ALKALINE PHOSPHATASE AND EMBRYOGENESIS IN TWO URODELE AMPHIBIAN SPECIES

by

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ABSTRACT

The development of alkaline phosphatase (AP) has been studied in two species of Urodele amphibian, Ambystoma gracile and Taricha torosa. The enzyme is present in embryo homogenates at gastrulation and increases immensely in activity as development proceeds to the free-swimming stages. The activity level is a product of two isozymic forms that change quantitatively. Using histochemical detection methods, it was possible to correlate the specific activity and electrophoretic data with histological AP development. Some function of AP were related to the available data. A correlation between substrate specificities and function is proposed which may assist in understanding the role of AP in the process of differentiation.

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INTRODUCTION

It has been proposed that non-specific phosphatases are involved in the basic biochemical mechanisms underlying and essential for overt, or morphological, differentiation (Moog, 1944 and 1952). Karzmer and Berg (1951), also, suggest that a differentiating cell may have to pass through an "alkaline phosphatase-rich transition phase". Numerous studies have been concerned with the molecular differentiation of alkaline phosphatases during normal development and have demonstrated changes in the multimolecular forms of this enzyme (Moog et al, 1965; Pfohl, 1965; Schneiderman, 1967; Solomon et al, 1964). To date, no attempt has been made to correlate the multimolecular forms of alkaline phosphatase with its role in the processes of differentiation. Recent papers still cite this differentiation function (Piatka and Gibley, 1967; Shah and Chakko, 1967) although the mechanism of action remains to be elucidated. As well, alkaline phosphatase has been implicated in transport processes (Danielli, 1952; Matthiessen, 1966; Tosteson et al,1961), in protein synthesis (Osawa, 1951; Vorbrodt, 1958), in mucopolysaccharide formation (Moog and Wenger, 1952; Matthiessen, 1966), in bone formation (Matthiessen, 1966; Stadtman, 1961; Schmidt and Laskowski, 1961) and, also, in the control of DNA synthesis (Rubini et al,1964; Baserga, 1968).

The present study involved an analysis of the development of alkaline phosphatase in salamander embryos, a situation that has been touched on (Karzmer and Berg, 1951; Krugelis,

1950; Lovtrup, 1953; and Osawa, 1951) but not adequately defined in any one species. The use of histochemical detection methods coupled with biochemical analyses, specific activity determinations and electrophoretic analysis, served here to define the ontogeny of the alkaline phosphatases in Ambystoma gracile and Taricha torosa embryos.

MATERIALS AND METHODS

I. Preparation of Embryos:

Ambystoma gracile embryo masses were collected in early stages of development from natural pools in the vicinity of Vancouver, B.C.. Thermos jugs containing *Taricha torosa* egg cluthces were received air express from Stanford and St. Marys, California. Both species were allowed to develop at 8 $\pm$ 1.5 ^{o}C. in a controlled temperature refrigerator until the desired stages of development were attained. For *A. gracile* (A.g.) staging was by the schema of Harrison for *A. punctatum*, as described in Rugh (1962). *T. torosa* (T.t.) embryos were staged using the method of Twitty and Bodenstein (Rugh, 1962) for this species. After stage 40, *T. torosa* was staged on the basis of limb development, using *A. punctatum* (Rugh, 1962) as a standard for comparison. Morphologically, the stages for the two species were almost identical.

A. Specific activity determinations:

Groups of fifteen embryos from each of the major developmental periods were frozen in small plastic capped test-tubes in liquid nitrogen and stored at -20 ^{o}C. until required. Fresh material was also used for comparative purposes. Fifteen embryos were routinely homogenized in 1.0 ml. of borate buffer (0.3 M, pH 8.6) in a Bellco plastic homogenizer with teflon pestle, maintained cold in an ice-bath. To this brei 1.5 volumes of n-butanol were added. This step was followed with further homogenization as the brei was allowed to attain room temperature (after Morton, 1954). Subsequently, centri-

fugation was carried out in an International refrigerated centrifuge (model HR-1, Head 856) at 16,000 rpm. and 0-3 °C. for fifteen minutes. The aqueous layer, lying above the precipitated cell debris and below the light butanol and fatty layers, was removed and stored at -20°C. In certain cases, the brei alone was stored for later determinations on activity loss in the precipitate. It was noted that frozen samples could be stored for months with little detectable loss of alkaline phosphatase activity, as compared to fresh samples. However, samples were usually used within one week. For comparative purposes an identical buffer extraction without butanol was done.

For the developmental analysis of specific alkaline phosphatase activity, the extracts were prepared for each of the following periods of development: blastula (T.t. stage 8), gastrula (A.g. st. 12; T.t. st. 13), neurula (A.g. st. 16; T.t. st. 16 and st. 18), closed neural folds (T.t. st. 21), tail bud (A.g. st. 25; T.t. st. 28), late tail bud (A.g. st. 31; T.t. st. 32), C-flexure (A.g. st. 33), S-flexure (A.g. st. 35; T.t. st. 36), and free-swimming larvae (A.g. st.38, st. 41/42 and st. 45/46; T.t. st. 39, st. 42/42+ and st. 45/46. These extracts were then frozen and stored until all stages were accumulated. Three separate extractions were done for each stage analyzed.

B. Electrophoresis:

Both butanol and buffer extraction procedures were utilized for samples to be subjected to electrophoretic analysis. Here, 30 embryos were homogenized in 0.2 ml. of buffer. If

the number of embryos was increased the buffer added was also increased proportionately. With butanol extracts it was necessary to dialyze the samples against sucrose (20 % in 0.3 M borate buffer) before electrophoresis, to remove the butanol. Routine dialysis of buffer extracts was unnecessary since preliminary runs demonstrated it did not qualitatively change the results.

C. Histochemistry:

Live embryos were fixed overnight (approx. 12 hours) in 80% ethanol and, after the fixed embryos were taken to xylene through 95% and absolute ethanol, they were embedded in a low melting point wax (Paraplast, M.P. 42-44^{o}C.). The total time in the wax was one hour. The paraffin blocks were stored at -20^{o}C., for the period when the stages to be studied were accumulated. The blocks were then trimmed and sectioned at 7-10 microns and the sections mounted on albumenized slides.

II. Specific Activity Assays:

In these experiments two different substrates were used for the analysis of alkaline phosphatase activity, sodium beta-glycerophosphate (BGP; Nutritional Biochemicals) and para-nitrophenyl phosphate (PNPP; Calbiochem). The specific activity for BGP was defined as micromoles of phosphate released per minute per milligram of protein per milliliter. For PNPP, the specific activity was defined as 1 X 10^{-3} mg. PNP released per min. per mg. protein per ml.. The specific activity values were then converted to percent of maximum activity for graphic representation. For comparison of

developmental changes, they were converted to the amount of substrate utilized, expressed in micromoles (μM), to allow a direct comparison of the hydrolysis of the two substrates by the embryo extracts. These data too were plotted as percent of maximum values.

A. Enzyme activity assays:

The standard reaction mixture , for each substrate, was modified from the method of Moog and Grey (1967) for beta-glycerophosphate and consisted of:

0.25 ml. of 0.25 M substrate (BGP or PNPP)

1.50 ml. of 0.10 M carbonate-bicarbonate buffer (pH 10.0)

0.25 ml. of 0.10 M $MgCl_2$

The concentrations of each of the reagents in this standard reaction mixture had been determined as optimal by preliminary experiments. Free-swimming larvae because of their abundance were used in most cases for the determination of the optimal conditions and characteristics of the alkaline phosphatase activity. The concentration of the substrate or $MgCl_2$, the pH of the buffer and temperature were varied in accordance with the parameter under observation. For measurements of activity below pH 9.0, Tris/HCl buffer (0.1 M) was used.

To the reaction mixture, 0.2 ml. of sample was added, followed by rapid mixing on a Vortex Genie. The reaction was carried out at room temperature and was "killed" with 0.8 ml. of 10% trichloracetic acid (TCA), after one hour unless otherwise indicated. When BGP was used the released

inorganic phosphate was measured by the method of Fiske and Subbarow (1925) using a Technicon Autoanalyzer. Standards of known inorganic phosphate concentration (KH_2PO_4) were run with each set of determinations. For PNPP the yellow colour of the released para-nitrophenol (PNP) was measured at 420 mμ in a Spectronic 20 spectrophotometer. Since TCA destroys the colour, by pH depression, it was redeveloped with 2.0 ml. 0.1 N NaOH (after Pfohl and Guidice, 1966). The amount of PNP released was determined from a standard curve prepared from a series of known concentrations of this product (Calbiochem).

Controls consisted of reaction mixtures to which TCA was added prior to the addition of the sample (0-time control) and of substrate-free mixtures.

B. Effect of inhibitors:

The two amino acids, phenylalanine and tryptophan, and inorganic phosphate (KH_2PO_4) were used, in concentrations ranging from 1.25×10^{-3} to 1.5×10^{-2} Molar, to study the inhibition of larval phosphatase.

C. Protein determination:

Protein contents were determined by the technique of Lowry et al (1951) using the Folin Ciocalteau Phenol Reagent (Fischer) as described by Adams (1964). In this case 0.2 ml. of sample rather than 0.4 ml. was used so as to make possible the direct correlation with the enzyme activity assays. No sample dilution was necessary for these determinations. A standard curve was prepared with egg albumen treated the same as the experimental samples, ranging in concentration from

4.0×10^{-2} to 4.0 mg. of protein per ml.. Blanks for zeroing the spectrophotometer consisted of all the reagents with water in place of the embryo extracts. A Spectronic 20 spectrophotometer was used in all cases.

III. Electrophoresis:

A. Starch gel electrophoresis:

An attempt to reveal multimolecular forms of alkaline phosphatase was made using the Tsyuki et al (1962, 1963) modification of the starch gel method of Smithies (1955, 1959). Samples were run in 12% gels in the borate buffer system for 1½ to 2 hours at 3°C. and 3 mA per gel. Sample dilution had no effect except to lower total activity.

B. Disc electrophoresis:

Disc electrophoresis in 7% acrylamide gels as described by Ornstein (1962) and Davis (1962) was also used to reveal the alkaline phosphatase patterns of salamander embryo extracts. Samples were run in a Canalco disc electrophoresis apparatus at 3 mA per gel for approximately 1 to 1¼ hours depending on the rate of migration of the more electronegative of the two embryonic pigment bands. The length of the run corresponded closely to the time required for a bromphenol blue dye marker to migrate to the end of the gel. When the most rapidly migrating band reached the end of the running gel the run was terminated. Sample volumes of 100 µl. were layered directly on the surface of each stacking gel.

C. Gel staining:

Both starch and acrylamide gels were stained for localization of alkaline phosphatase activity using sodium alpha-

naphthyl phosphate (100 mg.) and Fast blue RR (50 mg.) in carbonate-bicarbonate buffer (100 mls. of 0.1 M at pH 10), containing 1.0 ml. of 0.1 M $MgCl_2$. Staining was done at room temperature after which the gels were stored in distilled water (acrylamide gels) or in Saran Wrap (starch gels), until photographed. The gels were photographed by transmitted light using Kodak Plus X Pan film. The reddish-brown, azo-dye complex depicts sites of alkaline phosphatase activity and is stable indefinitely, although darkening in time, both at room temperature and in the refrigerator.

