 18 19 20 21

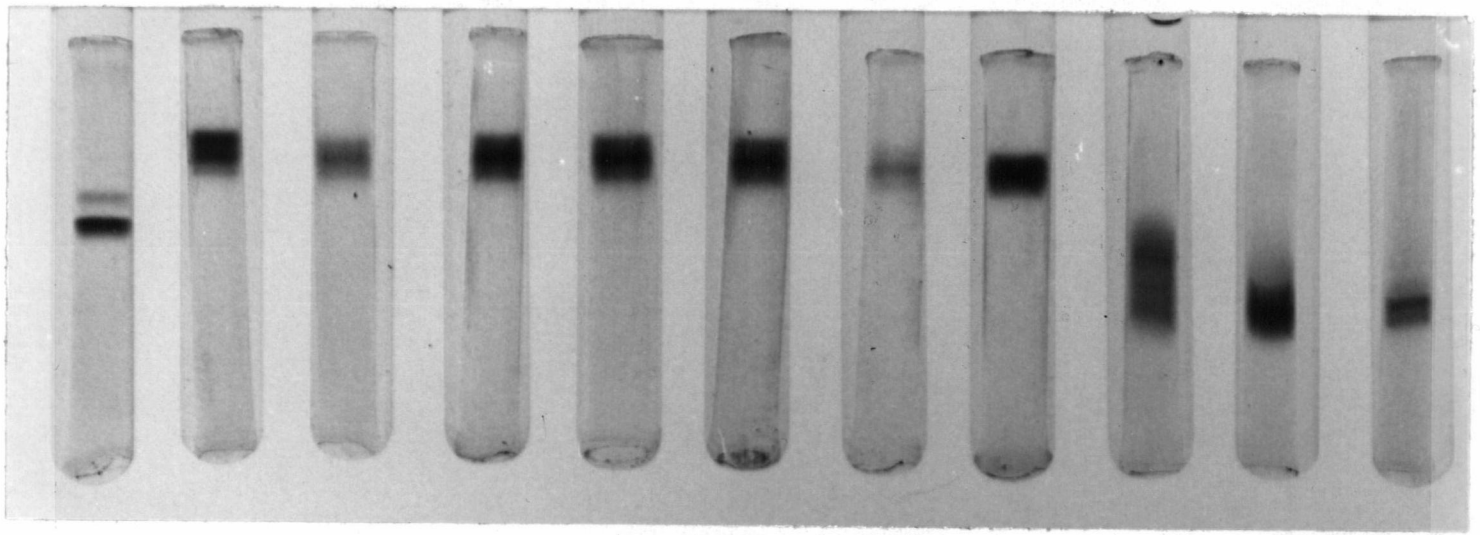
in stages 10-20± but after stage 20± the patterns seem to show a loss of bands from the end of the pattern lying closest to the origin. For purposes of further reference the bands in the A. gracile patterns are numbered 1, 2, ..., 12, as they progress from the origin to the cathodal end of the starch gel. It should be pointed out that: (a) the loss of bands from a pattern occurs from the extreme ends of the total spectrum, and not from the middle portion of it, and (b) all the bands in the patterns appear to be equally spaced from one another. The band pattern obtained when A. gracile samples of equal specific enzyme activity were run was too faint for analysis, so attention was focused on those patterns obtained from samples run at full strength. A comparison of the A. gracile LDH patterns with the rabbit muscle pattern reveals that there is little correspondence between the two, as can be seen in Fig. 2. Of the 6 bands visible in rabbit muscle LDH, one lies on the anodal side of the origin, the remaining 5 on the cathodal side. The bands at the two extreme ends of the rabbit muscle LDH pattern do not appear to have any counterparts among the A. gracile bands, while the intermediate 4 bands appear to match bands 4, 5, 10, and 11 of the A. gracile pattern.

The isozyme patterns obtained for R. aurora are less complex. One cathodally migrating band appears in all of the stages studied. It may correspond to band 1 or band 2 of the A. gracile pattern, but not to any of those in the rabbit muscle LDH pattern. A second, anodally migrating band seems to appear at stage 21. It lacks a counterpart in A. gracile and its relationship to the anodally migrating band in the rabbit muscle LDH pattern is not at all clear.

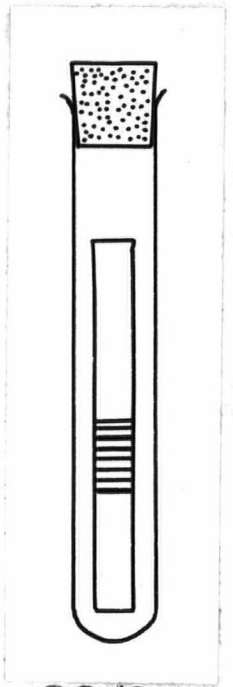
III Disc Electrophoresis:

In the clearest isozyme patterns obtained for A. gracile by disc electrophoresis (see Fig. 4) it can be seen that the number of isozyme bands visible is smaller than the number observed on starch gel (see Fig. 2). The pattern for stage 7 consists of 2 bands (one of which may coincide with the second band in the rabbit muscle LDH pattern) which persist throughout the examined stages, while a sample from stage 33-34 (run for 2½ hours, 5 times concentrated) seems to consist of at least 8 bands and is thus more reminiscent of the patterns obtained on starch gel (see Fig. 4 and the accompanying diagram). Other developmental stages failed to give as clear a resolution of their bands. No bands appeared in gels which had been run in reverse, thus indicating an absence of anodally migrating isozymes in both amphibian species and in rabbit muscle LDH.

Fig. 4. LDH Isozyme Patterns of Amblystoma gracile on Polyacrylamide Gel. RM: rabbit muscle; 7, 10, ...40: consecutive developmental stages. 7, 10: run at full strength; RM, 12-20, 30: run at 2 times full strength; 25: run at 3 times full strength; 33-34, 36-37, 40: run at 5 times full strength. RM - 30: run for 2 hours; 33-34, 36-37, 40: run for 2½ hours. The diagram shows more clearly the band pattern in stage 33-34.



