CHANGES IN SERUM PROTEINS ASSOCIATED WITH METAMORPHOSIS IN SALAMANDERS OF THE FAMILY AMBYSTOMATIDAE

RONALD A. NUSSBAUM

Museum of Zoology and Department of Zoology, University of Michigan,
Ann Arbor, MI 48104, U.S.A.

Abstract—1. The effect of metamorphosis on the serum proteins of three genera and five species of ambystomatid salamanders was examined by electrophoresis.

2. Four of the five species showed a significant increase in the fastest fraction upon metamorphosis.

3. Three of the five species showed a significant metamorphic increase in the second fastest fraction.

4. Four of the five exhibited a significant metamorphic increase in total protein.

5. The results support earlier hypotheses that a metamorphic increase in the fast components of amphibian blood serum serves to maintain blood volume in the desiccating, terrestrial environment of the transformed organism.

INTRODUCTION

ALMOST every homeostatic mechanism which has been adequately studied is seen to adjust during amphibian metamorphosis in such a manner as to enable the organsim to cope with the new physiological problems attendant with life on land. The morphological and physiological changes incurred at metamorphosis are accompanied with, and indeed most if not all are driven by, biochemical changes. In regard to biochemical changes, Wald (1945, 1952) and Crescitelli (1958) demonstrated metamorphic changes in visual pigments in frogs and salamanders; Munro (1953) and later authors found a switch from ammonotelism to ureotelism in amphibians, with correlative alterations in enzyme systems; and McCutcheon (1936) and Riggs (1951) described hemoglobin metamorphosis in frogs. These authors and others have argued convincingly for the specific adaptive nature of each of these biochemical changes. Wald (1960, 1961) has admirably summarized the literature.

Metamophosis of serum proteins in anurans was discovered by Frieden *et al.* (1957), and subsequent supportive results were summarized by Herner & Frieden (1960) and Frieden (1961). Frieden and his associates found that concomitant with metamorphosis in *Rana catesbeiana*, *R. hecksheri*, *R. grylio* and *Xenopus laevis* there was: (1) a redistribution of the globulin fractions including either appearance of a fraction(s) for the first time, increase or decrease in quantity of certain fractions, or change in electrophoretic mobility; (2) no change in the mobility of albumin, the fastest fraction; (3) an increase in the total protein concentration; and (4) most notably, an increase in the amount of albumin relative to total globulin (A/G ratio).

In 1956, Manelli found a metamorphic increase in the total serum protein concentration of the axolotl, *Ambystoma mexicanum*. As with frogs the fastest fraction, which Manelli (1958) equated with human albumin, increased the greatest amount at metamorphosis.

Manelli showed that neither sex nor maturity affected the metamorphic relationships of the proteins. Hahn (1962) demonstrated identical results for the tiger salamander, *Ambystoma tigrinum*, but suggested the fastest fraction was α-globulin rather than albumin. Hahn thought that if the fraction was α-globulin it served the same physiological function as the albumin of frogs, and he invoked the same explanation as to the adaptive significance of the metamorphic increase proposed by Frieden *et al.* (1957) for frogs.

It is well known that one important function of serum proteins is to maintain osmotic equilibrium between blood and tissue fluids (Bell *et al.*, 1965). An increase in the total protein concentration at metamorphosis serves to increase the osmotic pressure of the blood and hence its water-retaining capacity, an important adaptive switch considering the transition from the aquatic environment of the larva to the drying, terrestrial habitat of the transformed frog or salamander. As pointed out by Frieden (1961) and others, albumin, with its lower molecular weight, exerts two to three times the osmotic pressure per unit weight than the globulins; and it is the albumin fraction (or at least the fastest fraction) which increases proportionately the greatest amount during metamorphosis. At the pH of blood, albumin is highly charged and therefore has a great ion-binding capacity. An increase in the ion-binding capacity per unit volume of blood at metamorphosis fulfills the greater transport needs associated with metabolism and excretion in the terrestrial environment (Frieden, 1961).

