Genetic and Metabolic Bases of Two "Albino" Phenotypes in the Leopard Frog, *Rana pipiens*

SANDRA J. SMITH-GILL,[1] CHRISTINA M. RICHARDS AND
GEORGE W. NACE
*Department of Zoology and Center for Human Growth and Development,
The University of Michigan, Ann Arbor, Michigan 48104*

ABSTRACT   Preliminary characterizations of albino *Rana pipiens* from two widely separated geographic origins, Mexico and Michigan, indicated that different metabolic bases underlie the albino phenotype.

Albinism in the Mexican frogs was shown to result from a single Mendelian recessive gene, *am* (amelanotic), and appeared to involve a biochemical failure of the pigment synthesizing system. Melanophores were present in *am/am* integument which exhibited tyrosinase activity when provided with exogenous tyrosine or L-DOPA. The tyrosinase activity of *am/am* melanophores showed greater thermolability than did that of wild type malanophores.

The "albinism" of tadpoles collected in Michigan was only partial, and was associated with a highly pleiotropic syndrome, abnormal patterning of chromatophore distributions, and metamorphic failure. Poor pigmentation of integumental melanophores appeared associated in part with inhibitors of melanogenesis. Genetic data on the possible mode of inheritance are not yet available.

The value of pigment forming systems in vertebrates as models of gene expression during development is well known and is particularly great in amphibians because of the accessibility of the neural crest — the source of chromatophores (DuShane, '35; Rawles, '47) — to manipulation (Lehman and Youngs, '59; Niu, '59). The final pigmentary pattern of amphibians is a result of the interactions of four pigment cell types — the silver reflecting iridophores, the yellow xanthophores or red erythrophores, the dermal melanophores, and the epidermal melanocytes — with each other and with their milieu. Biochemical mutants differentially affecting one or more of these chromatophore types (Humphrey, '67; Humphrey and Bagnara, '67; Browder, '67, '68, '72; Richards, Tartof and Nace, '69; Benjamin, '70; Berns and Narayan, '70; Lyerla and Dalton, '71) can provide insight into the biochemical and developmental relationships between these cell types. One class of such mutants includes those affecting the production of melanin.

The occurrence of albinism in the *R. pipiens* complex has been reported sporadically (reviewed by Hensley, '59), but such animals have been exploited experimentally on only a limited basis (Browder, '67, '72). Recently, albino *R. pipiens* have come to us from two sources. Albino adults have been obtained from Mexican populations on several occasions and, in the summer of 1970, albino tadpoles were found in Michigan. Because melanogenic deficiencies in other vertebrates can result from such diverse causes as lack or inhibition of the enzyme tyrosinase (reviewed by Foster, '65), defective pigment granules (Rittenhouse, '65, '68a,b; Weiss, '70), or failure of neural crest migration (Dalton, '50), we have taken advantage of our resources (Nace, '68) to investigate the developmental basis of the albino phenotype in *R. pipiens* from these two sources. Preliminary investigations of melanogenesis indicated that different metabolic bases underlie the albino phenotypes in these Mexican-obtained and Michigan-obtained groups (Gill, Richards and Nace, '70). It is the purpose of this paper to describe these two types of pigmentary defects in the *R. pipiens* complex, and report on the genetic basis of one, that derived from Mexican populations.

[1] Present address: Department of Biology, Swarthmore College, Swarthmore, Pennsylvania 19081.

MATERIALS AND METHODS

1. *Animals.* Wild type adult *R. pipiens* were obtained from commercial dealers in Vermont and Wisconsin, and collected by us in the vicinity of Whitmore Lake, Michigan. Adult *R. pipiens berlandieri*, wild type and albino, were received over a number of months from The Lemberger Company and E. G. Steinhilber and Co., Inc., both of whom reported the animals originated in Mexico. Seven albino tadpoles were collected in a shallow pond between two railroad tracks near Wyandotte, Michigan, and brought to the Amphibian Facility.

Ovulation and biparental or gynogenetic reproduction was accomplished according to standard procedures (Rugh, '62; Nace, '68; Nace, Richards and Asher, '70).

All adults and their progeny were maintained under standard laboratory conditions at the Amphibian Facility of The University of Michigan (Nace, '68). However, because of their particular sensitivity to light, albino progeny were maintained in shallow enamel pans in partial darkness through the completion of metamorphosis.