RM 7 10 12 16 20± 25 30 33/34 36/37 40



33/34

On the other hand, better resolution of the isozyme patterns was obtained for R. aurora by the disc method (see Fig. 5) and a larger number of isozyme bands separated out than did by starch gel electrophoresis. A relatively heavy band at the migration front appears in the over-ripe eggs and persists throughout the developmental stages examined. Two bands located immediately behind the first one appear in the over-ripe eggs, disappear at stage 12+, reappear at stage 14 and persist thereafter. The pattern obtained with a duplicate sample of stage 12+ also failed to show more than the single heavy band. A fourth band located just behind the other three appears at stage 17 and remains visible through stage 21. It should be noted that where patterns consist of fewer than 4 bands, the loss of bands is from the extreme end of the spectrum rather than from the middle. Here, also, the bands appear to be equally spaced from one another and none of the bands seem to correspond to any bands in the rabbit muscle LDH pattern or in the A. gracile pattern.

In order to establish the effect of duration of electrophoretic run on resolution of the isozymes and spreading of their bands, the R. aurora samples were subjected, at full strength, to a 2 hour run. The results (see Fig. 6) show the same bands appearing in Fig. 5 to be present, but the greater separation between the bands and the spreading of the bands themselves resulted in a pattern less sharp. However, as noted above, runs of longer duration than 1½ hours did increase resolution in some of the A. gracile samples.

Possible deterioration of the samples in terms of loss of enzymatic activity with time or with repeated freezing and thawing was investigated by running certain samples on two different occasions. The results are presented in Fig. 7. Rabbit muscle LDH which had been frozen and thawed 3 times or so produced a 4-band pattern, but in all subsequent patterns only 3 bands were visible. Similarly, the pattern obtained for a sample of A. gracile stage 20± frozen and thawed only twice showed a faint band to be present at the migration front which is missing from the patterns of later runs of stage 20±. The patterns obtained for two samples of R. aurora stage 20 examined before and after having been frozen and thawed several times indicate that while the same bands are present in both, the intensity of the staining in the middle two bands has decreased in the sample frozen and thawed more times. On this basis then, it would seem that a quantitative evaluation of the data is not warranted.

Fig. 5. LDH Isozyme Patterns of Rana aurora on Polyacrilamide Gel. O-R eggs, 12+, ... 21: consecutive developmental stages; RM: rabbit muscle. All samples run at full strength for 1½ hours.

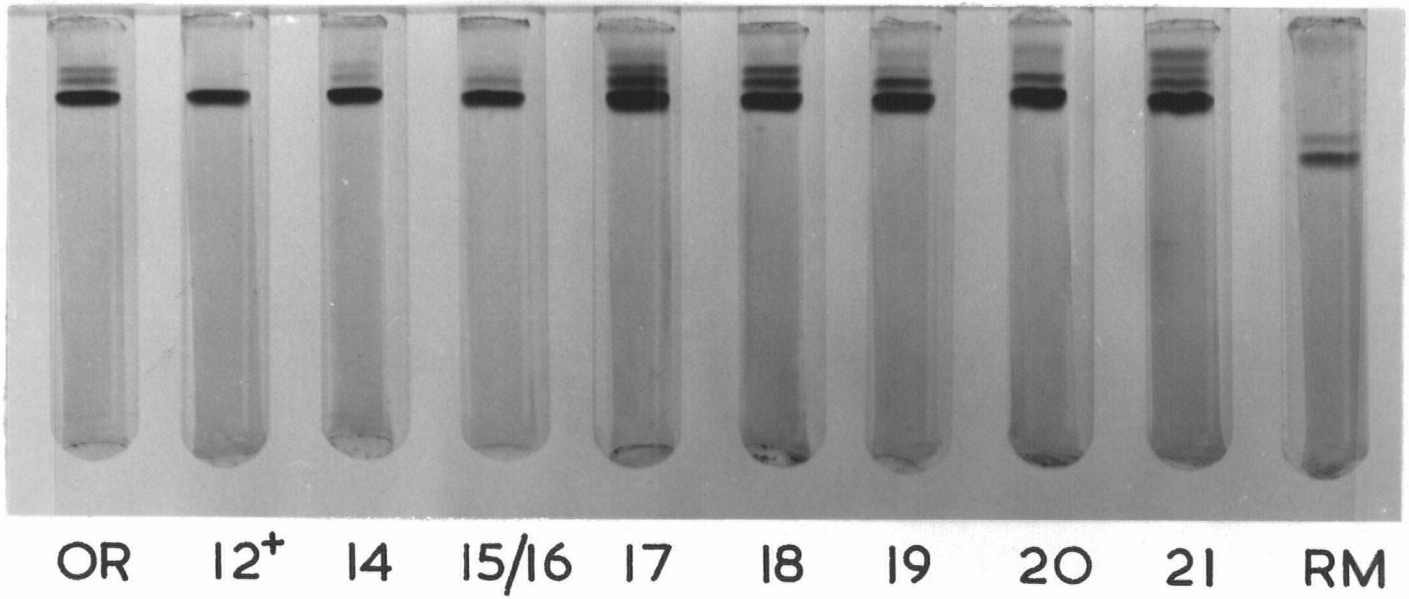

OR
12+
14
15/16
17
18
19
20
21
RM

Fig. 6. LDH Isozyme Patterns of Rana aurora on Polyacrylamide Gel after an Extended Run. O-R eggs, 12+, ... 21: consecutive developmental stages; RM: rabbit muscle. All samples run at full strength for 2 hours.