IV. Histochemistry:

Histochemical localization of alkaline phosphatase was by the Burstone (1962) Naphthol AS Phosphate Method, which is similar to that used in the gel staining procedure. Staining was performed at pH 9.0 because the stain precipitates out at higher pHs and this region of pH is specified in the method. On the basis of a developmental analysis of specific alkaline phosphatase activity carried out in this laboratory, the use of this pH seems legitimate, although not optimal (see Figure 1). After staining for one hour at 37°C., the sections were mounted in Aquamount and covered with a coverslip. Sections were viewed with a Wild microscope and activity values given in arbitrary units (+ to ++++) based on a comparison with maximal gut activity of free-swimming larvae. Selected sections were photographed on a Zeiss Photomicroscope using Kodak High Speed Ektachrome (Type B).

RESULTS

I. SPECIFIC ACTIVITY:

A. Optimal conditions for enzyme activity:

1. pH optima- Buffer extracts of free-swimming larvae (st. 40-46) demonstrated a single peak of activity within the range of pH from 9.75-10.0 (Fig. 1. A,C). This held true for both T. torosa and A. gracile. Below pH 9.0 buffer extracts showed a low level of activity which dropped off slowly as the pH moved towards neutrality. Butanol extracts revealed (Fig. 1. B,D,E, and F) the same major activity peak, in both species, as had the buffer extracts. No peak of activity occurred below pH 9.0 in butanol extracts. Butanol extracts, of both species, revealed a 6-fold increase in specific activity above that for buffer extracts. Morton (1954) has suggested that the increased specific activity in butanol extracts is due to increased enzyme release. However, the data in Table I show that although butanol extraction produces a modest increase in absolute activity (column 3), the major increase in specific activity is due to the much lower non-enzyme protein in butanol extracts. This interpretation is confirmed by comparing the amounts of AP activity lost to the precipitate in buffer and butanol preparations (calculated from data in Table II). The comparison of T. torosa butanol extract activity, by volume (column 3, Table I), to brei activity (Column 2, Table II) revealed that only about one-third (33.8%) of the total enzyme activity was lost in the precipitated layer on centrifugation.

2. Substrate optima- In A. gracile varying the substrate

FIGURE 1. The effect of pH on the alkaline phosphatase activity of extracts of free-swimming larvae.
A. Buffer extract of A. gracile (st. 42/44) embryos (substrate:PNPP).
B. Butanol extract of A. gracile (st. 42/44) embryos (substrate:PNPP).
C. Buffer extract of T. torosa (st. 42/44) embryos (substrate:PNPP).
D. Butanol extract of T. torosa (st. 42/44) embryos (substrate:PNPP).
E. Butanol extract of A. gracile (st. 42/44) embryos (substrate: BGP).
F. Butanol extract of T. torosa (st. 42/44) embryos (substrate: BGP).

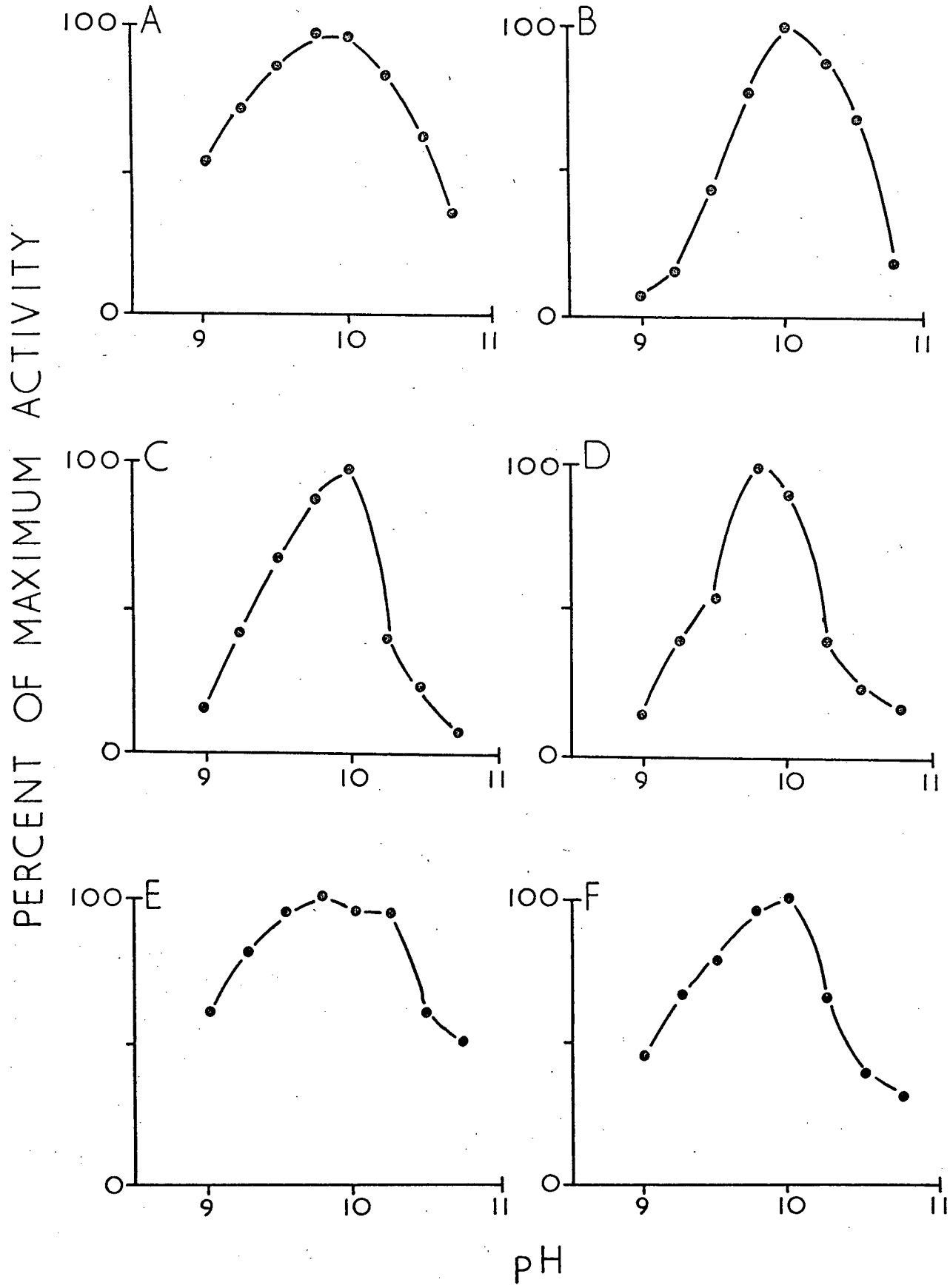

A
B
C
D
E
F
100
0
9
10
11
PERCENT OF MAXIMUM ACTIVITY
pH

TABLE I. Comparison of specific activities with different extraction methods (A. gracile and T. torosa stage 42 embryos; data represents the results of one set of experiments).

TABLE II. Comparison of crude homogenate activity with supernatent activity (T. torosa stage 42; buffer extraction).

TABLE I

COMPARISON OF SPECIFIC ACTIVITIES WITH DIFFERENT EXTRACTION METHODS

SPECIES	EXTRACTION METHOD	PNP X 10^{-3} RELEASED	PROTEIN CONC.	SPECIFIC ACTIVITY	ACTIVITY INCREASE
A. gracile	buffer	.235	1.30	0.181	5.82
	butanol	.310	0.29	1.05	
T. torosa	buffer	.399	1.10	0.362	5.85
	butanol	.448	0.21	2.13	

TABLE II

COMPARISON OF CRUDE HOMOGENATE ACTIVITY WITH SUPERNATANT ACTIVITY*

SAMPLE	PNP X 10^{-3} RELEASED	PROTEIN CONC.	SPECIFIC ACTIVITY
Brei	.676 $\pm$ .006	2.4 $\pm$ .20	.273
Supernatant	.373 $\pm$ .064	.98 $\pm$ .19	.380

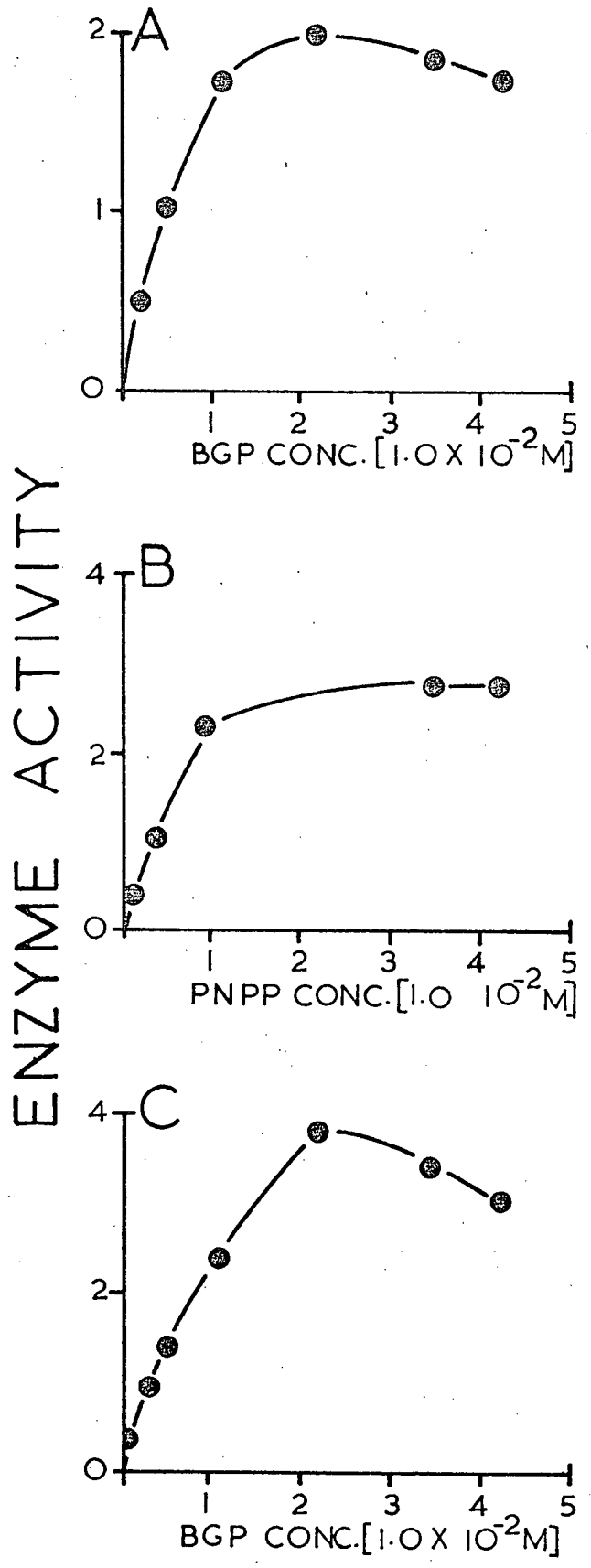
* Data represents means ($\pm$ S.D.) of three experiments.

concentration of both PNPP and BGP gave maximal activity at 2.2×10^{-2} Molar (Figure 2). Some inhibition of enzyme activity occurred at higher substrate levels. This might be a result of the free inorganic phosphate present in the substrates. The high level of inorganic phosphate present in PNPP gave very high zero-time control readings on the autoanalyzer and, thus, it was more accurate to use the absorbance of the PNP as an index of enzyme activity rather than the release of inorganic phosphate. Although T. torosa extracts have a higher specific activity at st. 42 (approx. 2 times that of gracile, Table I), the plot of enzyme activity versus substrate concentration (for PNPP) appeared the same as that for A. gracile (Fig. 2, C).