2. *Analysis of Pigmentation.* Pieces of integument approximately 25 mm² were removed from between the dorsal folds of unanesthetized adult frogs. Unless otherwise noted, interspot areas of integument were used. Wild type and Mexican-derived albino tadpoles were decapitated, and the dorsal integument removed. The Michigan albino tadpoles, however, did not survive metamorphosis. Consequently, dorsal integument was removed immediately following death. To bring the chromatophores to a standard physiological state, all integument samples were treated with amphibian Ringer's solution for one hour at room temperature, or for several hours at 4°C. Using representative samples of integument, dermis was separated from epidermis by Hadley's ('66) modification of the NaBr method of Staricco and Pinkus ('57). The integument was incubated for 30 to 60 minutes in 2 M NaBr. The dermis and epidermis were then teased apart and held in Ringer's solution until fixed or mounted. For the observation of iridophores and xanthophores, whole mounts of integument were made in Karo syrup (Bagnara, '58).

Mutant integuments were compared to wild type controls at various developmental stages for their ability to darken when supplied with exogenous substrates, and for the thermostability of their DOPA oxidase reactions. Studies of substrate incorporation were by methods modified from Pearse ('60). The tissue samples were fixed overnight in 10% formaldehyde at 2°C, rinsed in 0.01 M phosphate buffer, pH 7.4, and incubated in the substrates or the control buffer for 12 to 16 hours at 25°C, a normal temperature for non-hibernating *R. pipiens*. The substrates were 0.1% L-tyrosine (A grade, Calbiochem), or 0.1% L-dihydroxyphenylalanine (DOPA) (A grade, Sigma). For the inhibition studies, 10⁻²M phenylthiourea (PTU) (Eastman, twice recrystallized from ethanol) was added to the preparations. To study temperature effects, 30 minute treatments at 55°C, 37°C, and 2°C were applied just prior to incubation with the substrates. This protocol was modeled after that of Foster ('65, '67). Following incubation, the tissues were finally fixed either in 10% formaldehyde or in a solution of 1 formaldehyde:2 acetic acid:17 ethanol (FAA), dehydrated, cleared, and mounted whole in permount. When the FAA solution was used for postfixation, the pigment of the iridophores was preserved. Pigment cell counts were made by scoring chromatophores under an ocular grid 0.08191 mm² in three randomly chosen microscopic fields per sample.

RESULTS

A. *Genetics*

1. Mexican albinos

Tables 1–3 summarize the genetic data on Mexican albinism. Dichotomous phenotyping of larvae as albino or non-albino was possible by Shumway stage 21 (SS 21; Shumway, '40) in those crosses using albino eggs because melanization, where present, was apparent in the eyes and integumental melanophores shortly after hatching which occurs at SS 20. This was not true of crosses using pigmented females because the egg pigment obscures

TABLE 1

Production of F_1 from albino parents

Cross type	No. cases	Parental phenotypes [1]	No. of progeny [2]	
			Albino	Nonalbino
1	1	albino, gynogenesis	17	0
2	2	albino × albino	992	0
		Total	1009	0
3	2	albino × burnsi	0	168
4	1	albino × blue burnsi	0	54
5	2	albino × melanoid	0	551
6	1	albino × kandiyohi	0	236
7	1	albino × Wisc. +/+	0	180
8	1	albino × Mexican +/+	0	30
		Total	0	1219
9	4	Vt. +/+ × albino	0	180
10	1	kandihoyi × albino	0	60
		Total	0	240

[1] Two albino females and three albino males are represented.
[2] Progeny from albino ♀ scored at Shumway stage 21; those from nonalbino ♀ scored at metamorphosis.

TABLE 2

Backcross of F_1 to albino parents

Parental origin of F_1 (cross type)	Presumed parental genotypes		No. of progeny			
			Observed		Expected	
	P_1	F_1	Albino	Nonalbino	Albino	Nonalbino
9	am/am ×	am/+	2	1	1.5	1.5
9	am/am ×	am/+	21	19	20	20
9	am/am ×	am/+	12	13	12.5	12.5
9	am/am ×	am/+	38	28	33	33
10	am/am; +/+ ×	am/+ ;K/+	5	5	5	5
	Total		78	66	72.0	72.0

Test for homogeneity: $\chi^2 = 1.55$ 7 df, N.S.
Test for 1:1 ratio: $\chi^2 = 3.08$ 7 df, N.S.