There is a phylogenetic counterpart to these ontogenetic changes. Permanently aquatic vertebrates such as fish have relatively low serum protein concentrations, especially in regard to the faster fractions, compared to fully terrestrial vertebrates (Prosser, 1950). The correlation between ontogeny and phylogeny for this and other biochemical changes associated with metamorphosis has been used to support

arguments concerning the proposed adaptive signifi-
cance of these changes (Wald, 1960).

The two species of *Ambystoma* studied by Manelli
(1958) and Hahn (1962) are the only salamanders ana-
lyzed to date. The purpose of this study was to exa-
mine the metamorphic pattern of serum proteins in
three genera of ambystomatid salamanders represent-
ing a wide variety of ecological types with different
life history patterns in order to test the generality
of the metamorphic pattern, and to inquire further
into the validity of the proposed adaptive value of
the observed phenomena.

MATERIALS AND METHODS

Five species of ambystomatid salamanders from the
Pacific Northwest were studied. Collecting sites were:
Ambystoma macrodactylum, Summit Spring and Bear
Spring, Cascade Mountains, Jefferson Co., Oregon;
Ambystoma gracile, Willamette Valley, Polk and Benton
Cos., Oregon; *Dicamptodon ensatus*, Cowlitz Co., Wash-
ington and Benewah and Valley Cos., Idaho; *Dicamptodon
copei*, Cowlitz and Skamania Cos., Washington; *Rhyacotri-
ton olympicus*, Columbia River Gorge, Multnomah Co.,
Oregon. All specimens are deposited in the Museum of
Zoology, University of Michigan.

Samples of larval and transformed *A. gracile*, *A. macro-
dactylum*, and *R. olympicus* were collected and tested with
no previous treatment. Larval and transformed *D. ensatus*
were also collected and tested with no treatment, but an
additional sample of larvae was treated with thyroxine in
order to observe the sequence of changes in serum protein
patterns relative to the metamorphic process. This also
allowed comparison between transformed animals from the
field and those obtained by forced metamorphosis. *D. copei*
apparently do not normally metamorphose (Nussbaum,
1970). A sample of larvae of this latter species was treated
with thyroxine to force metamorphosis as far as possible.
"Transformed" *D. copei* were used only after 12 continuous
months of thyroxine treatment.

Blood was drawn from the heart of anesthetized animals
into capillary tubes, allowed to clot, and the serum separ-
ated by centrifugation. Hemolytic samples were discarded.
Samples were run within 1 hr of the time blood was drawn.
Aliquots (0·25 μl) were placed on biologically inert cellulose
acetate membranes, and the proteins separated with a
Beckman Microzone Cell, Model R-101. The proteins were
allowed to migrate for 20 min at 250 V, with a barbital
buffer solution of pH 8·6 and an ionic strength of 0·075.
Migration was stopped by placing the membrane in a fixi-
tive dye (0·2% ponceau-S, 3·0% trichloroacetic acid, 3·0%
sulfosalicylic acid) for 8 min. Excess dye was rinsed away
with 5% acetic acid. The membranes were then dehydrated
for 1 min in 95% ethanol, transferred to a clearing solution
of 25% glacial acetic acid and 75% ethanol (95%) for 1 min.
The dried membrane was placed in a clear envelope, and
scanned with a Microzone densitometer. Beckman Model
R-110. The densitometer is equipped with an automatic
integrator, and the relative amounts of the protein frac-
tions can easily be determined through its use. Migration
rates were compared to human albumin.

Samples were grouped by species, sex, stage of sexual
maturity, and locality. Thyroxine-treated *D. ensatus* were
compared to those already metamorphosed when collected.
Standard statistical tests were made which showed no
effects of sex, thyroxine treatment, or state of maturity.
so the samples were combined to compare only larval
versus transformed individuals within and between species.
The two samples of *D. ensatus* from Idaho and Washington
were different enough to warrant separate statistical treat-
ment.