3. Temperature optimum- The temperature optimum for A. gracile occurred around 37°C. (Fig. 3, A). However, activity is still present at 5°C., at 10% of the maximum. The increase in activity with temperature is rather sharp after 30°C. as is the decrease beyond 37°C.. Since the temperature at which maximum activity was present was so much higher than the temperatures these embryos normally encounter, it was decided to use room temperature (approx. 22°C.) for the temperature at which enzyme activity analysis would be done, though even this is a substantial increase over the usual temperature at which the embryos develop.

4. Enzyme dilution- Dilution of embryo extracts of A. gracile revealed a linear increase in activity with increasing enzyme concentration (Fig. 3, B).

FIGURE 2. The effect of substrate concentration on the rate of hydrolysis of substrate by butanol extracts of free-swimming salamander larvae:
A. BGP hydrolysis by A. gracile (st. 42) extracts.
B. PNPP hydrolysis by A. gracile (st. 42) extracts.
C. BGP hydrolysis by T. torosa (st. 42) extracts.
The enzyme activity units refer to PNP X 10^{-3} released per hour per ml. (for PNPP) and micromoles of inorganic phosphate released per hour per ml. (for BGP).


A
B
C
ENZYME ACTIVITY
BGP CONC. [1·0 X 10-2M]
PNPP CONC. [1·0 10-2M]
BGP CONC. [1·0 X 10-2M]
0
1
2
3
4
5

5. Time course- In extracts of A. gracile the rate of reaction is linear with time for at least two hours (Fig. 3, C). This was true for both A. gracile and T. torosa from gastrulae to free-swimming larvae.

6. Effect of magnesium ions- In many cases magnesium ions are essential for the activity of alkaline phosphatase. No effect on enzyme activity was noted here when a range of concentrations of $MgCl_2$ from 9.0×10^{-2} to 9.0×10^{-4} Molar was used.

B. Effect of inhibitors on phosphatase activity:

Both tryptophan and phenylalanine, which are known inhibitors of other alkaline phosphatases (Fishman et al, 1963; Ghosh and Kotowitz, 1969; Griffin and Cox, 1966), inhibit the phosphatase activity present in extracts of free-swimming larvae of A. gracile and T. torosa. The level of inhibition (50% at 7.5×10^{-3} Molar) seems too low to be considered significant (Fig. 4). Inorganic phosphate, too, inhibits the salamander alkaline phosphatase (Fig. 4) and is less efficient in this respect than the amino acid inhibitors (50% inhibition at about 1.25×10^{-2} Molar).

C. Developmental changes in specific activity:

As shown in Figures 5 and 6 the alkaline phosphatase activity of the salamander embryo extracts show a general overall increase with development. This trend holds true for both species with both substrates.

When PNPP was used as a substrate with A. gracile extracts (Table III; Figure 5) the increase in specific AP activity was gradual up to the appearance of a definite tail

FIGURE 3. Some parameters of alkaline phosphatase activity in butanol extracts of A. gracile embryos:
A. Effect of temperature on PNPP hydrolysis by st. 42/44 extracts.
B. Effect of dilution of enzyme extracts on activity of st. 42/44 embryo extracts.
C. Time course of PNPP hydrolysis by st. 35 embryo extracts.

(● and ▲ represent results with different extracts).

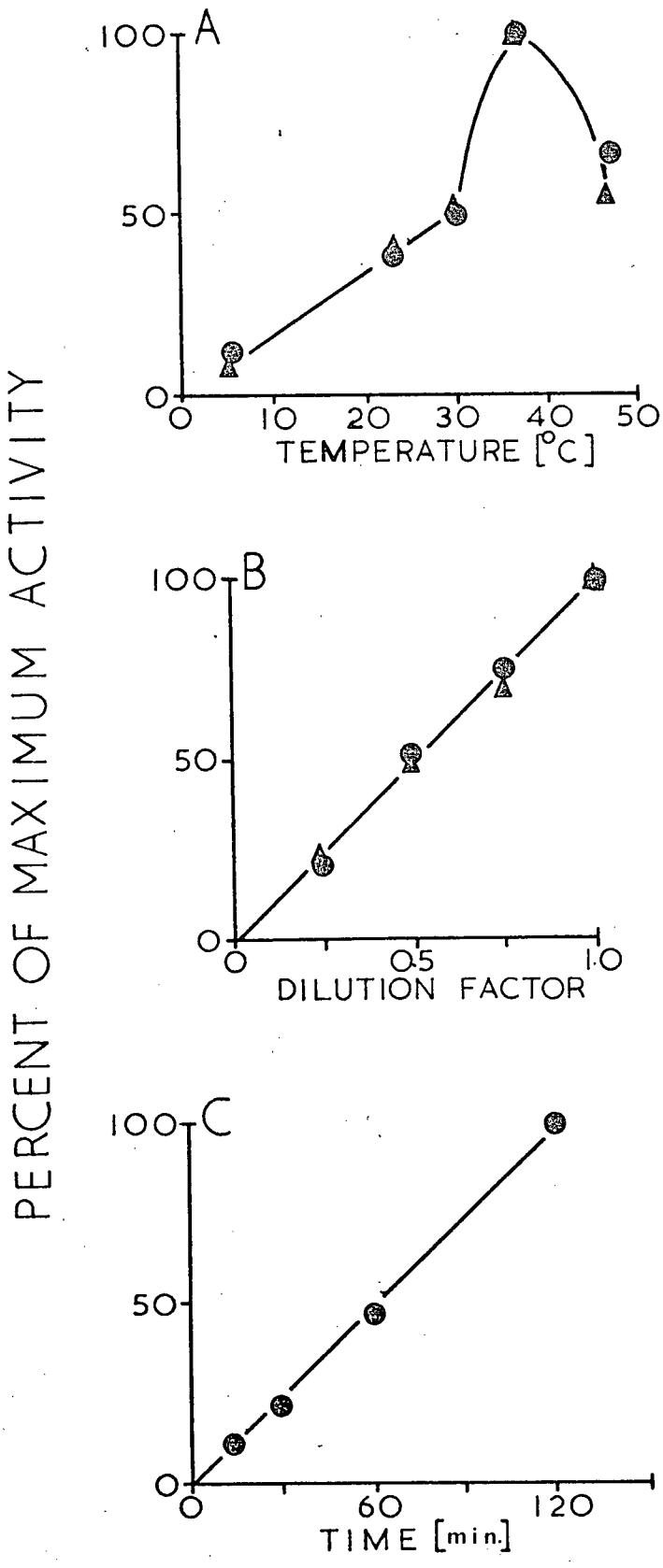
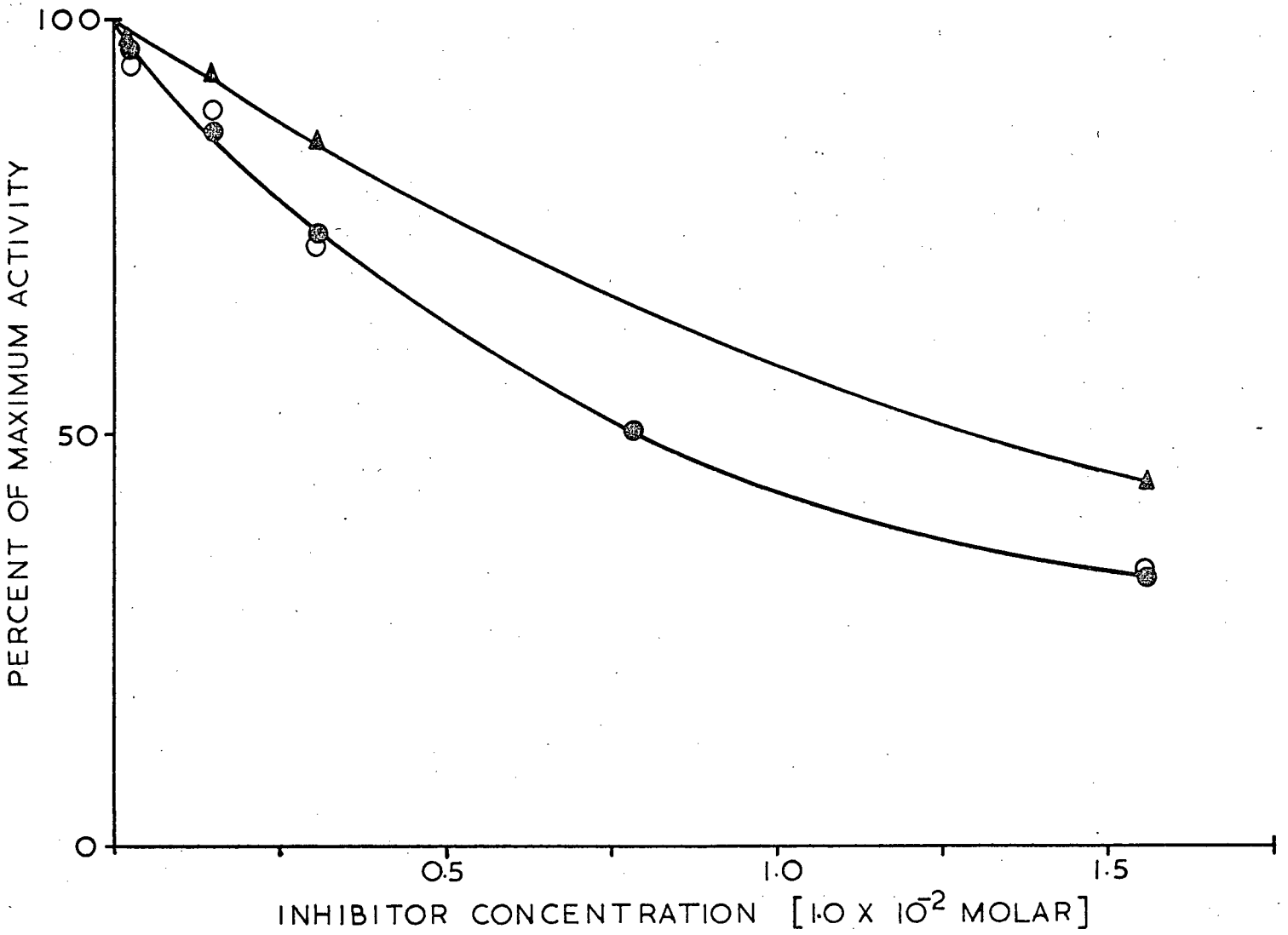

A
100
50
0
0
10
20
30
40
50
TEMPERATURE [°C]
B
100
50
0
0
0.5
1.0
DILUTION FACTOR
C
100
50
0
0
60
120
TIME [min.]
PERCENT OF MAXIMUM ACTIVITY

FIGURE 4. Effect of known inhibitors of alkaline phosphatase on PNPP hydrolysis by buffer extracts of T. torosa free-swimming larvae (st. 40):
O Tyrosine
● Tryptophan
▲ Inorganic Phosphate (KH_2PO_4).