TABLE 3

Backcross of F_1 to nonalbinos

Parental origin of F_1 (cross type)	No. cases	Presumed parental genotypes		Progeny	
		P_1	F_1	Albino	Nonalbino
9	5	+/+ ;K/+ ×	am/+ ; +/+	0	56
9	4	Vt. +/+ ×	am/+	0	27
9	2	+/+ ;B/+ ×	am/+ ; +/+	0	7
9	2	Mich. +/+ ×	am/+	0	16
9	1	Mex. +/+ ×	am/+	0	13
10	1	Mex. +/+ ; +/+ ×	am/+ ;K/+	0	4
			Total	0	193

Fig. 1   Wild type, heterozygous $am/+$, and homozygous am/am embryos. The slight developmental abnormalities often exhibited by the heterozygotes and albinos did not interfere with scoring the phenotype with respect to albinism.

early embryonic pigmentation (see fig. 1). In these cases the phenotypic ratios of progeny were obtained at metamorphic climax (Taylor-Kollros stage XX, TK XX; Taylor and Kollros, '46).

Albino females crossed to albino males or reproduced gynogenetically produced all albino progeny (table 1), while reciprocal crosses of albino ♀ × wild ♂ and wild ♀ × albino ♂ gave all non-albino offspring (table 1). This suggested a recessive inheritance of the phenotype. To date we have been unable to detect any obvious interaction of albinism in the

heterozygous state with any of the genetic markers used in the crosses. The number of progeny from F_1 heterozygotes backcrossed to albino ♀♀ was generally low (tables 2,3), primarily because sperm suspensions from heterozygous males contained very few motile sperm, but five successful cases produced albino:non-albino progeny in a 1:1 ratio, while backcrosses of F_1 to non-albinos yielded only non-albino progeny. This confirmed our hypothesis of a single Mendelian recessive mode of inheritance. No F_2 have yet been obtained, as all but one of the seventy-two F_1 individuals surviving to sexual maturity were males. Hormone treatment is currently being used to assure both sexes in future offspring (see Richards and Nace, '70).

2. Michigan albinos

No genetic data are available for the Michigan albinos, as none of the individuals survived to sexual maturity (see table 4). All the tadpoles died prior to or during metamorphic climax (TK XX–XXV), with one individual advancing as far as TK XXIV. Two tadpoles remained at TK XX–XXI, normally transient stages (Rugh, '62), for five days before death. Since all these tadpoles were collected from the same small pond and were found in association with wild type tadpoles, it is reasonable to speculate that the tadpoles were siblings. However, the possible genetic or environmental agents underlying the phenotype cannot be evaluated at this time.

B. *Pigmentation characteristics*

1. Mexican albinos

No melanization was visible in either the integument, the eyes, or the internal organs of Mexican albino larvae or adults. A color illustration of typical Mexican albino and wild type adults has previously been published by Nace ('68); wild type adults were characterized by dark brown or black dorsolateral spots, surrounded by yellow halos, on a brown background or interspot area, while albino adults were cream colored with darker yellow dorsolateral spots. Both phenotypes were sliv-

TABLE 4

Summary of developmental stages achieved by Michigan partial albino tadpoles. Larval staging that of Taylor and Kollros

Animal	Stage at death
06997	XIII
19832	XIX
19835	XX
19834	XX [1]
06799	XXI [1]
06798	XXIII
19833	XXIV

[1] Five days after attaining stage XX.

ery-white ventrally. Metamorphosed albino juveniles had pink eyes, and pinkish skin which became yellower as they grew older.

Iridophores and xanthophores were present in interspot regions in normal numbers, morphologies, and distributional patterns as judged by comparison with the wild type (table 5).

In the comparison of albino spot with interspot areas, the same density of xanthophores but fewer iridophores were found (table 5). In addition, spot xanthophores were much more expanded and more highly clumped than those of the interspot regions. Thus the yellow spots in the albino result from a combination of altered morphology of xanthophores and density of iridophores.