RESULTS

Five protein fractions were resolved, except that the
sample of *D. ensatus* from Idaho had a sixth fraction
labeled 3' in Fig. 4. The homologies of these proteins
with those of higher vertebrates are not certain. They
were therefore numbered in order, from the fastest
to slowest fractions. Fraction 1 is comparable to
human albumin in position; $R_f = 0.93$ for *Dicampto-
don* and *Rhyacotriton*, and $R_f = 0.87$ for *Ambystoma*.
Fraction 2 is in the same relative position as α_1-glo-
bulin, fraction 3 is comparable to α_2-globulin, fraction
4 to β-globulin, and 5 to γ-globulin.

The electrophoretic patterns for larval and trans-
formed *A. macrodactylum* are shown in Fig. 1. It is
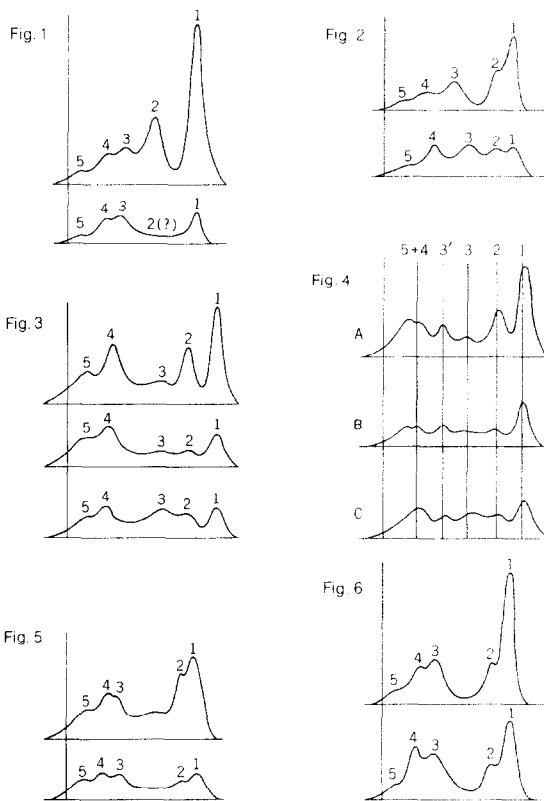evident that the relative amount of fraction 1 in-

Fig. 1. Electrophorograms of larval (lower) and trans-
formed (upper) *Ambystoma macrodactylum*. Vertical line
marks point of application. Fractions 1 and 2 increase tre-
mendously concurrent with metamorphosis.

Fig. 2. Electrophorograms of larval (lower) and trans-
formed (upper) *Ambystoma gracile*.

Fig. 3. Electrophorograms of immature larva (lower), neo-
tenic larva (middle), and immature but transformed (upper)
Dicamptodon ensatus from Washington. This figure shows
that the pattern of increase in fractions 1 and 2 is not
dependent on sexual maturity.

Fig. 4. Electrophorograms of larval (lower) and trans-
formed (upper) *Dicamptodon ensatus* from Idaho. The mid-
dle pattern is for an individual in the process of metamor-
phosing. Fraction 3' is not found in *D. ensatus* from Wash-
ington (Fig. 3).

Fig. 5. Electrophorograms of larval (lower) and trans-
formed (upper) *Dicamptodon copei* from Washington.

Fig. 6. Electrophorograms of larval (lower) and trans-
formed (upper) *Rhyacotriton olympicus*.

Table 1. Proportional changes in serum proteins associated with metamorphosis. L = Larval; T = transformed; W = Washington population; I = Idaho population; A/G = albumin/total globulin; ⍺-G/G' = alpha-globulin/total remaining globulin; A +⍺-G/G' = albumin + alpha-globulin/remaining globulin. Means and standard errors in parenthesis are given