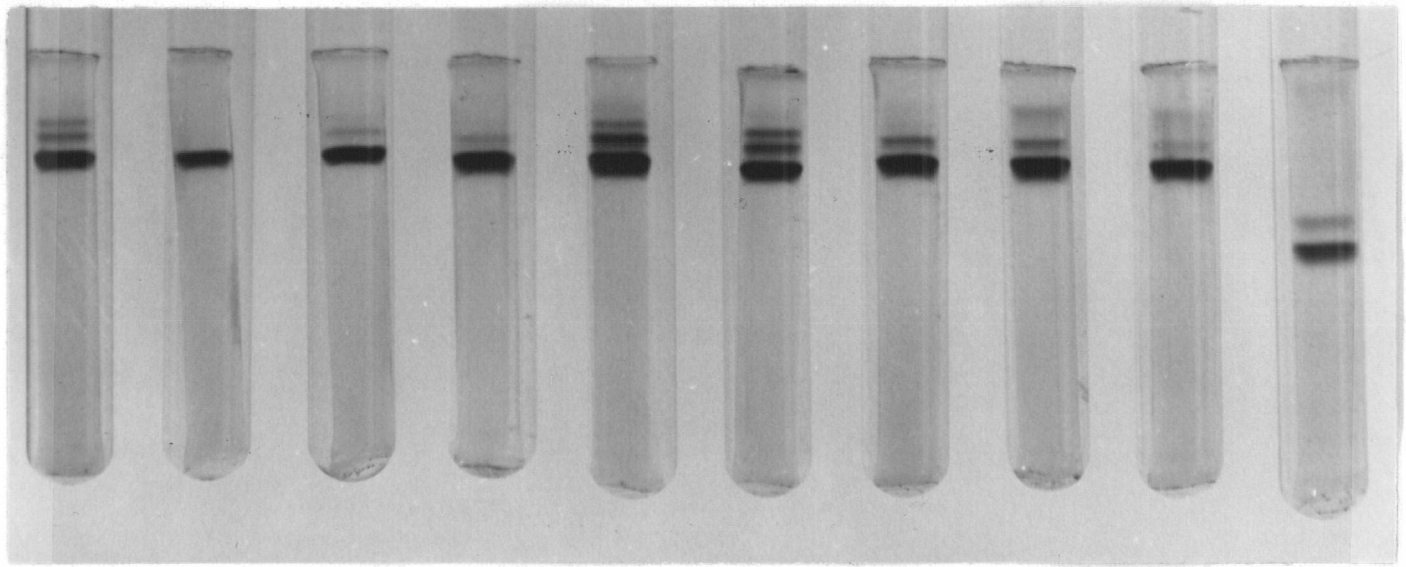
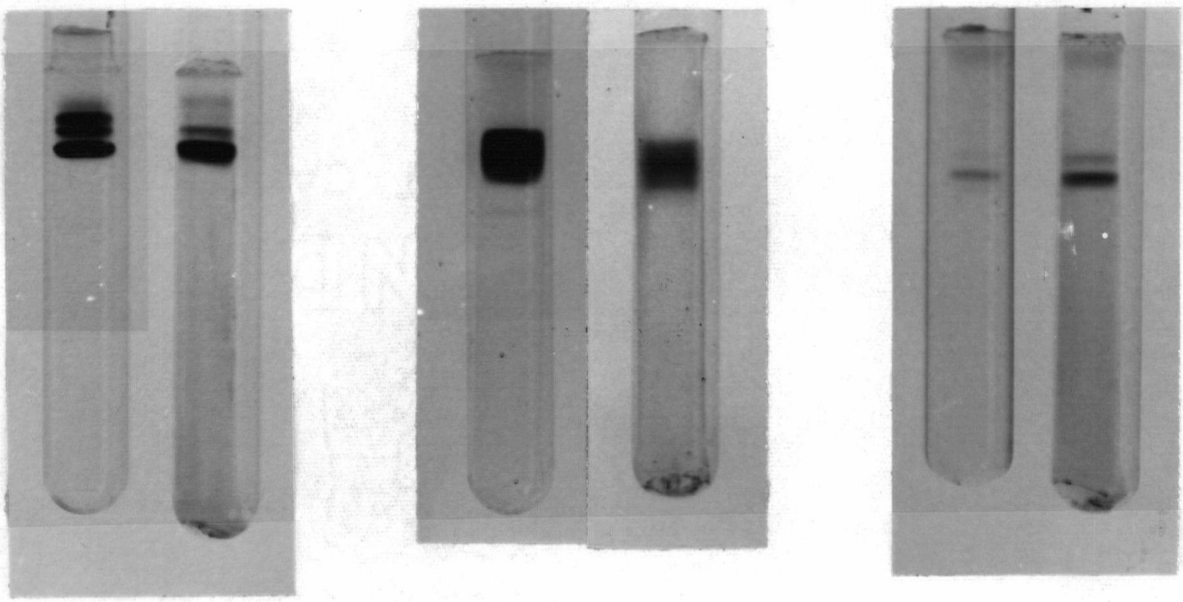

OR
12+
14
15/16
17
18
19
20
21
RM

Fig. 7. LDH Isozyme Patterns of Test Material Before and After Repeated Freezing and Thawing. 1, 2 : R. aurora stage 20; 3, 4 : A. gracile stage 20±; 5, 6 : rabbit muscle. 2, 4, 6 were frozen and thawed more times than were 1, 3, 5 (see text). All samples run at full strength for 1½ hours.