Eggs from albino females were creamy white, lacking any visible melanin. The only dark pigment in the albinos appeared in the black labial teeth of the tadpoles from SS 25 to metamorphic climax (TK XX), and a dark but presumably non-melanin pigment in the tadpoles' irises which persisted through metamorphosis.

In the course of substrate incorporation studies, adult albino integument showed only modest pigmentation when incubated with tyrosine. Whether already melanized wild type adult controls incorporated tyrosine could not be determined under our conditions, but DOPA utilization was evident by increased melanization of the wild type melanophores, giving a more dendritic appearance (figs. 2A,B). Both epidermal and dermal melanophores of albino and wild type animals incorporated DOPA into melanin (fig. 2), and this was completely inhibited by phenyl-

TABLE 5

Comparison of nonmelanin chromatophores in Mexican albino and wild type adult dorsal integument [1]

Area	Chromatophore number [2]					
	Xanthophores	t [3]	p [4]	Number Iridophores	t	p
Wild type interspot	61.67 ± 5.51			142.00 ± 4.58		
		0.354	0.737		2.539	0.126
Albino interspot	59.67 ± 7.64			122.70 ± 11.93		
		2.409	0.095		3.310	0.046
Albino spot	93.00 ± 24.56			74.00 ± 19.97		

[1] Unfixed integument mounted whole in Karo.
[2] Average $\pm$ one standard deviation of one field 0.08191 mm^2 in each of three individuals per genotype.
[3] Students t test, performed on transformed data.
[4] Attained probability level indicated.
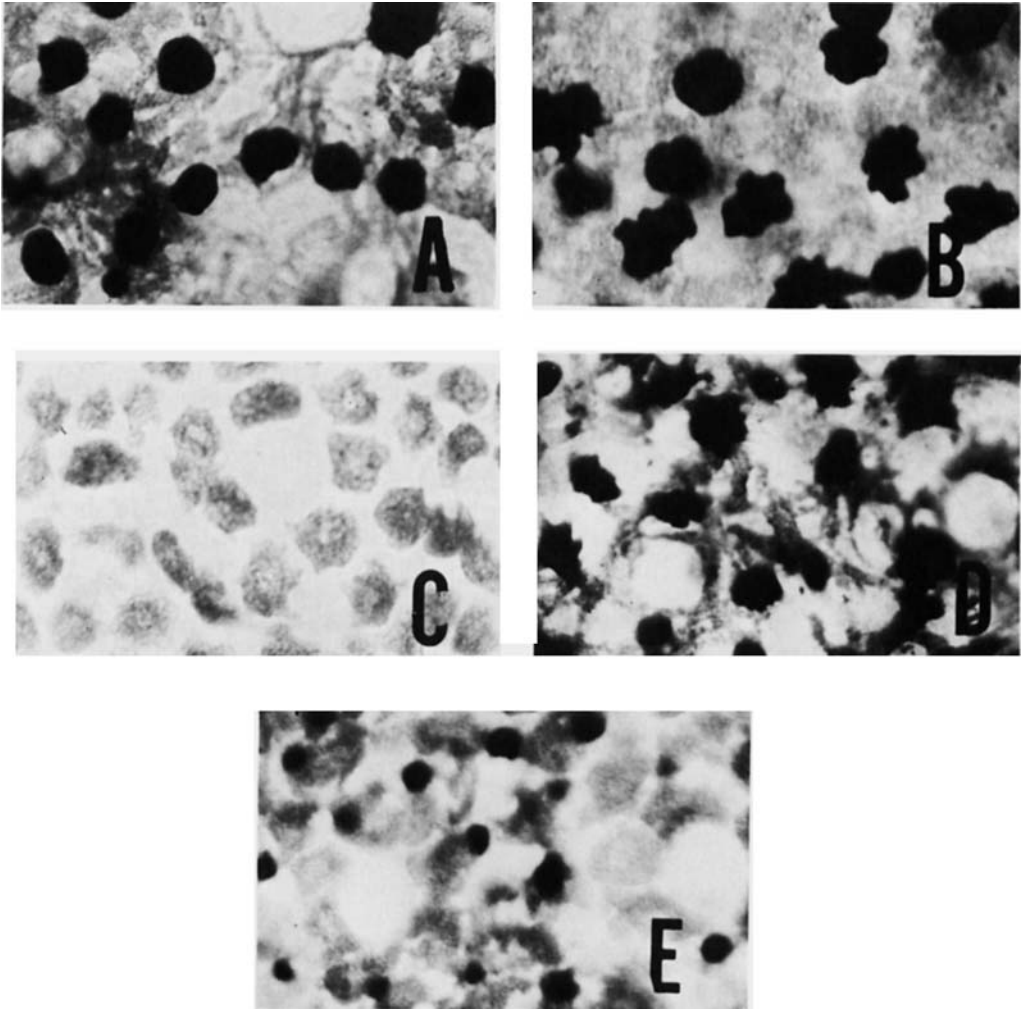
Fig. 2   Melanogenesis in Mexican adult dermis. A–B, Wild type: A, Control; B, L–DOPA, with or without heat. C–E, Albino: C, Control; D, L–DOPA; E, 55° + L–DOPA. FAA post-fixation.