Species	A/G	⍺-G/G'	A + ⍺-G/G'	Total Protein	n
A. macrodactylum (L)	.576(.024)	.000(−)	.576(.024)	61.0(2.47)	16
A. macrodactylum (T)	.883(.032)	.216(.011)	2.862(.115)	220.0(9.22)	12
A. gracile (L)	.191(.020)	.110(.011)	.523(.043)	126.7(10.20)	10
A. gracile (T)	.505(.070)	.142(.021)	1.139(.072)	96.9(9.51)	8
D. ensatus (W,L)	.264(.015)	.169(.014)	.551(.040)	133.6(5.67)	9
D. ensatus (W,T)	.364(.011)	.168(.014)	.697(.033)	150.3(10.37)	8
D. ensatus (I,L)	.271(.026)	.178(.009)	.575(.046)	116.2(5.65)	5
D. ensatus (I.T)	.462(.025)	.226(.004)	1.003(.045)	162.2(10.88)	6
D. copei (L)	.339(.021)	.095(.005)	.683(.041)	100.2(5.20)	11
D. copei (T)	.397(.022)	.204(.017)	1.388(.153)	145.8(7.61)	6
R. olympicus (L)	.428(.016)	.103(.006)	.850(.047)	154.8(7.30)	8
R. olympicus (T)	.621(.025)	.108(.007)	1.181(.049)	187.1(7.32)	17

creases significantly at metamorphosis. Thus the A/G (albumin/globulin) ratio increases from 0·576 to 0·883 (Table 1). Just as striking is the metamorphic change in fraction 2. This protein was not detectable in larvae, but amounts to 21·6% of the globulin component of transformed individuals. The total protein concentration was 3·61 times higher in transformed, as compared to larval individuals.

There was also a significant metamorphic increase in fraction 1 for *A. gracile*, but fraction 2 did not increase significantly (Fig. 2, Table 1). Fraction 2 is present in larval *A. gracile*, whereas it was not in *A. macrodactylum*. There was a decrease in total protein concomitant with metamorphosis (Table 1). Compared to other data for frogs (Frieden, 1961) and salamanders (Manelli, 1958; Hahn, 1962; this paper) this result seems anomalous, and cannot be explained completely. Samples of serum of transformed *A. gracile* were less viscous than any other samples. Since the applicator works on the principle of surface tension, a less viscous serum would tend to be applied to the membrane in smaller quantities, and this sampling error could in part account for the anomaly.

D. ensatus from Washington (Fig. 3) showed a significant, metamorphic increase in the relative amount of fraction 1, but not of fraction 2 (Table 1). A subsample of five, large, neotenic larvae from Washington showed results indistinguishable from those of smaller, immature larvae. *D. ensatus* from Idaho (Fig. 4) had an even greater increase in the A/G ratio associated with metamorphosis, and there was a significant increase in fraction 2 (α-G/G'). *D. ensatus* from Idaho showed a significant increase in total protein, but those from Washington did not.

The metamorphic increase in the ratio A/G for *D. copei* (Fig. 5) is not significant at $P < 0.05$. This is the only species examined for which this is true. As noted above, this species does not normally transform. Morphological changes do not go to completion by administration of thyroxine (Nussbaum, 1970), and perhaps the present result indicates a similarly incomplete biochemical metamorphosis. There

is a significant increase in the relative amount of fraction 2 after thyroxine treatment, as well as a significant increase in total protein (Fig. 5, Table 1).

There is a significant increase in the relative amount of fraction 1 upon transformation of *R. olympicus* (Fig. 6, Table 1), but not of fraction 2. There is likewise a significant increase in total protein.

DISCUSSION

The proposal by Frieden *et al.* (1957) that the increase in albumin attendant with metamorphosis is designed by natural selection to meet the osmotic needs of the organism in the terrestrial environment, is by no means proven. The hypothesis is logical and attractive, but so far is supported only by the fact that there is an increase in the fast fraction of a few species of frogs and salamanders associated with transformation. It is proposed now to examine the evidence more closely with special attention given to the live histories and habitats of the species involved.

It was shown above that in some species of salamanders there is a metamorphic increase in the relative amount of fraction 2 as well as fraction 1. No reason can be thought of why this second fastest fraction should not be given the same consideration as potentially important in osmotic control, and consideration will be given to both fractions from this point-of-view.

Since the metamorphic change is not all-or-none, it is possible to search for patterns, across and within species, in the degree of change in relation to the degree of independence of water of the terrestrial stage. Hence if the theory is correct, the most terrestrial species should show the greatest metamorphic increase in fractions 1, 2 or both. Similar arguments can be advanced for populations within species.