1 2 3 4 5 6

DISCUSSION

Løvtrup (1959) has pointed out that carbohydrate is the principal energy source during early amphibian development (i.e., the developmental stages studied here), and Richterich et al (1963) note that the presence of the enzyme LDH in a tissue is indicative of glycolysis reactions occurring there. However, Markert (1962) has suggested that the significant role of LDH may be not mediation of the transformation between lactate and pyruvate so much as generation of NAD^+ or NADH, thereby regulating the $NADH/NAD^+$ ratio which is important in controlling the rates of many different reactions in the cell. In a review of developmental metabolism Boell (1955) reports that oxygen consumption increases during the early amphibian developmental stages of concern in this study, and this trend would be expected to be accompanied by corresponding changes in those enzymes through which such respiration is mediated. The enzyme activity assay results reported here demonstrate that LDH occurs in all of the developmental stages of Amblystoma and Rana studied but when LDH activities are measured against total protein (see Fig. 1) it is not possible to establish a continuing increase in specific enzymatic activity. The steady state of LDH specific activity maintained through cleavage, gastrulation and neurulation in A. gracile suggests that the rate of LDH synthesis is approximately equal to the rate of synthesis of all other protein occurring at this time. The large decrease in LDH specific activity observed between neurulation and stage 30 implies that while LDH synthesis may be continuing, its rate is slower than that of general protein synthesis at this time, when the anterior-posterior axis is elongating and a large increase in mass and therefore in total protein is occurring. The increase in LDH activity which takes place between stages 30 and 35 suggests that the rate of total protein synthesis drops below that of LDH synthesis, and this coincides with the time when differentiation of myoblasts and the onset of muscular function occur, phenomena possibly requiring a large expenditure of energy. This trend may reflect also Boell's (1955) observation of a break occurring in semilogarithmic plots of oxygen consumption during urodele development at about the same time. He reports a similar break at the time of gastrulation for anurans, and it may be this trend which is reflected in the initial decrease in LDH activity observed during early neurulation in R. aurora where it appears that the rate of total protein synthesis exceeds that of LDH synthesis. It should be remembered that induction and differentiation of the neural plate is occuring at this time. The increase in LDH activity observed to occur between

stages 14 and 15-16 in the frog suggests that the rate of synthesis of LDH exceeds that of other proteins, which may reflect an energy requirement as closure of the neural folds takes place. The decrease in LDH activity occurring between stages 15-16 and 17, i.e., at the time of anterior-posterior axis elongation, appears to be similar to the trend occurring at the same time in A. gracile. Also, the gradual but significant rise in LDH activity which is observed from stage 17 through stage 20 in R. aurora may parallel the similar trend in A. gracile (through stage 35) when the onset of muscular function probably requires the expenditure of energy.

It should also be remembered that the specific LDH activities obtained are a reflection of the total potential activity of LDH at a particular developmental stage, since the assay is performed under essentially optimal conditions for most of the isozymes. Such measurements may provide little information on the degree to which the enzyme functions in vivo, since suboptimal conditions, inhibitors, etc., could greatly alter the functional expression of an enzyme within the intact developmental system. In this connection it has been demonstrated by Kaplan and his associates (1960-1964) that high levels of pyruvate prove stimulatory to some isozymes of LDH and inhibitory to others and that isozymes vary from one another with respect to their kinetic properties when tested with different substrates and with analogues of NAD^+ or NADH. Such investigations have led to the concept that the types of isozymes found in a tissue reflect that tissue's state of energy metabolism, but from the work presented here it is not possible to make any comments on the individual contributions of the LDH isozymes to the amphibian developmental pattern as a whole.

The constancy of the isozyme patterns obtained for A. gracile on starch gel (see Fig. 2) implies that no increase in complexity of the isozyme repertory is occurring during the developmental stages studied but the patterns themselves are complex, consisting of 10 or 12 distinct bands. The isozyme pattern typically found in tissues of mammals and birds consists of 5 bands and has been accounted for within the framework of an hypothesis put forward by Appella and Markert (1961), Markert (1962), and Cahn et al (1962). Their studies have established that the functional isozymes of LDH each consist of 4 subunits of which there are two types, A and B, each with a molecular weight of ca. 34,000. The two subunits can assort randomly (Markert, 1963) to produce 5 isozymes in which the 4 subunits are held together by H bonds. Of the 5 isozymes, 3 are hybrid forms of the 2 "pure", parental types: A_4, A_3B_1,

A_2B_2, A_1B_3, and B_4. Amino acid (Cahn et al, 1962; Markert, 1963; Fondy et al, 1964) and immunochemical (Cahn et al, 1962; Nace, 1963; Kaplan and White, 1963; Markert and Appella, 1963) analyses of the two types of subunits show the two to differ from one another. This has led these authors and Nance et al (1963), and Shaw and Barto (1963) to suggest that the production of each type of subunit is under the control of a separate gene, which genes are also denoted A and B. Other studies by Tsao (1960), Goldberg (1963), and Nitowsky and Soderman (1964) have shown that the two genes are operative within a single cell and therefore isozyme heterogeneity is not dependent upon the presence of a heterogeneous cell population. As Cahn et al (1962) emphasize, the observation that an enzyme is resolvable into several isozyme bands is not in itself sufficient evidence to conclude that some of the isozymes represent hybrid forms of parental types unless it can be demonstrated that at least 2 parental types of isozyme are also present. It may be that in the A. gracile pattern the isozymes which band at positions intermediate to the slowest and fastest migrating ones do represent hybrids of parental types (represented themselves by the bands at the extreme ends of the spectrum) as is suggested by the fact that when a pattern consists of fewer than the maximum number of bands for the species studied the loss is from one end rather than from the middle of the spectrum. On the assumption that hybridization of subunits to produce isozymes does occur in A. gracile and in order to account for the 10 or 12 bands observed an hypothesis is suggested which involves a simple mechanism compatible with the general LDH isozyme hypothesis and which is basically similar to the explanations offered by Boyer et al (1963), Nance et al (1963), Barto and Shaw (1963), and Kraus and Neely (1964), for their data on the genetics of LDH isozyme control.