100
50
0
PERCENT OF MAXIMUM ACTIVITY
0.5
1.0
1.5
INHIBITOR CONCENTRATION [1.0 X 10⁻² MOLAR]

bud (st. 25), which possessed a level of activity 5 times that of the gastrular level (st. 12), but became by stage 38 about 25 times the gastrular level. The increase was even more obvious within the next few stages of development, reaching ultimately (st. 45/46) about 150-fold the activity possessed by the gastrulae. Similar results were obtained when BGP was used as substrate (Table IV; Figure 6). However, the increase between gastrula and free-swimming stages reached a maximum of only 35-fold when BGP was used.

The results with T. torosa extracts correlated very well with those for A. gracile, although the former species usually possessed a greater activity in the later stages of development. Comparing free-swimming activity (st. 42/42+) to gastrular specific AP activity there was a 60-fold increase when PNPP was used and a 25-fold increase when BGP was used as substrate. After stage 42/42+ a decrease in specific activity was noted with both substrates.

The foregoing discussion of results has been based on mean values calculated from three data points for each stage of development. Confidence limits (5%) were calculated, with the aid of a computer, and demonstrated large overlap below stages 35 (A.g.) and 36 (T.t.) but showed significant differences past these stages. This would indicate that between gastrulation and the onset of autonomous muscle movement in these salamander embryos no significant difference in enzyme activity exists. On the basis of work in other Urodele species (Krugelis, 1950; Lovtrup, 1953), in which similar results were obtained, it would seem that this situation

TABLE III. Hydrolysis of PNPP by butanol extracts of A. gracile embryos of different developmental stages.

TABLE IV. Hydrolysis of BGP by butanol extracts of A. gracile embryos of different developmental stages.

TABLE III

STAGE	MEAN SPECIFIC ACTIVITY	INCREASE	% MAXIMUM	SUBSTRATE USED (μM)
12	0.007		0.600	0.051
		0.002		
16	0.009		0.720	0.065
		0.017		
18	0.026		2.200	0.187
		0.013		
25	0.039		3.300	0.280
		0.061		
31	0.100		8.450	0.719
		-0.026		
33	0.074		6.920	0.530
		0.022		
35	0.096		8.120	0.690
		0.092		
38	0.188		15.90	1.350
		0.730		
41/42	0.918		73.90	6.600
		0.362		
45/46	1.180		100.0	8.500

TABLE IV

STAGE	MEAN SPECIFIC ACTIVITY	INCREASE	% MAXIMUM	SUBSTRATE USED (μM)
12	0.300		2.820	0.300
		0.150		
16	0.450		4.230	0.450
		0.100		
18	0.460		4.320	0.460
		0.230		
25	0.690		6.490	0.690
		0.170		
31	0.860		8.080	0.860
		-0.190		
33	0.670		5.490	0.670
		0.210		
35	0.880		8.260	0.880
		0.270		
38	1.150		10.80	1.150
		5.460		
41/42	6.180		63.94	6.810
		3.840		
45/46	10.65		100.0	10.65

TABLE V. Hydrolysis of PNPP by butanol extracts of T. torosa embryos of different developmental stages.

TABLE VI. Hydrolysis of BGP by butanol extracts of T. torosa embryos of different developmental stages.

TABLE V

STAGE	MEAN SPECIFIC ACTIVITY	INCREASE	% MAXIMUM	SUBSTRATE USED (μM)
13	0.035		1.660	0.250
		-0.007		
16	0.028		1.460	0.220
		0.009		
21	0.037		1.800	0.270
		0.051		
28	0.088		4.190	0.630
		0.141		
32	0.147		7.050	1.060
		0.147		
36	0.294		14.20	2.120
		0.502		
39	0.796		38.00	5.720
		1.295		
42/42+	2.091		100.0	15.05
		-0.175		
45/46	1.916		91.70	13.80

TABLE VI

STAGE	MEAN SPECIFIC ACTIVITY	INCREASE	% MAXIMUM	SUBSTRATE USED (μM)
8	0.310		2.540	0.310
		0.200		
13	0.510		4.180	0.510
		-0.060		
16	0.450		3.690	0.450
		-0.060		
21	0.390		3.190	0.390
		-0.010		
28	0.380		3.110	0.380
		0.210		
32	0.590		4.830	0.590
		0.920		
36	1.510		12.36	1.510
		1.150		
39	3.020		24.73	3.020
		9.190		
42/42+	12.21		100.0	12.21
		-0.640		
45/46	11.57		94.75	11.57

FIGURE 5. Development of alkaline phosphatase specific activity as revealed by the hydrolysis of PNPP.

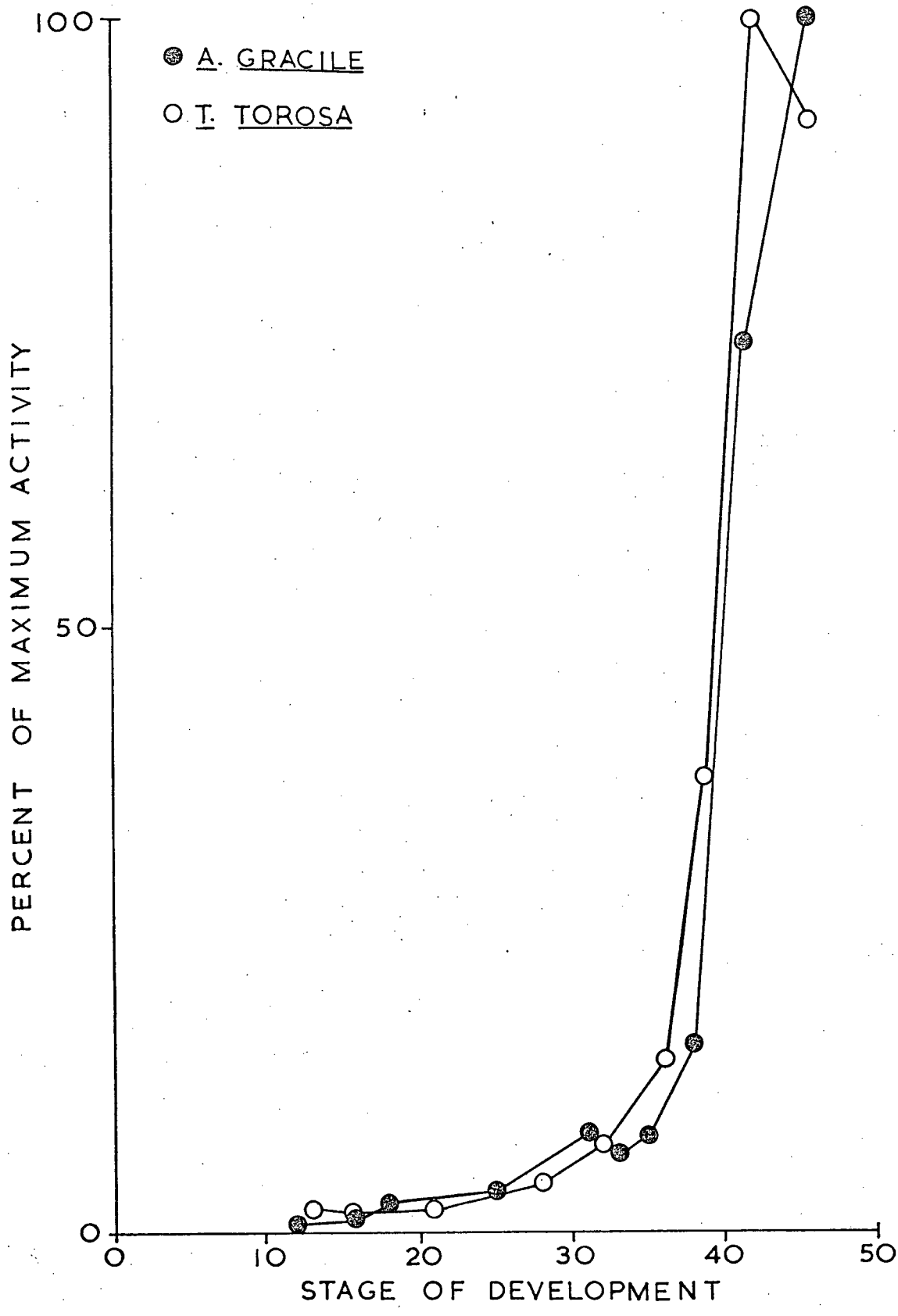
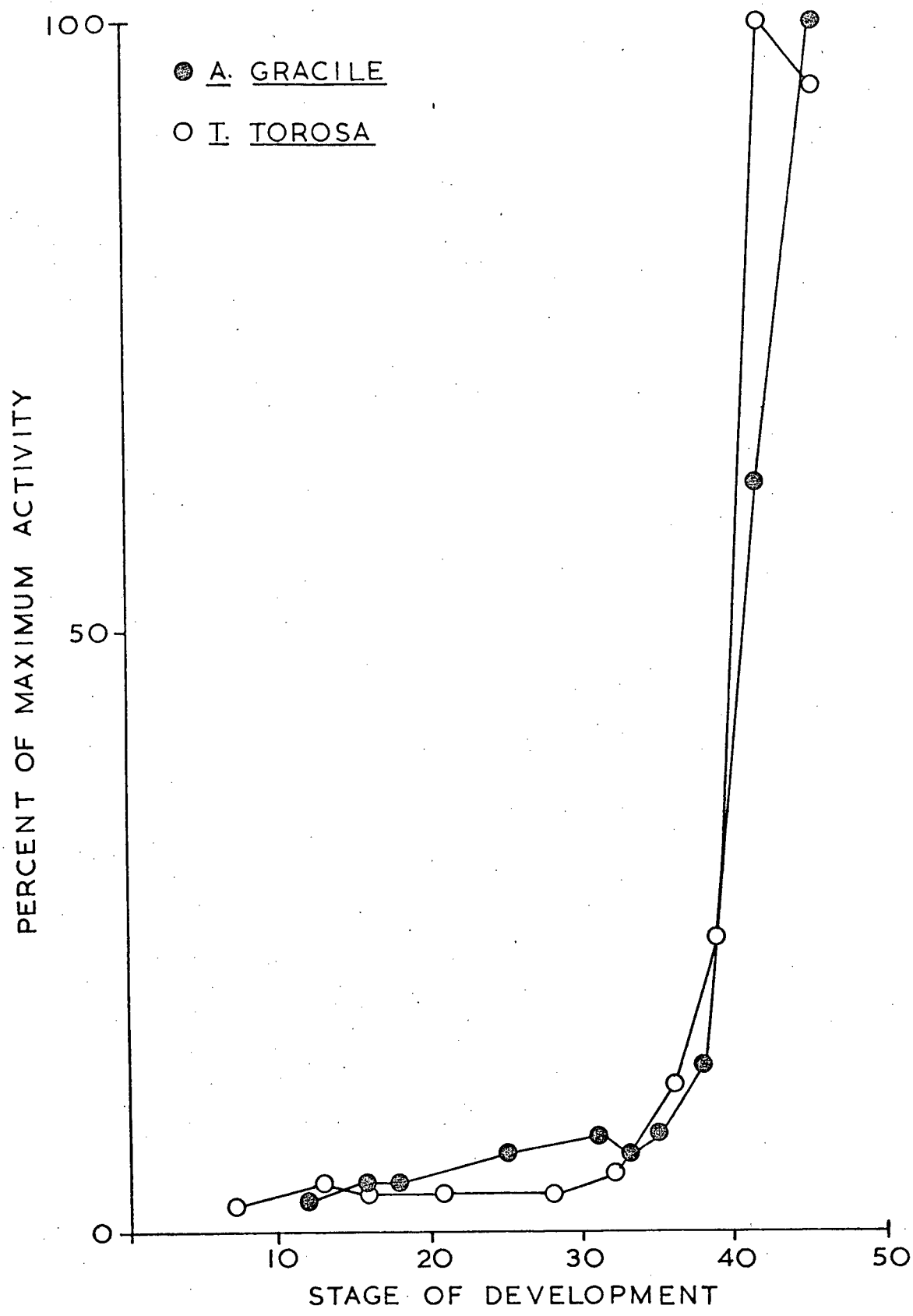

100
50
0
A. GRACILE
T. TOROSA
PERCENT OF MAXIMUM ACTIVITY
0
10
20
30
40
50
STAGE OF DEVELOPMENT

FIGURE 6. Development of alkaline phosphatase specific activity as revealed by the hydrolysis of the substrate BGP.