A. macrodactylum is the most widespread geographically, and occupies a greater diversity of habitats than any of the species studied. It breeds in forested, montane situations as well as in the xeric, sagebrush plains of the Columbia Plateau. Neotenic

populations are unknown. Of the six species studied, the terrestrial adults of *A. macrodactylum* are the most independent of water, except of course during the breeding season. Fractions 1 and 2 have been combined in relation to the remaining globulins (A + α G/G') for comparison of the metamorphic pattern across species. During metamorphosis of *A. macrodactylum* this ratio changes from 0·576 to 2·862, an increase of 4·97 times.

A. gracile is restricted in distribution to the humid, Coastal and Cascade forests of the Pacific Coast of western North America. Permanently aquatic, neotenic populations are known. Terrestrial individuals are usually found near permanent water. The distribution, life history, and habitat of terrestrial individuals suggest this species has a greater moisture dependency than *A. macrodactylum*. The ratio A + α G/G' for *A. gracile* increases metamorphically from 0·523 to 1·139 or 2·18 times.

D. ensatus is similar to *A. gracile* in that both species are restricted primarily to moist, montane or coastal regions. *D. ensatus* from coastal California, Oregon and Washington are similar to sympatric *A. gracile* in that both species have neotenic or partially neotenic populations. However, *D. ensatus* from the inland, more xeric forests of the Rocky Mountains of Idaho are not known to have neotenic populations (Nussbaum, unpubl.); and terrestrial individuals often occur in relatively dry circumstances compared to the coastal situation. As can be seen from Table 1, *D. ensatus* from Idaho show a greater metamorphic response in the ratio A + αG/G' than do those from Washington (1·74 as opposed to 1·26). Since *D. copei* do not normally metamorphose, they will not be considered in this part of the discussion.

R. olympicus is restricted to the humid, coastal forests of the Pacific Northwest. Transformed individuals are seldom found more than a meter from a permanent seep or stream, and usually they are found in the splash or spray zone, their bodies in contact with the water film. There are no neotenes. In this salamander the ratio A + α G/G' increases from 0·850 to 1·181 during metamorphosis for an increase of 1·39 times.

It was contended that *A. macrodactylum* and *R. olympicus* occupy opposite ends of a dry–wet, ecological spectrum. Terrestrial individuals of both species can be found on saturated substrate, especially during the breeding season or after rains. However, the critical factor is the extremes of drought to which individuals may be exposed. Outside the breeding season, *A. macrodactylum* are often found on relatively dry substrate, whereas *R. olympicus* are always collected in saturated microhabitats. These field observations are supported by laboratory studies. Ray (1958) compared the dehydration–hydration rate of *R. olympicus* to more terrestrial plethodontid salamanders and found that *R. olympicus*: (1) exhibits great irritation on contact with dry air; (2) lacks any protective behavioral mechanism to prevent desiccation (such as reducing body surface area by coiling); and (3) has a relatively fast dehydration rate. *R. olympicus* also has a relatively low tolerance of water loss (unpubl. data supplied by Dr. Stanley Anderson, Kenyon College). Alvarado (1967) noticed that both *A. macrodactylum* and *A. tigrinum* tended to form clumps if

exposed to dehydrating conditions in the laboratory. His experiments showed that individuals in clumps dehydrated much more slowly than single individuals, and he hypothesized that aggregative behavior would have survival value in desiccating environments. Field observations taken support Alvarado's ideas in that several aestival aggregations of *A. macrodactylum* of up to 33 individuals were found. This discussion has been belabored because acceptance of these arguments in conjunction with the relatively greater metamorphic increase in A + α G/G' for *A. macrodactylum* (4·97 times) compared to *R. olympicus* (1·39 times) greatly strengthens the hypothesis of Frieden *et al.* (1957) concerning the adaptive significance of this phenomenon.