It is assumed that the population of salamanders studied was heterozygous, due to a mutation, with respect to one of the genes controlling the synthesis of one of the LDH subunits, e.g., A, so that the genotype of the salamanders was $\frac{A \quad B}{\alpha \quad B}$. Such a heterozygous population is not unlikely to occur, as pointed out by Volpe and Dasgupta (1962). The two normal genes, A and B, plus the mutant α would give rise to 3 types of products (subunits) which

would differ from one another in their amino acid composition and possibly also in their net electrical charge. The charges which the polypeptides could carry are +, o, and -, but since 3 polypeptides must be considered, the simplest comprehensive analysis requires that each be allowed to carry as many as 3 like charges, e.g., +++ or ---. For each subunit then, 7 possible charges can be assigned: +++, ++, +, o, -, --, ---. If triplication and duplication of net charges on the 3 subunits is allowed then the number of possible random combinations of net charges, when the 3 subunits assort 4 at a time, is 343 (see Table II). Three subunits assorting 4 at a time can produce 15 isozymes, but if separation of the isozymes is based on net charge, as in electrophoresis, then 1, 5, 9, 12, 14, or 15 bands may be distinguished, depending on how many of the 15 isozymes bear similar net charges, migrate to the same positions, and therefore produce a pattern appearing to contain fewer than 15 bands (see Table II). The equal spacing observed to occur between bands in a pattern (see Fig. 2) suggests that the isozymes differ from one another in their net charge by an equal increment. It would be expected that a change in pH would cause a pattern to be produced which would consist of a different number of bands or one in which the spacing between bands would be altered since chemical modifications of the reactive groups on the polypeptide subunits could result in a change in the net charge of the isozyme. Such changes in spacing of bands in a pattern with alterations in pH have been noted by Markert and Ursprung (1962), and by Cahn et al (1962). This might explain why the clearest pattern obtained for A. gracile by disc electrophoresis at pH = 9.4 contained only 8 isozyme bands rather than the 10 or 12 bands found on starch gel at pH = 8.5, even though the results obtained on R. aurora seemed to indicate that the disc method is the more sensitive of the two, at least for the latter species. Incidentally, this pH-dependent discrepancy in the results obtained for a single species by the use of the two electrophoretic techniques causes one to wonder whether conclusions based on either method can have more than a comparative meaning within the context of the method used.

The other ways in which it is possible to account for the 10 or 12-band pattern found in A. gracile are that either: (a) 2 subunits assort more than 4 at a time, or (b) more than 2 subunits assort 2, 3, ... at a time. Zinkham et al (1963) have in fact postulated a control by more than 2 genes in order to account for the 1 or more "X bands" which they find in postpubertal testes. Patterns consisting of more than 5 isozymes have also been found by Wieland and Pfleiderer (1961), Allen (1961), and Ebert (1964) which,

Table II. The Number of Electrically Resolvable Isozymes under the Genetic Hypothesis Proposed.

No. of bands resolvable	No. of combinations giving that no. of bands	% of total no. of combinations
1	7	2
5	126	37
9	54	16
12	60	17
14	36	11
15	60	17

Explanation of Table.

Using the hypothesis of a mutant gene, the number of distinct net charges on the isozymes were computed, allowing each of the A, α, and B genes to assume any charge between +3 (+++) and -3 (---).

For example, if the charges were A, +3; α, +1; and B, -3, the net charges on the 15 possible isozymes would be:

Isozyme	A_4	A_3B_1	A_2B_2	A_1B_3	B_4	α_1B_3	α_2B_2	α_3B_1	α_4	$A_1\alpha_3$
Net charge	+12	+6	0	-6	-12	-8	-4	0	+4	+6

$A_2\alpha_2$	$A_3\alpha_1$	$A_1\alpha_1B_2$	$A_2\alpha_1B_1$	$A_1\alpha_2B_1$
+8	+10	-2	+4	+2

The 15 isozymes have only 12 distinct charges in this case and thus would be resolved into 12 distinct bands. There were 60 combinations in all which yielded 12 distinct bands, including, as a further example, A, +3; α, 0; B, +2 and A, +3; α, +1; B, 0.

contrary to Cahn et al (1962), do not seem to be artifacts and thus seem to require an extension of the original hypothesis. It would appear that Allen's (1961) report of 9 LDH isozymes in the tissues of the mouse can be explained by the hypothesis suggested here.

Since the staining method used to visualize the isozyme patterns is based on the assay system for LDH it follows that any bands detected in this manner consist of functional LDH. This, together with the observation that where many bands occur in a single pattern they appear to be equally spaced from one another (see Fig. 2 and 5) argues against the notion that the LDH being detected is in some way dissociated, because the subunits, and thus the isozymes, are all of the same molecular weight, whence the difference from one band to the next in a pattern would seem to be a function of net charge only. However, Markert (1963) reports that monomers obtained by dissociation of the enzymes with 1 M NaCl may retain enzymatic activity, and Millar (1962) has reported an LDH to be enzymatically active which had a molecular weight corresponding to the dimer form. But, Cahn et al (1962) maintain that the intact molecule consisting of 4 subunits is required for enzymatic activity.

The resolution of the LDH isozymes of A. gracile stages on polyacrylamide gel was relatively poor (see Fig. 4) and it is difficult to interpret the patterns in terms of a possible increase in complexity during development or in terms of a relationship with the bands obtained on starch gel.