100
50
0
A. GRACILE
T. TOROSA
PERCENT OF MAXIMUM ACTIVITY
10
20
30
40
50
STAGE OF DEVELOPMENT

is widespread in developing salamanders.

With A. gracile the ratio calculations showed two peaks of high ratio (PNPP/BGP) corresponding to late tail bud (st. 31) and early free-swimming larvae (st. 38)(Table VII; Figure 7). The results of the activity ratios, when plotted as percent of maximum, were comparable between the two species, with the T. torosa values overlapping the A. gracile values continuously from gastrulation to the free-swimming stage (Table VIII; Figure 7).

II. GEL ELECTROPHORESIS:

A. Starch gel electrophoresis:

The results with Tsyuki's (1962, 1963) microstarch gel electrophoretic system were discouraging. It was necessary to use butanol extracts to obtain migration. However only a dense band streaking out approximately one-half inch from the origin occurred (Figure 8). Some sub-banding was evident within the streak, but was not reproducible. An analysis of the general protein patterns (stained with Amido Black 10B) revealed many clearly delineated bands and commercial intestinal phosphatase (lyophilized, bovine; Calbiochem) gave a single band of activity. Therefore, the failure to obtain banding of AP lies with the embryo extracts rather than the starch gel technique.

B. Acrylamide gel electrophoresis:

Clear results were obtained with acrylamide gel electrophoresis. It was found that butanol extracts, after dialysis, gave the same electrophoretic pattern as buffer extracts of any one stage. This is shown in Figure 9. Migration was

TABLE VII. Activity ratios (PNPP/BGP) of butanol extracts of A. gracile from different developmental stages.

TABLE VIII. Activity ratios (PNPP/BGP) of butanol extracts of T. torosa from different developmental stages.

TABLE VII

STAGE	ACTIVITY RATIO (PNPP/BGP)	% MAXIMUM OF RATIO
12	0.170	14.50
16	0.140	11.95
18	0.410	35.05
25	0.420	35.90
31	0.840	71.80
33	0.790	67.50
35	0.780	66.70
38	1.170	100.0
41/42	0.970	82.80
45/46	0.800	68.30

TABLE VIII

STAGE	ACTIVITY RATIO (PNPP/BGP)	% MAXIMUM OF RATIO
13	0.490	26.05
16	0.490	26.05
21	0.690	36.70
28	1.650	87.80
32	1.800	95.50
36	1.400	74.50
39	1.880	100.0
42/42+	1.230	65.40
45/46	1.190	63.20

FIGURE 7. Change in activity ratio (PNPP/BGP) during development of A. gracile and T. torosa.

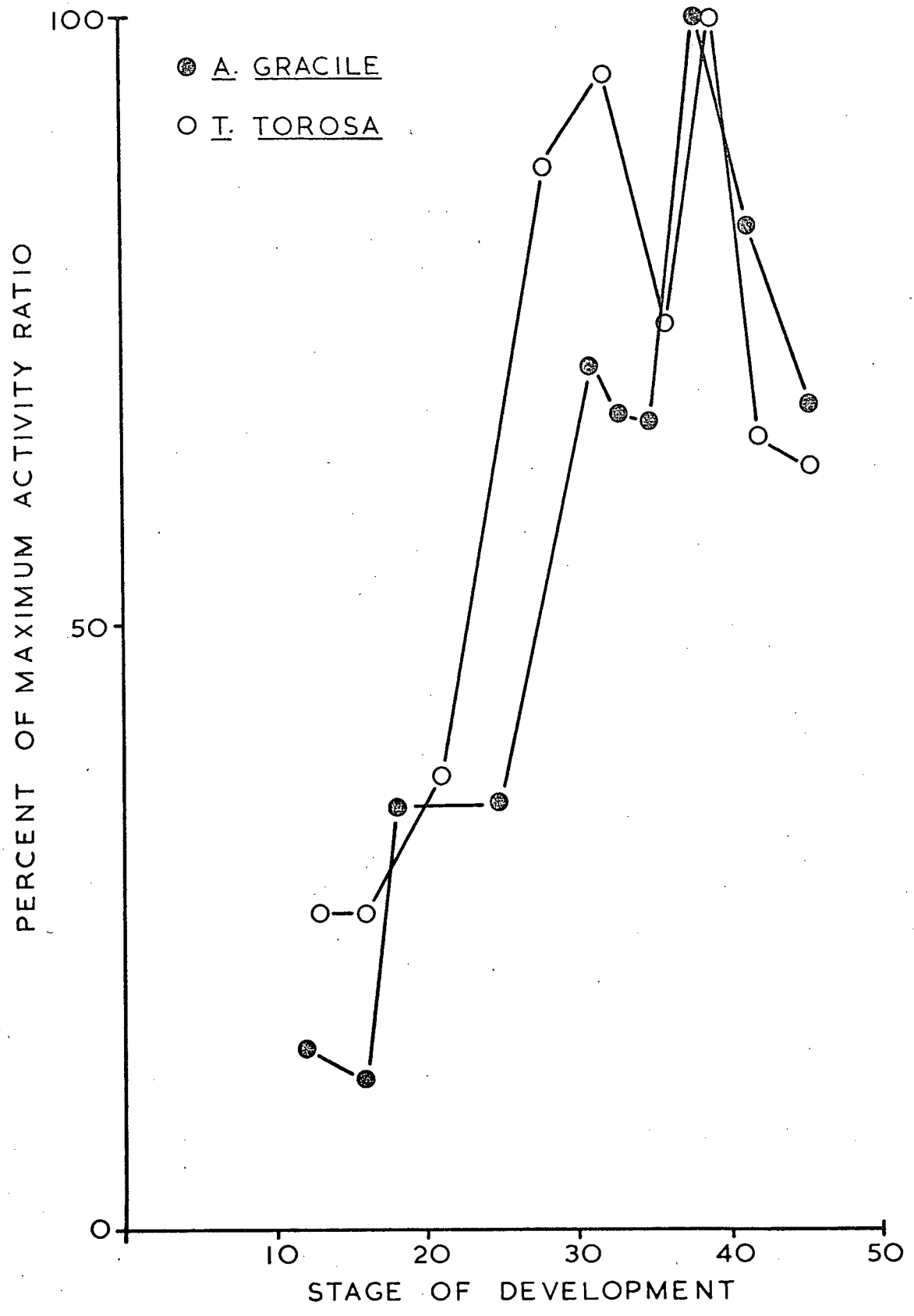
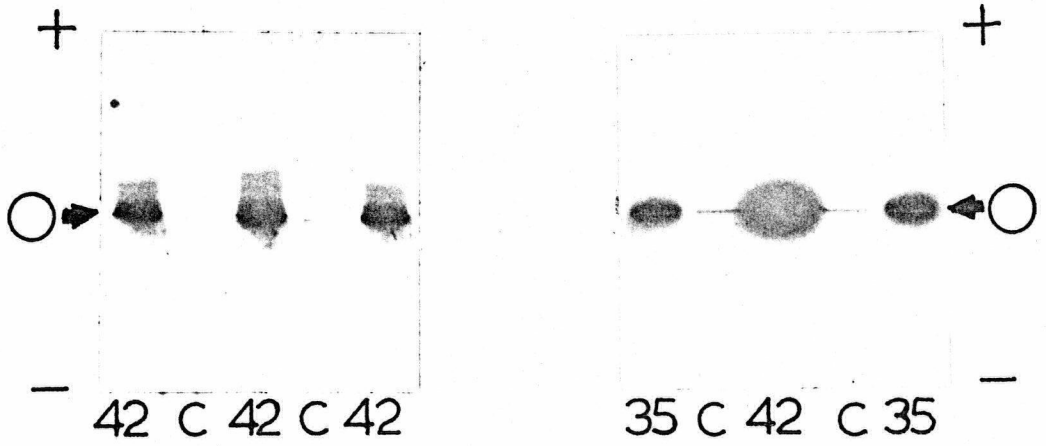

100
50
0
A. GRACILE
T. TOROSA
PERCENT OF MAXIMUM ACTIVITY RATIO
10
20
30
40
50
STAGE OF DEVELOPMENT

FIGURE 8. Effect of different extraction methods on electrophoretic patterns of alkaline phosphatases in starch gels. (all are A. gracile embryo extracts; 35 and 42 refer to stages of development; c stands for control).

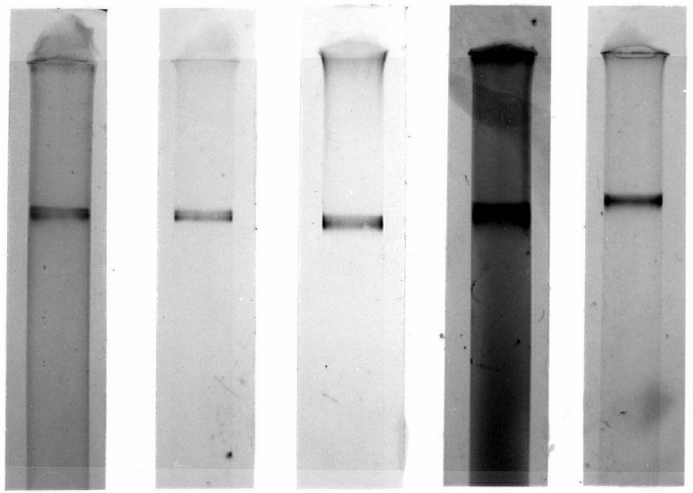

+
−
42 C 42 C 42
BUTANOL EXTRACT
+
−
35 C 42 C 35
BUFFER EXTRACT

slower with butanol extracts than with buffer extracts, which may reflect the lower protein content of the butanol extracts. As a result, buffer extracts were used routinely. The maximum number of alkaline phosphatase bands was two. These components migrated very close together making it difficult to quantify the individual bands. Microdensitometer tracings of the stained gels have been made and such tracings reveal the slower component of the zymogram as a shoulder of the faster, more dense band. That the activity of the bands increased, and markedly with development, was evident. The same results were obtained for both species and a summary of the development of the enzyme patterns is shown in Figure 10. Designating the more electronegative band as band 2 and the more electropositive as band 1 the developmental trend can be described as an increase in band 2 activity and a decrease of band 1. By stage 40 the activity is so great that separation of the bands visually is difficult. It was impossible to detect any subtle changes in the band activities for a comparison of their relative activity, and increased running time, up to 2½ hours, did not aid electrophoretic band separation.