Bertini & Cei (1960) reported that transformed toads, *Bufo arenarum*, from two arid localities in South America had significantly higher amounts of serum albumin than did individuals from three separate humid localities. They suggested the higher albumin concentration was an adaptation for resistance to ambient aridity, but gave no reason for this assertion and did not seem to be aware of Frieden's hypothesis. They also showed a geographic increase in the second fastest protein (their G') corresponding to the more arid localities, but did not discuss this result.

In summary, there is both interspecific (*A. macrodactylum* and *R. olympicus*) and intraspecific (*D. ensatus* and *B. arenarum*) evidence that higher concentrations of albumin (or at least the fast fraction) are characteristic of transformed individuals of populations adapted to more xeric conditions. There are several groups of amphibians perfect for further testing this generality, the best of which is perhaps the desmognathine salamanders which form a linear series from aquatic- to terrestrial-adapted species (Hairston, 1949, 1973).

Another approach is to examine species which metamorphose but nevertheless remain aquatic. If resistance to desiccation is the main adaptive advantage to increased albumin or α-globulin then there should be no reason for these fractions to increase if transformation is not accompanied with transition to land. Rall *et al.* (1961) demonstrated that a new, fast component appeared in the serum proteins of the sea lamprey, *Petromyzon marinus* upon transformation. The authors thought the new component was α-globulin, but did not speculate on the functional significance of their finding. Since lampreys are aquatic throughout their life cycle, it would seem that the metamorphic appearance (increase?) of a fast fraction must serve some function other than to maintain blood bolume in desiccating environments. In the case of *P. marinus*, however, it could be argued that since the larvae live in fresh water and the adults are historically inhabitants of the sea (freshwater, landlocked populations are known), then the increased concentration of the fast fraction may serve the same function; i.e. prevention of dehydration (maintenance of blood volume), but in this case imposed by a hypertonic, aquatic environment rather than a drying atmosphere.

Herner & Frieden (1960) studied the metamorphic pattern of the pipid frog, *Xenopus laevis*, a species well known by experimental biologists to remain

aquatic throughout the life cycle. Yet *X. laevis* exhibits a marked increase in the A/G ratio upon metamorphosis. The authors showed that the pattern for *X. laevis* differed from *Rana* in that both the A/G ratio and total protein concentration increased steadily as the transformed froglet grew; whereas the metamorphic change in the more terrestrial *Rana* was more abrupt and changed little after metamorphosis. These observations are consistent with the hypothesis in that *Rana* froglets are immediately confronted with a dehydrating environment whereas *X. laevis* froglets are not. The fact that there is a gradual increase in the A/G ratio of *X. laevis* is not inconsistent if one considers this frog in its natural habitat. *X. laevis* occurs in the temporary pond or "vlei" habitats of South Africa. Here the species is often confronted with desiccation during droughts when their pools dry up. The tadpoles always succumb when this happens, but the transformed frogs often survive until the next rain by aestivating in the soil of the dried pond bottom (Rose, 1929).

The salamander *Cryptobranchus alleghaniensis* transforms from larvae to permanently aquatic adults, apparently with no concomitant change in the serum proteins (Stone, 1971; cited by Nickerson & Mays, 1973).

The metamorphic increase in A/G, α-G/G', and total serum protein in amphibians and other vertebrates likely has multiple functions. Previous evidence and data presented here suggest that a major function is related to water conservation and maintenance of blood volume in desiccating environments. The relative importance of this particular function could be measured with a species such as the newt, *Notophthalmus viridescens* which undergoes a "secondary metamorphosis" and returns to a permanently aquatic existence. The terrestrial eft stage should have higher A/G and total protein values than both the aquatic larvae and the aquatic adults. The differences between the larvae and aquatic adults, if there are differences, should reflect adaptive changes unrelated to osmoregulatory problems imposed by the environment.

Whipple (1956) suggested that biosynthesis of albumin may be regulated in a homeostatic manner by the osmotic needs of the animal. This then should be measurable in some species of newts of the genera *Taricha* and *Triturus* in which after metamorphosis and growth, individuals alternate between periods of "aquatic" and "terrestrial" morphology in relation to the breeding cycle.