The results obtained by starch gel electrophoresis for the developmental stages of R. aurora show the isozyme pattern to consist of 1, or possibly by stage 21, of 2 bands (see Fig. 3). If the second band visible by stage 21 does represent a new isozyme then it would seem that an increase in isozyme repertory, and therefore in complexity, was occurring during development. A clear isozyme pattern consisting of more bands was obtained for R. aurora by disc electrophoresis and it does show an increase in isozyme repertory as development proceeds (see Fig. 5). The pattern obtained for stage 12+, from which 2 bands found in the earlier stage and in all later stages studied are absent, constitutes an exception to this trend. Since duplicate samples of stage 12+ both showed the same 1-band pattern, it seems unlikely that the absence of the other 2 bands was due to the preferential inactivation of the isozymes contained in them, unless the inactivation took place during the initial preparation of that embryonic stage. This does not seem a likely explanation as the procedure followed was the same for all of the embryo samples. That the absence of the 2 bands at state 12+ may be due to the absence of the isozymes themselves at that stage in development is suggested by

the fact that Goldberg and Cather (1963), in a study of LDH in the early development of the snail Argobuccinum, found that 2 isozyme bands present in eggs and early cleavage stages disappeared as blastulation commenced but that 2 new LDH forms appeared sequentially in veliger stages. Also, Nace et al (1961), using the Echo immunochemical technique to study LDH in R. pipiens, found 4 isozymes to be present in summer ovary and fertilized eggs, while, by stage 18, only 2 isozymes remained.

Since the maximum number of isozyme bands found for R. aurora by either electrophoretic method employed in this study is 4, there is no reason to doubt the direct applicability of Markert's and Cahn's hypothesis to this species. It is not clear whether or not the 4^{th} band appearing at stage 17 in R. aurora on polyacrylamide gel bears any relationship to the 2^{nd} band appearing at stage 21 on starch gel, in view of the possible pH-dependence of the isozyme pattern. The lack of correspondence generally noted between bands in the patterns of developmental stages of the two amphibians and the bands in the rabbit muscle LDH pattern serves to emphasize the fact that the polypeptide fragments which confer species specificity to the isozymes differ from one another in their net charges, thus altering the mobilities of the whole isozymes.

Neither electrophoretic method for separating isozymes seems to point to any similarity between the two species of amphibians studied, nor of the LDH of either to that of rabbit muscle. Markert (1961) does not think there is any similarity, in a fundamental sense, between the isozyme patterns of an amphibian, a chicken, and a mammal, and states that whatever occasional similarities may have been observed are probably just coincidental. In a comparative analysis of the LDH's from the brains of vertebrates, Bonavita and Guarneri (1963) reported finding that by starch gel electrophoresis hardly any difference was noted between the brain isozymes of Discoglossus pictus (an Anuran) and of Triturus cristatus carnifex (a Urodele). However, they found that when agar-gel electrophoresis was used a discrimination between the two species could be made, in keeping with recent zoological theory which considers Anurans and Urodeles as possibly derived from different phyla (subclasses of Lepospondyli and Aspidospondyli, respectively).

The results of the investigation into the problem of loss of enzyme activity from the samples after repeated freezing and thawing given in Fig. 7 do indicate that certain of the isozymes are more labile than others to inactivating conditions, thus making a discussion of patterns from which bands appear to be missing somewhat ambiguous. Evidence which shows that the hybrid

forms of LDH are in fact less stable than the pure forms has been presented by Fondy et al (1964) and the implication is that the isozymes differ from one another in their tertiary structure, a fact borne out by heat inactivation studies on the isozymes by Fondy et al (1964), by Markert (1963), and by Lindsay (1963). Opinions in the literature on the effects of freezing and thawing on LDH are varied and include reports of a loss of bands (Zondag, 1963), of a reduction in isozyme pattern intensity (Lindsay, 1963), and of no difference in results obtained on fresh or frozen tissues (Flexner et al, 1960; Wiggert and Villee, 1964; Ebert, 1964). However, it should also be remembered that an apparent loss of bands could be caused by the enzyme being present in an amount below the detection sensitivity of the assay system.

SUMMARY

1. The occurrence of LDH in early developmental stages of A. gracile and R. aurora was investigated by assaying for enzyme activity in embryo homogenates and it was found present in all of the stages examined. Changes in specific enzyme activity were correlated with the morphological changes occurring at particular stages.
2. The LDH in each developmental stage studied was resolved into an isozymic pattern by the methods of starch gel and disc electrophoresis.
3. Using disc electrophoresis the LDH isozyme patterns in R. aurora were shown to undergo an increase in complexity as development proceeded, resulting finally in a maximum of 4 bands.
4. For the 10-12 band pattern of LDH found on starch gel for most A. gracile stages an hypothesis was suggested which was correlated with the general LDH isozyme hypothesis of Markert and Cahn.
5. Characteristics and limitations of the methods used were discussed.

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APPENDIX A

Enzyme Activity Assays

The reaction catalyzed by LDH is the following:

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The assay for LDH depends on the spectrophotometric measurement of the rate of appearance of the absorption band at 340 mμ as NAD^+ is reduced to NADH. The band is absent in the oxidized form, i.e., in NAD^+. Activity is assayed at pH = 10 since the reaction produces one equivalent of acid. The method may be applied directly to crude extracts without danger of nonspecific reduction of NAD^+ by contaminating substrates as the amount of enzyme needed for the test is only of the order of 0.1 μg. Substrate and NAD^+ are present in excess amounts in the assay mixture for at least the first minute of reaction and thus are not rate-limiting to it. In the presence of excess substrate the rate of reduction of NAD^+ is proportional to the amount of enzyme present, so the reaction follows first order kinetics.

Preparation of reagents:

(a) 0.1 M glycine-NaOH buffer, pH = 10.0
Prepared after Gomori (1955)

(b) 0.5 M Na-D,L-lactate.
Commercial 85% lactic acid was diluted with an equal volume of water. 5 N NaOH was added to 10 ml. of the diluted solution in 2.0 ml. portions until the solution tested alkaline to pH indicator paper. The solution was then heated to 80°C in order to hydrolyze the inner ester. More NaOH was added cautiously while the heating was continued until the solution remained neutral. The solution was then diluted to 94 ml.

(c) 2×10^{-2} M NAD^+.
133 mg. of β-NAD^+ were dissolved in 5 ml. of water. NaOH was added carefully from a 0.1 ml. pipette until the pH was about 6.0. The solution was diluted to 10.0 ml. and stored in a refrigerator at about 5°C.