III. HISTOCHEMISTRY: A. A. gracile:

The first histochemically detectable alkaline phosphatase activity occurred during tail bud stages (st. 27) and was localized in the yet undifferentiatied endoderm, two regions of ectoderm (the stomodeal and proctodeal regions) and in the forebrain (composed at this time of the telencephalon and diencephalon). The general activity of the gut and

FIGURE 9. Migration of alkaline phosphatases in acrylamide gel (10/12, 22/24,.....40 refer to developmental stages; 10/12 buffer and 10/12 butanol compare results with different extraction methods. 50 µl. of extract were applied to each gel.

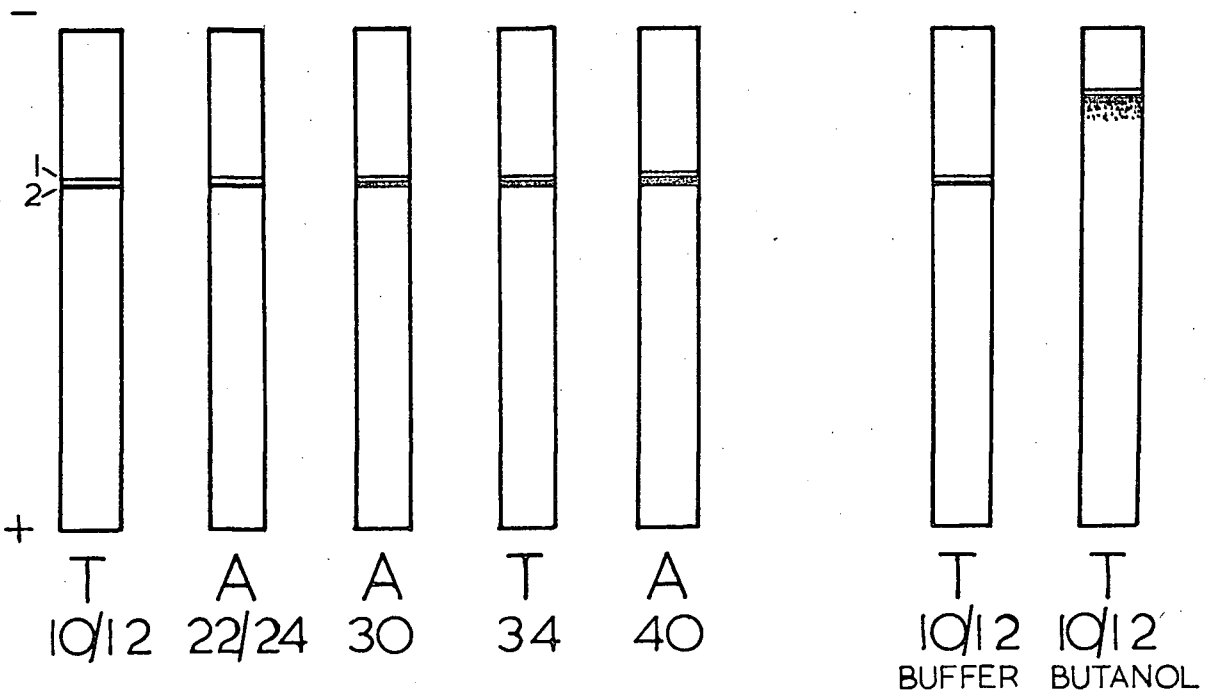


T 10/12 | A 22/24 | A 30 | T 34 | A 40

DEVELOPMENTAL PATTERN

T 10/12 BUFFER | T 10/12 BUTANOL

FIGURE 10. Diagrammatic representation of the developmental sequence of alkaline phosphatase differentiation and a comparison of the extraction procedures as pictured in Figure 9. (symbols as for Fig. 9).



DEVELOPMENTAL PATTERN

brain regions was low, whereas that of the developing stomodeum and proctodeum regions was higher but restricted, only occurring in a few sections.

As the embryos continued to develop into the late tail bud stage (st. 30/31), alkaline phosphatase activity became evident in other areas. The liver diverticulum, which first appeared as a simple ventral extension of the foregut into the yolk, had a relatively high level of activity initially, with the major amount of activity disposed dorsally towards the foregut. The pronephric tubules, which at this time have just become morphologically distinct, had little activity. Although little differentiation of the heart had occurred there was a detectable level of activity and the head and branchial mesenchymes showed low levels of AP activity as well. The only other change by stage 30/31 was a slight increase in the stomodeal activity.

As development proceeds, evidenced externally by an elongation of the embryo and an increase in the size of the gill buds, some new tissues are added to the ensemble of alkaline phosphatase producers. By stage 33, the spinal cord possessed a detectable level of the enzyme whereas the optic cup and stalk had a higher level of AP activity. It was not until this time that AP enzyme differentiation of the optic stalk and cup became obvious, yet the initial morphological differentiation had started some time during mid-tail bud. The mid-brain region (mesencephalon), too, had a low level of enzyme activity. Concomitant with the appearance of these new enzyme sources, certain other tissues had been

increasing their levels of activity. The AP activity of both ectodermal sites had increased, as had the anterior-most region of the pharynx. The regions of the gut as well as the liver diverticulum (transverse duodenum) had not undergone any detectable change. The pronephric tubules (Plate II, D), now with a central lumen, had a high level of activity disposed towards the lumen. Specific tissue level localization of enzyme activity had been evident only in the pronephric tubules. The heart at this time was still only in its early stages of differentiation but did show increased activity.

By the time the embryos had the capacity for full S-flexure (st. 36), all of the neurectodermal components demonstrated alkaline phosphatase activity histochemically. This activity was highest in the diencephalon with more intense localizations where the optic stalks associated. Although the telencephalon possessed a very low level of AP activity the remaining regions of the brain, as well as the spinal cord, had a general, slightly higher level of activity and the optic cup had its maximal amount of activity (see T. torosa, Plate III, H.). Although activity in the ectodermal derivatives became reduced (especially in the proctodeum) the hypophysis, now morphologically evident, demonstrated a fairly high level of activity where it associated with the diencephalon. The amount of activity in the ventral prosencephalon seemed to be higher than other regions as well. While the endoderm had not improved as an alkaline phosphatase contributor the yolk activity began to show an increase. The heart, with an increase in activity as well as in its

morphological development, now revealed that the major activity was associated with the endocardium though other restricted areas of light staining did occur within the heart. The head mesenchyme at this stage generally demonstrated a low level of activity that was more intense where it associated with the optic cup and stalk. The branchial mesenchyme AP activity was very intense. With the appearance of the limb bud, a low level of activity was associated with the dense mesenchyme of the limb. In the region of the future axial skeleton the axial mesenchyme also demonstrated a low level of activity.

All the tissues, other than endodermal or yolk, that had not previously reached their maximal level attained it by stage 38. However, the stomodeal activity was reduced and the proctodeal activity totally lost as these openings became morphologically complete. The hypophysis, whose association with the infundibulum was now more intimate, showed a very high level of activity (Plate III, J). In general the enzyme activities in the diencephalon and telencephalon had increased to a fairly high level while increases in the mesencephalon, rhombencephalon and spinal cord were not so marked. Specifically the telencephalon had a general, overall staining whereas the diencephalon activity was located in the region of the optic stalks. The staining of the optic stalk was all-inclusive but the cup activity was localized in the cells around the lumen. The AP activity of the mesencephalon , rhombencephalon, and spinal cord was more intense in the region of the white matter and was dis-

posed more ventrally than dorsally. The foregut enzyme activity was at a fairly high level yet the midgut activity was slight and the liver diverticulum showed a decline in enzyme activity. The well developed pronephric tubules of these embryos possessed a brilliant scarlet staining indicating very high levels of enzyme activity. This kidney activity demonstrated the precise localization afforded by the method, as the staining in the tubule cells was localized at the surface towards the lumen and no diffusion was observed. The heart at this time had a fairly high level of activity and the localizations remained as previously described. The activity of the head mesenchyme had increased markedly as had that of the axial mesenchyme, which included regions around the notochord, spinal cord and dorsal aorta (Plate II, F). Yolk staining remained about the same.

In the free-swimming larvae (st. 42) a reduced number of tissues possessing alkaline phosphatase activity were seen, and most of these demonstrated a decline in activity. The only active ectodermal derivative was the hypophysis which had lost a noticeable amount. In the brain, the telencephalon and diencephalon possessed very low levels of phosphatase activity, which was associated with the white matter (see T. torosa, Plate III, I) and the remaining regions of the brain did not demonstrate the presence of any enzyme activity. The activity of the optic cup had dropped to almost nothing, whereas, in contrast, the optic stalk was still fairly intense. The eye by this time has undergone extensive differentiation and the specific local-

izations were in two regions; one was in the region between the nerve fiber layer and retinal neuroblasts and the other was in the region between the inner and outer nuclear layers of retinal neuroblasts (see T. torosa, Plate III, I). In contrast, the optic stalk (nerve) was stained throughout (see T.torosa, Plate III, I). The endoderm, too, had undergone a great deal of histological differentiation. The pharynx had no demonstrable activity. The fore-gut remained as previously described, while ventrally and posteriorly in the transverse duodenum, a decrease in activity had occurred. Where the transverse duodenum associated with the rest of the intestine the activity increased again and became very intense posterior to the transverse duodenum (Plate I, B). The original gut cavity (the archenteron) was no longer visible and the region where it had been present no longer demonstrated AP activity. Posteriorly, where the intestine was present as simply a split in the yolk material, the activity bordering the lumen was just as intense as that of the more anterior, clearly formed, gut regions and the general yolk staining in this region was also very intense (see T. torosa, Plate I, C). The pronephric tubules possessed an intense though slightly lower activity than the stage 38 embryos while the heart activity remained the same (for tubule activity see T. torosa, Plate II, E). Both the axial and head mesenchyme had decreased to a just detectable level. The limb activity, seemingly, had increased, and proximal to the embryo a fair level of activity was present in the central limb mesenchyme. Moving distally in the limb

an increase in activity was followed by a decrease, although activity was still present as far distal as the digits. Within the limb was a central dense region, which seemed to be cartilage, around which the staining was most intense but lateral to this central region the activity dropped (see T. torosa, Plate III, G). Another region where only light staining occurred was the somite tip. At this stage, a staining of the mesenchyme associated with the fin was at a higher level than previously.

B. T. torosa:

The alkaline phosphatase histochemical picture is basically the same for T. torosa, so that the A. gracile description, in general, should suffice for the California species as well. However, slight differences, all associated with ectoderm or ectodermal derivatives, were noted.