REFERENCES

ALVARADO R. H. (1967) The significance of grouping on water conservation in *Ambystoma. Copeia* 1967(3), 667–668.

BELL G. H., DAVIDSON J. N. & SCARBOROUGH H. (1965) *Textbook of Physiology and Biochemistry* i-vii + 1140 pp. Williams & Wilkins. Baltimore.

BERTINI F. & CEI J. M. (1960) Observaciones electroforeticas en seroproteinas de poblaciones Argentinas de *Bufo arenarum. Rev. Soc. argent. Biol.* 36, 355–362.

CRESCITELLI F. (1958) The natural history of visual pigments. *Ann. N.Y. Acad. Sci.* 74, 230.

FRIEDEN E. (1961) Biochemical adaptation and anuran metamorphosis. *Am. Zoologist* 1, 115–149.

FRIEDEN E., HERNER A. E., FISH L. & LEWIS E. J. C. (1957) Changes in serum proteins in amphibian metamorphosis. *Science, N.Y.* 126, 559–560.

HAHN W. E. (1962) Serum protein and erythrocyte changes during metamorphosis in paedogenic *Ambystoma tigrinum mavortium. Comp. Biochem. Physiol.* 7, 55–61.

HAIRSTON N. G. (1949) The local distribution and ecology of the plethodontid salamanders of the Southern Appalachians. *Ecol. Monogr.* 19, 47–73.

HAIRSTON N. G. (1973) Ecology, selection, and systematics. *Breviora* 414, 1–21.

HERNER A. E. & FRIEDEN E. (1960) Biochemistry of anuran metamorphosis. VII. changes in serum proteins during spontaneous and induced metamorphosis *J. biol. Chem.* 235(10), 2845–2851.

MANELLI H. (1958) Changes of the electrophoretic serum pattern of *Amblystoma* according to age, sex, and metamorphosis. *Riv. Biol.* 50, 15–26. (Italian)

MCCUTCHEON F. H. (1936) Hemoglobin function during the life history of the bullfrog. *J. cell. comp. Physiol.* 8. 63–81.

MUNRO A. F. (1953) The ammonia and urea excretion of different species of amphibians during their development and metamorphosis. *Biochem. J.* 54, 29–36.

NICKERSON M. A. & MAYS C. E. (1973) The hellbenders. Milwaukee Public Museum, *Publ. Biol. Geol.* 1, 1–106.

NUSSBAUM R. A. (1970) *Dicamptodon copei,* n. sp., from the Pacific Northwest, U.S.A. (Amphibia: Caudata: Ambystomatidae). *Copeia* 1970. 506–514.

PROSSER C. L. (ED.). (1950) *Comparative Animal Physiology.* W. B. Saunders. Philadelphia.

RALL D. P., SCHWAB R. & ZUBROAD C. G. (1961) Alteration of plasma proteins at metamorphosis in the lamprey (*Petromyzon marinus dosatus*). *Science, N.Y.* 133. 279–280.

RAY C. (1958) Vital limits and rates of desiccation in salamanders. *Ecology* 39(1), 75–83.

RIGGS A. F. (1951) The metamorphosis of hemoglobin in the bullfrog. *J. gen. Physiol.* 35. 23–40.

ROSE W. (1929) *Veld and Vlei* i-xxiii + 240 pp. Specialty Press of South Africa, Capetown.

WALD G. (1945) The chemical evolution of vision. *Harvey Lectures* 41. 117.

WALD G. (1952) Biochemical evolution. In *Modern Trends in Physiology and Biochemistry* (Edited by BARRON E. S. G.). Academic Press, New York.

WALD G. (1960) The significance of vertebrate metamorphosis. *Circulation* 21, 916.

WALD G. (1961) Phylogeny and ontogeny at the molecular level. *Evolutionary Biochemistry* (Edited by OPARIN, A.), Proc. Fifth International Congress of Biochemistry, Vol. 3.

WHIPPLE C. H. (1956) *The Dynamic Equilibrium of Body Proteins.* Charles G. Thomas, Springfield, Illinois.