(d) rabbit muscle LDH (standard).
A commercial preparation of rabbit muscle LDH reportedly containing 5 mg. protein/ml. was diluted serially with 0.1 M phosphate buffer, pH = 7.0 to yield seven samples containing 1.00, 0.50, 0.25, 0.125, 0.05, 0.025, and 0.0125 mg. protein/ml.

APPENDIX B

Protein Determinations

The method of Lowry et al (1951) is a convenient and sensitive way of determining protein concentration. They claim it to be as sensitive as digestion and subsequent nesslerization; to be 10-20 times more sensitive than the method based on uv absorption at 280 mμ and 260 mμ because it is more specific and less liable to inaccuracy due to turbidity or the presence of RNA; and to be 100 times as sensitive as the biuret reaction alone. Its only disadvantages lie in the fact that the intensity of colour produced varies with different proteins and is not strictly proportional to the protein concentration. The final colour produced is a result of the biuret reaction of protein with copper ion in alkali and the reduction of the phosphomolybdic-phosphotungstic (Folin) reagent by the tyrosine and tryptophane present in the protein sample.

Preparation of reagents:

(a) crystalline egg albumin standard.

A stock solution containing 0.005 gm. crystalline egg albumin/ml. of water was prepared. For each series of protein determinations a suitable volume of the stock solution was diluted 1:10 to give a concentration of 500 μg./ml. and was further diluted serially to yield 5 samples containing 50, 100, 200, 300, and 400 μg./ml.

APPENDIX C

Starch Gel Electrophoresis

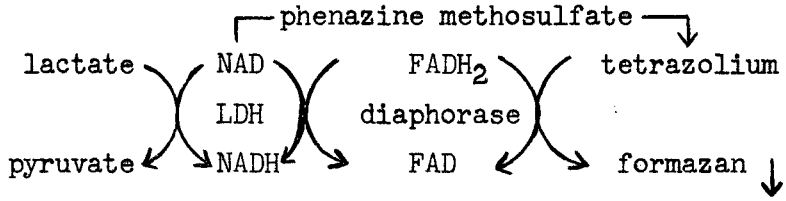
The starch gel method of zone electrophoresis has a greater resolving power for separating proteins than do previous electrophoretic methods, probably because the pore size of the gel support approaches the molecular dimensions of proteins in solution. The method seems to combine resolution by free solution mobilities with resolution according to molecular size. However, the pore size of the gels constitutes the chief limitation of the method because it is not suitable for separation of high molecular weight substances. For this reason it should not be considered a replacement for other electrophoretic methods. The use of low ionic strength buffers in the gels allows high potential gradients to be applied without troublesome heating effects. This may also contribute to the high resolution of the bands since anion-binding by the various proteins is reduced. Also, the bands appear sharper because of the greater mobilities of the proteins in the sample introduced into the slot, as compared to their mobilities in the gel. It seems that the relative values of the starch concentration and the ionic strength of the buffer are the fundamental determinants of the detailed pattern produced.

Starch gels were prepared by dissolving hydrolyzed starch (Connaught Laboratories, Toronto, Ont.) in 0.023 M borate-NaOH buffer, pH = 8.5, usually at a concentration of 11.3 gm. starch/100 ml. of buffer. This gives a gel of considerable strength and the hot solution is not too viscous to handle during its preparation. The starch solution was heated to 90-95°C with constant mechanical stirring in an evacuation flask for about 20 minutes or until the gel had become viscous and then fluid again as the starch grains ruptured. At this point heating was discontinued and the gel was degassed by application of negative pressure with a filter pump until the contents boiled vigorously and the bubbles were large and uniform. The boiling time was kept as short as possible in order to minimize the amount of water lost from the gel. The gel was then cast in a 16.5 in. x 8.5 in x 4 mm. pre-oiled Plexiglass migration tray and covered with another sheet of Plexiglass which had 12 tabs across its centre for making sample slots in the gel, at right angles to the gel's greatest length. Air bubbles were avoided by lowering the sheet carefully from the centre towards the ends. Excess gel in the tray was squeezed out by pressing on the Plexiglass sheet with six 10 lb. lead weights. The gel was allowed to cool to room temperature and then to 0°C under these weights. It was then trimmed, and, except for a small section around the

slots, sealed in Saran Wrap to prevent drying during the electrophoretic run. Then it was mounted in the apparatus, the side wells of which each contained 300 ml. of 0.3 M borate-NaOH buffer, pH = 8.5. Samples were inoculated into the central slots in the gel by syringe and capillary tube. The slots were then sealed with a strip of Saran Wrap so that no air bubbles were trapped in the slots and no excess sample lay on top of the gel. Electrical contact was made directly by having the ends of the gel dip into the bridge buffer in the side wells. The samples were allowed to run for 18 hours at 0°C and 250 volts. The apparatus used was one which had been custom built for the Fisheries Research Board of Canada Technological Station in Vancouver. After completion of the run the Saran Wrap was removed and the portions of the gel in contact with the buffer were cut off. The gels were removed by first freeing them from the tray and then causing them to fall out by inverting the tray onto another Plexiglass sheet. At this time they were ready to be stained.

Isozyme Staining

The specificity of the staining solution depends, as did the enzyme activity assays, on the oxidation of lactate to pyruvate in the presence of NAD^+. It provides, essentially then, a colourimetric test for the production of NADH. Upon the reduction of NAD^+ to NADH, an H^+ is transferred from NADH to the phenazine methosulfate, which in turn carries an electron to the nitro blue tetrazolium, causing the precipitation of the dark purple formazan dye at the site of enzymatic activity. The following reaction sequence was proposed as being the probable one occurring by Dewey and Conklin (1960):



The intensity of the stain serves as a relative index of the amount of functional enzyme present.