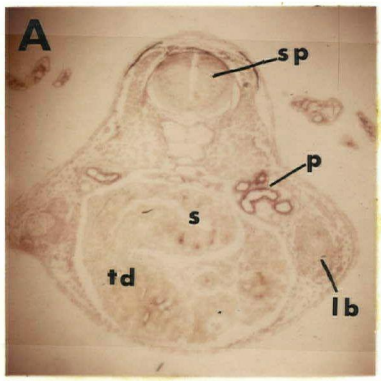
The T. torosa embryos possessed no staining at any stage in the stomodeum, proctodeum or hypophysis. Localizations of AP did occur in the otic placode, which was not seen in the A. gracile embryos, and was present in torosa in only low levels at two stages (St. 36 and 38). The embryo epithelium possessed no detectable activity until stage 42, when there appeared a general low level of staining with more intense localizations in the epithelium lying adjacent to the eye. Finally, the AP staining at any one stage, in all responding torosa tissues, was more intense than the counterpart staining in A. gracile embryos though the temporal changes, as well as the localizations and relative changes

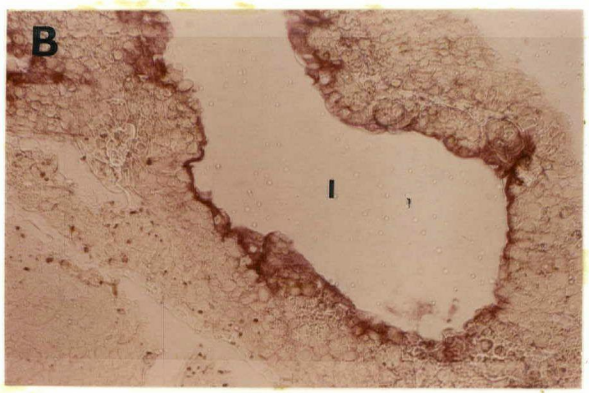
PLATE I

A. Cross-section of st. 42 T. torosa embryo revealling sites of AP activity in the pronephric tubules (p), stomach (s), transverse duodenum (td), limb bud (lb) and spinal cord (sp). (X 34).

B. Cross-section of St. 42 A. gracile free-swimming larva showing intense localization towards the intestinal lumen (l). (X160).

C. Cross-section of st. 42 T. torosa in region of the presumptive intestine (pi) where activity is intense. Activity in the spinal cord and yolk (y) is also noticeable here. (X 55).


A
sp
p
s
td
lb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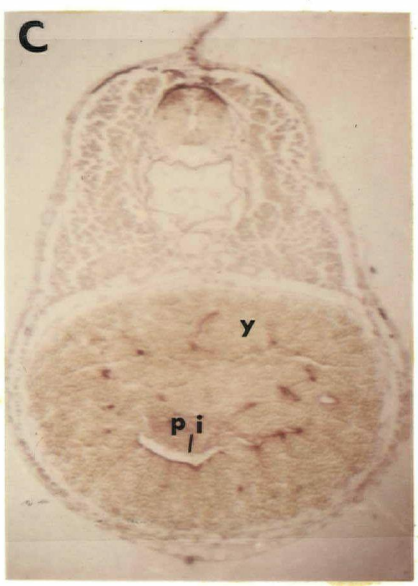
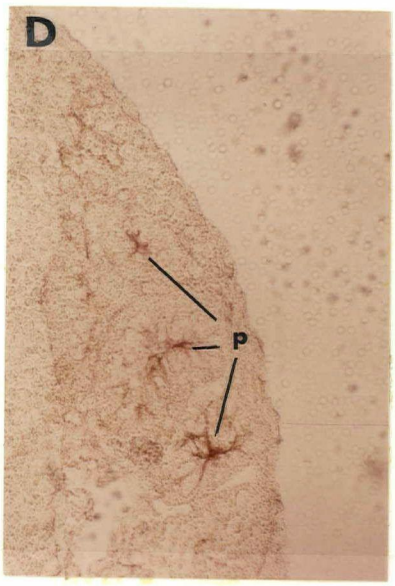

C
y
pi

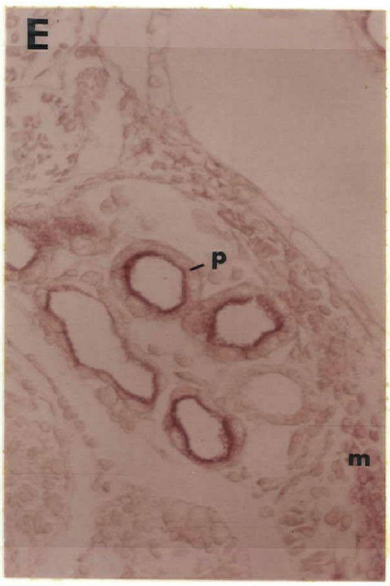
PLATE II

D. Cross-section of pronephric tubules (p) of st. 32 A. gracile embryo showing localization towards the lumen. (X 100).

E. Well developed pronephric tubules (p) of st. 42 T. torosa in cross-section, showing localization towards lumen. Activity in the adjacent mesenchyme (m) is also evident. (X 110).

F. Cross-section of st. 38 A. gracile showing activity of axial mesenchyme surrounding spinal cord (sp), notochord (n), and dorsal aorta (da). (X 100).


D
P


E
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m

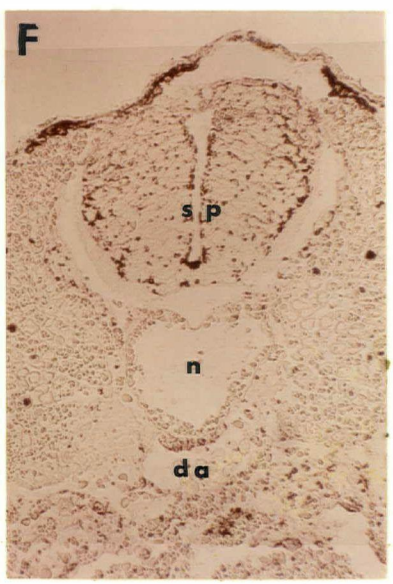

F
s
P
n
da

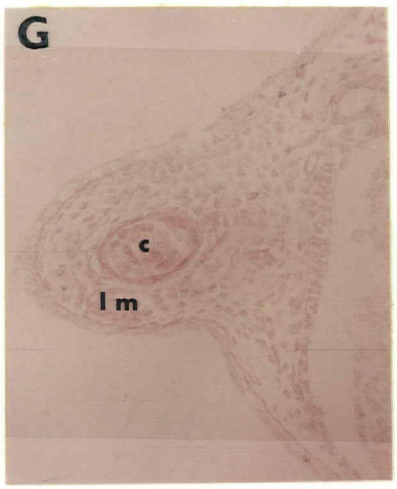
PLATE III

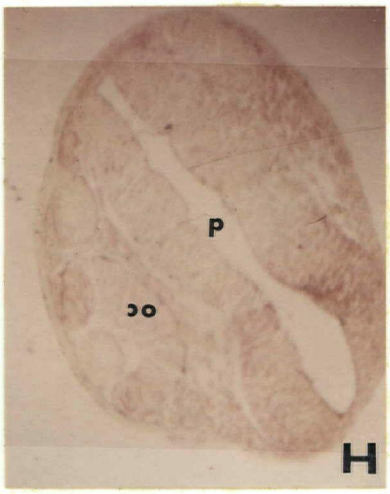
G. A cross-section in the limb region (anterior) of st. 42 T. torosa showing localization in central dense region (c) (presumed to be cartilage) and in surrounding mesenchyme (m). (X 100).

H. Cross-section revealing activity of diencephalon (d) region of brain and optic cup (oc) of an early T. torosa embryo (st. 35/36). (X 45).

I. A cross-section through the well developed eye of st. 42 T. torosa showing the localization in the optic stalk (os) and in two layers within the eye (shown by arrows; described in results); activity in the diencephalon (d) and head mesenchyme (hm) is also evident. (X 100).

J. Activity of the hypophysis of st. 38 A. gracile embryo as revealed by cross-section. (X 100).


G
c
lm


p
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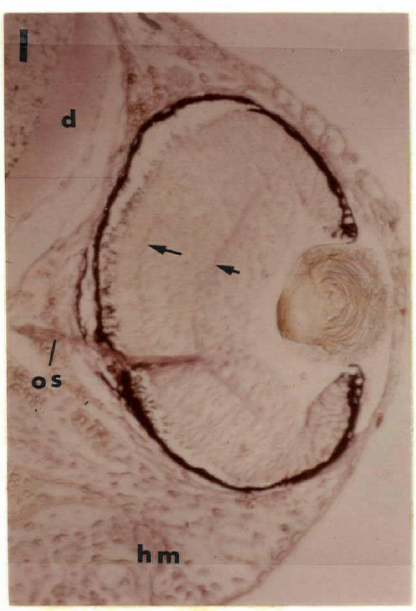
I
d
os
hm

were the same.

The histochemical results are summarized in Table IX.

TABLE IX. A table of the relative AP activity (as compared to maximal gut activity of st. 42 larvae) of embryonic tissues at different stages of development.
(data refers to both species, A.g. and T.t., except where indicated).

TISSUE	RELATIVE ACTIVITY					
	STAGE 27/28	STAGE 30/32	STAGE 33/34	STAGE 36	STAGE 38	STAGE 42
ECTODERM:						
[a]Stomodeum	+-	+	++-	+-	-	-
[a]Proctodeum	+	+	++-	++-	+-	-
[a]Hypophysis	-	-	-	++	+++	++-
[b]Epithelium	-	-	-	-	-	+-
[b]Otic placode	-	-	-	+-	+-	-
NEURECTODERM:						
Telencephalon	+-	+-	+-	+	++	+
Diencephalon	+-	+-	+	++	++	+-
Mesencephalon	-	-	+	++	++-	+-
Rhombencephalon	-	-	-	+	+	+-
Spinal cord	-	-	-	-	+-	+-
Optic cup	-	-	+	++-	++	+-
Optic stalk	-	-	+	++-	++	++
ENDODERM:						
Archenteron-						
foregut	+-	+-	+-	+-	+	++-
midgut	+-	+	+	+-	-	-
hindgut	+-	+-	+-	-	-	-
liver divert.	-	+	++-	++	++	+
Intestine	-	-	+	++-	+++	++++
MESODERM:						
Pronephros	-	+	++	+++	++++	+++
Heart	-	+-	+	++	+	+
Mesenchyme-						
head	-	+-	+	++	+++-	++-
branchial	-	+	+	++-	+++-	+
limb	-	-	-	+	++	+++-
axial	-	-	-	+-	++-	++-
fin	-	-	-	-	+-	+
YOLK:	+-	+	+	++-	++	+++

a) only detected in A. gracile
b) only detected in T. torosa

DISCUSSION

It has been stated (Moog, 1967) that an analysis of the specific enzyme activity of whole embryo or tissue homogenates requires an associated analysis of the enzyme activity at the histochemical level. It is evident in the present investigation that, although the general trend of specific AP activity of homogenates of developing salamanders is towards a higher level as development proceeds, this does not indicate a general tissue increase but, instead, is the cumulative expression of a multitude of regularly increasing and decreasing enzyme levels within individual tissues. This is especially evident in these salamander embryos after stage 38, when the total number of tissues contributing to the measured AP activity begins to drop rapidly while the actual homogenate activity increases prodigiously. The major source of the increased homogenate activity is the differentiating endoderm (gut).

Lovtrup (1953) suggested, since the intestinal mucosa is one of the richest sources of AP, wherein it may play a role in transport (Danielli, 1952; Matthiessen, 1966; Tosteson *et al*, 1961) and fat absorption (Warnock, 1968), that the increase or "break" noted at this time in specific activity of Urodele embryo homogenates (*A. punctatum*, Lovtrup, 1953; *A. mexicanum*, Krugelis, 1951) was due to endodermal differentiation. More recently Harris (1967), in a study of the morphogenesis of the stomach and intestine of *A. maculatum* (*A. punctatum*), revealed that about the time of the described AP increase, the gut begins its differentiation,

but, no histochemical data were made available. The present work shows that the assumption of Lovtrup (1953) was indeed correct, and further reveals that the enzyme activity, as expected, is low in the stomach but becomes very intense as one progresses ventrally and posteriorly past the transverse duodenum (liver diverticulum) and into the intestine of free-swimming larvae. However important the gut increase may be, the contribution of other tissues should not be ignored since certain of them show their greatest activity at this time. The best example of this is found in the limb components but localization in the yolk was also intense indicating, most likely, the increased utilization of this material by the free-swimming embryos. There is evidence that AP is involved in the mobilization of these yolk components that serve as a source of raw materials and free-energy for embryonic development (Williams, 1967). The A. gracile gut develops more slowly than that of A. maculatum, probably as a result of its higher yolk content which could undoubtedly retard this morphogenesis. This species variation in embryonal yolk content may explain the slightly earlier sharp increase (after st. 36) and the final activity decrease in the T. torosa specific activity curves. The torosa embryos possess less yolk than gracile and presumably gut differentiation would be completed earlier than in A. gracile embryos, an idea which was supported by observation of the histochemical sections. Further, the data could be considered to support the theory of non-archenteric gut formation proposed by

Balinsky (1947) and clarified by Harris (1967), since the presumptive intestine passage through the yolk was sharply demarcated by intense staining (indicating a high level of AP activity), a characteristic shared by both the differentiating and the mature gut, but not by the archenteron.

Piatka and Gibley (1967), using the Gomori technique, have studied the histochemical localization of alkaline phosphatase of the developing pronephros of the frog and Osawa (1952) described similar observations in two other amphibian species, the Urodele Hynobius tokyoensis and the Anuran Rhacophorus schelegleii. The present results correlate well with both of those observations of pronephric AP development. That the enzyme is concentrated at the luminal border of the tubules infers a role in the reabsorption and possible secretory function of the proximal tubules (Piatka and Gibley, 1967; Danielli, 1952). Specific localizations of high phosphatase activity in the limb mesenchyme during cartilage deposition, as seen in the present study, had been described previously in A. punctatum by Karzmer and Berg (1951), both during normal ontogeny and during regeneration. The enzyme can be implicated in the process of calcification (Karzmer and Berg, 1951; Matthiessen, 1966; Stadtman, 1961; Schmidt, 1961; Shah and Chakko, 1967) in which it seems to act by the production of high levels of phosphate ions facilitating the formation of calcium phosphate. This chondrogenic function would also explain the high levels of phosphatase present in the head mesenchyme, where the cranium will form and in the axial mesenchyme where the axial

skeleton will form. Moog and Wenger (1952) discovered histochemically that mucopolysaccharide is generally present in large amounts where high levels of AP are detected and the association of AP activity with cartilage in regions of future bone formation has interesting connotations in view of the presence of mucopolysaccharide in this connective tissue. Later work (Moog and Grey, 1966) revealed that mucopolysaccharide forms an integral part of the duodenal AP molecules and may play a significant role, via this moiety, in morphogenesis of the villi. This association might also explain the inability of McWhinnie and Saunders (1966) to obtain a solution of phosphatase of bone, even with butanol, if it is assumed that the carbohydrate constituent makes an undissociable bonding with the limb bone or cartilage. The presence of AP in the epidermis seems to vary with the species used. Shah and Chakko (1967) felt that where it is present it functions in the formation of fibrous proteins and in the passage of metabolites across the cell membrane.

Of importance to this study is the hypothesis that this enzyme plays a role in the processes of differentiation (Moog, 1944, 1952; Karzmer and Berg, 1951). The hypothesis is a product of evidence, at once plentiful and circumstantial, that AP is invariably associated with tissues during the early stages of their differentiation, but decreases in amount or is lost entirely on morphological or functional differentiation of these tissues. This idea would appear to have relevance in explaining the increase and subsequent decrease in levels of phosphatases in the hypophysis, the

various regions of the brain, the transverse duodenum, the heart, and the areas of the limb not involved in chondrogenesis. It also could explain the precocious AP increase in tissues where it plays a functional role in the mature tissue, such as transport in the kidney (Piatka and Gibley, 1967; Fortak et al, 1962). It is evident, in the present investigation, that AP does not show an increase and subsequent decrease in all tissues. In certain tissues, notably, the ectodermal sense placodes, AP shows no increasing activity prior to morphological differentiation. Indeed, many of these tissues possess no histochemically detectable amounts of AP so that the suggestion of Moog (1944 and 1952) should be modified. AP seems to be involved in certain biochemical events which precede, and might be essential for, the differentiation process in some but not all tissues. The cautiousness of such a statement is necessitated by the absence of any experimental data on inhibition of these enzymes in developing systems. It seems from the present work, that such data may not be immediately forthcoming, considering the nature and quantity of the inhibitors necessary for extensive inhibiton of AP activity.

In view of the postulated role for AP in development, taken with the increasing interest in multimolecular forms of enzymes, it is interesting that no attempt has been made to determine if the "differentiation-phosphatases" are unique forms of AP, or whether their importance lies in a type of dose-response mechanism as postulated by Karzmer and Berg (1952) and called a "phosphatase-rich transition phase".

The activity ratio data revealed, for both species, two peaks of PNPP-preference or high ratio. It should be understood that, as used in this context, "PNPP-prefering" and "BGP-prefering" enzymes are relative terms which serve only to indicate changes in substrate preference, since the enzymes have not been purified. The first peak occurred during the period of first autonomous muscle movements and the second occurred just prior to the free-swimming stage. Up to about stage 31 (A.g.) or 32 (T.t.), very little histological differentiation is obvious, the neurectoderm of both species shows some differentiation of the various major brain regions and most of these possess a low level of AP activity. This observation suggests that most phosphatases present at this time are those involved in the biochemical processes of differentiation and thus these enzymes can now be considered as PNPP-prefering enzymes. After stages 31 and 32, when a decrease in ratio is noted, the pronephric tubules are involved in their histogenesis which has associated with it an enzymic differentiation of AP revealed as an increase in activity. Since the tubules are histochemically the most reactive tissues at this stage and are relatively extensive, they are most likely the source of the AP enzymes with the BGP-preference. By stages 35 (A.g.) and 36 (T.t.) numerous tissues are contributing to the level of AP activity. Since most of these will show decreasing activity after stage 38 (A.g.) or 39 (T.t.), in association with increasing differentiation, we assume, as did Moog (1944 and 1952), that these enzymes are involved in the differentiation process.

The ratios observed for this period indicate these are PNPP-prefering enzymes. It has been observed that, after stages 38 and 39, the differentiation of the alimentary tract with its high AP levels becomes the dominant enzyme contributor. The ratio decrease noted at that time then must be a result of this differentiation and further indicates that the "mature" or differentiated enzymes are BGP-prefering enzymes.

It is evident from the foregoing that, on the basis of the observations made in this investigation, the AP enzymes affiliated with the differentiation process may be PNPP-prefering enzymes while those functioning in the gut and kidney are considered to have a BGP-preference. This view is supported by Moog's (1966) work on the AP of the mouse duodenum, in which the APs of this region have a BGP-preference (as compared to PNPP) at birth. No other quantitative work with both substrates which would further clarify this idea has come to our attention.

Since the acrylamide gel electropherograms revealed two molecular forms of alkaline phosphatase that were present throughout development, it is clear that the role of AP in differentiation is not a function of new AP molecular species. Coupling these data with the specific activity data it is evident that a simple developmental sequence in which band 1 decreases as band 2 increases is not valid. What is more likely is that the ratios of band 1 to 2 do change, but that such a change is masked by the proximity of the bands to each other. Thus the activity level, as well as the ratio change, may be important in the differentiation

process.

The literature on multimolecular forms of AP indicates that a general theme for the formation of new molecular forms, regardless of species or tissue source of the enzyme, exists. New molecular forms of alkaline phosphatase that appear during development can almost invariably be considered as products of modification of other AP species. Moog (1966) has indicated that the duodenal AP is synthesized in a moderately active (mice) or inactive (chick) form which at specific times in later development become activated by, in theory, the removal of a specific inhibitor which then allows the conversion of one molecular form to two other more active forms. In Drosophila (Schneiderman, 1967; Schneiderman et al, 1966), the development from larvae to pupae has associated with it a change in the types of AP molecules present in total homogenates which involves, in one case, the conversion of one molecular form to another. In this instance however, the conversion involves a tailoring of skin phosphatases by trypsin or "trypsin-like enzymes" after the entrapment of skin cells in the yellow body of mature larvae. Schlesinger and Anderson (1968) report that induction of AP in cultures of E. coli, by lowering of the external inorganic phosphate concentration, produces a high level of AP per cell which was due to the conversion of a slow migrating (in acrylamide gel) molecular component to a faster migrating form. Although no experimental evidence on this point is available for the salamander species studied it seems likely that AP development may include the formation of

multimolecular forms which, in many instances, would occur by a modification of enzyme species previously present or, more simply, by molecular conversion reactions or molecular tailoring. Since in the known cases these conversions involve the formation of a faster migrating form in starch or acrylamide gel electrophoresis (Schneiderman, 1967; Schlesinger and Anderson, 1968), it follows that the salamander situation observed in the present investigation could involve the formation initially of band one (1) which is then converted to band two (2). The rate of the conversion reaction then might determine the relative amounts of the two enzymes (the ratio determining factor) and thereby play a role in differentiation.

SUMMARY

1. The ontogeny of alkaline phosphatase (AP) in developing embryos of two species of Urodele amphibian, A. gracile and T. torosa, has been studied using biochemical assays (specific activity determinations, gel electrophoresis) and histochemical methods.
2. AP is present in embryo homogenates from gastrulation to free-swimming larvae, over which time it increases 150-fold (with PNPP) or 35-fold (with BGP) in A. gracile and 60-fold (with PNPP) or 25-fold (with BGP) in T. torosa. Plotting the specific activity data as a ratio of activity on the two different substrates (PNPP/BGP), two peaks of high ratio are seen during this period of development.
3. The AP level in the homogenates was found, by acrylamide gel electrophoresis, to be a product of two molecular forms.
4. The histological development of AP was related to the biochemical data and to proposed functions of the enzyme.
5. It was evident that the differentiation function of AP does not seem to be a product of new molecular forms. A correlation between substrate specificities and function was proposed which may allow a closer scrutiny of the role of AP in the process of differentiation.

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