Preparation of reagents:

(a) 0.5 M Na-D,L-lactate:
as given above.

(b) 0.1 M tris-HCl buffer, pH = 8.0:
prepared after Gomori (1955).

APPENDIX D

Disc Electrophoresis

Disc electrophoresis on polyacrylamide gels is reported to be 100 times more sensitive than the routine starch gel technique. It is capable of resolving 0.01 μg. of protein directly from a large volume of dilute protein solution. It achieves quick separation by concentrating the components of the sample into very thin starting zones and by using the frictional properties of the gel to aid separation by molecular sieving. It is said to be a more reproducible method than either starch or paper, and the gel does not react with the colourimetric reagents used for staining. The transparency of the gel is clearly advantageous for high-sensitivity photometric work. The composition of the gel can be adjusted for preferential separation of molecules larger or smaller than typical proteins. The standard 7½% polyacrylamide gel has such a pore size that it is capable of separating the majority of proteins ranging from 10,000 to 1,000,000 molecular weight, with maximum resolution from 30,000 to 300,000. The "large pore" 4% gel is reported to give about the same relative positioning of serum proteins as does starch gel. A regulated power supply is required as the resistance of the gel increases as the run proceeds and a constant voltage would pass less and less current through the gel columns.

The gel used in these studies was the standard 7½% gel, which stacks at pH = 8.3 and runs at pH = 9.4. The hard, small-pore lower gel in which actual separation of the proteins takes place was prepared by mixing together equal parts of lower gels A-1 and A-2, followed by enough lower gel B to exactly double the volume. Since lower gel B is the catalyst for A it was not mixed with A until just before the gel was to be used. The lower gel mixture was prepared and degassed in a large syringe and loaded via a polyethylene capillary tube into 12 stoppered cylindrical glass tubes 5 mm. x 70 mm. held by clamps against an illuminator. Each tube was filled to within ½ in. of the top with approximately 1 ml. of the gel. The gel was then carefully layered with water containing tracking dye (3 drops/20 ml. of water) by syringe, the illuminator was switched on, and the gel was allowed to photopolymerize for 45 minutes. Then the excess water was drained off, the tops of the tubes were rinsed with spacer gel solution, and the shallow (approximately 3/8 in.) layer of soft, large-pore spacer gel (upper gel diluted with an equal volume of water) in which electrophoretic concentrations of the protein sample takes place, was loaded into the tube, layered with water, and allowed to photopolymerize for 20 minutes. Excess water on top of the gel

was then carefully withdrawn by pipette, the soft, large-pore, anticonvection gel containing the protein sample was loaded in to completely fill the tube and was left to photopolymerize for 25-30 minutes. The sample gel was usually prepared by mixing 18 µl. of the embryo sample with 0.5 ml. of spacer gel, though the volume of sample added and therefore the volume of water used to dilute the upper gel were varied as the situation required. However, the proportions maintained were such as to have about 200 µg of protein in the total volume, and to have full-strength upper gel amounting to only one half of the total volume. After the last gel had polymerized, the base caps were pried from the sample tubes very slowly in order to avoid suction which might displace the gel column from the sample tube walls. The lower ends of the tubes were dipped in water and trapped bubbles were removed, and then the top ends of the tubes were firmly inserted from below into the tapered holes in the rubber fittings in the bottom of the upper buffer bath. 0.05 M tris-0.38 M glycine buffer, pH = 8.3 was pipetted into the tops of the tubes and then poured into the upper bath to avoid trapping air bubbles in the tubes. The lower buffer tank, filled with the same buffer, was raised so that about $\frac{1}{4}$ in. of each tube was immersed, electrical contact was made, and the run was allowed to proceed for $1\frac{1}{2}$-$2\frac{1}{2}$ hours at room temperature at a current of 2.5 ma./tube. The apparatus used was a Canalco Model 12 Disc Electrophoresis apparatus and a Heathkit Model PS-4 regulated power supply. At the end of the run the gels were carefully removed from the tubes by rimming around the gels to a depth of $\frac{1}{2}$-1 in. at both ends with a blunt hypodermic needle through which water trickled, followed by the application of hydraulic pressure to one end of the tube from a water-filled eye dropper bulb. The soft spacer and sample gels were discarded and the hard gel containing the proteins was dropped into a small test tube and stained.

Preparation of reagents:

The pre-mixed gel reagents used had been prepared as follows:

Stock solutions:

(a) 1 N HCl.....48.00 ml.
Tris.....36.30 gm.
N,N,N,N', tetramethylethylenediamine.. 0.46 ml.
H_2O to make.....100.00 ml.

(b) 1 M H_3PO_4.....25.6 ml.

Tris..... 5.7 gm.

H_2O to make.....100.0 ml.

(pH = 6.9)

(c) acrylamide.....30.0 gm.

N. N-methylenebisacrylamide monomer..... 0.8 gm.

$K_3Fe(CN)_6$.....15.0 mg.

H_2O to make.....100.0 ml.

(d) acrylamide.....10.0 gm.

N. N-methylenebisacrylamide monomer..... 2.5 gm.

H_2O to make.....100.0 ml.

(e) riboflavin.... 4.0 mg.

H_2O to make....100.0 ml.

Reagents:

(a) Lower Gel A (A-1 plus A-2)

1 part (a)

2 parts (c)

1 part H_2O

(pH = 8.7-8.9)

(b) Lower Gel B

ammonium persulfate..... 0.14 gm.

H_2O to make.....100.00 ml.

(c) Upper Gel

1 part (b)

2 parts (d)

1 part (e)

4 parts H_2O

(pH = 6.9)

(d) 0.05 M tris-0.38 M glycine buffer, pH = 8.3

tris..... 6.0 gm.

glycine..... 28.8 gm.

H_2O to make.....1000.0 ml.