TABLE 6

*Comparison of melanophore numbers in interspot areas of Mexican wild type
and albino integument after various treatments* [1]

| | | Melanophore number [2] | |
	Control	2° + DOPA	55° + DOPA
+/+	54.0 ± 12.0	51.33 ± 6.43	41.33 ± 1.16
am/+	36.0 ± 2.0	42.00 ± 8.72	42.67 ± 4.16
am/am	0	54.67 ± 1.53	55.33 ± 9.29

[1] Fixation as described in text.
[2] Average ± one standard deviation of one field 0.08191 mm² in each of three individuals per geno-
type. No significant differences (at a 5% probability level) were found between any of these values.

thiourea except for some incorporation
into clusters of cells in the vicinities of
skin glands in the albinos. There was
minor nonspecific utilization of these sub-
strates by cells surrounding the "melano-
phores" in both the dermis and epidermis,
but significant utilization of both DOPA
and tyrosine was limited to cells with
morphologies and distributional patterns
similar to those of wild type melanophores
(fig. 2, table 6). As can be seen in figure 2,
these melanophores were distinct from the
iridophores, although some melanin syn-
thesis seemed to occur in the iridophores.

Preincubation of integument at either
2°C or 37°C had no visible effect on
DOPA utilization by either albino or wild
type tissues. Following treatment at 55°C,
however, DOPA utilization was signifi-
cantly decreased in albino adult and tad-
pole integument (fig. 2E), though in the
integument of wild type controls it re-
mained unchanged or even increased
slightly. That this response was at the
cellular level was evident from data show-
ing that the numbers of melanophores
reacting following this heat treatment
did not decrease (table 6). The amount
of melanin deposited in each melanophore
was greatly decreased, and nonspecific
utilization by the surrounding tissue and
iridophores was completely inhibited by
55°C treatment. Heterozygous embryos
tested at SS 25 were intermediate between
the albinos and wild type as shown by
moderate reduction in DOPA utilization
following 55°C treatment. However, no
effect of the 55°C treatment could be
detected on heterozygous adults, and was
less striking in albino tadpoles tested at
metamorphic climax.

2. Michigan "albinos"

The Michigan "albinos" contained some
melanin and thus cannot be classified as
albinos. On examination, it was found
that the tadpoles contained only poorly
pigmented melanophores (fig. 3), and
these were sparsely distributed in the tail
and limbs. Most were limited to the epi-
dermis. The numbers of subcutaneous
chromatophores in general were greatly
reduced in comparison with those of wild
type tadpoles of the same stages; they
were most abundant anteriorly, decreas-
ing posteriorly and becoming almost en-
tirely absent on the tail. Their reduction
was even more pronounced along a dorsal-
ventral gradient. Many melanophores,
particularly those in subcutaneous sites,
had a brownish appearance, rather than
the typical black color seen in wild type
tadpoles, and all had abnormal morphol-
ogies. The number of peritoneal chromato-
phores was also greatly reduced, the only
significant internal pigmentation being
relatively well differentiated melanophores
on the lungs. Retinal pigment was absent
or greatly reduced, though some dark pig-
ment was present on the iris.

The partially pigmented skin of these
Michigan animals did not visibly incorpo-
rate tyrosine, but gave a weakly positive
DOPA reaction (fig. 4). Though the num-
ber of animals tested was few, the DOPA
reaction seemed to be more intense in
the integument from TK XX than from
TK XXIII individuals. Very few new epi-
dermal pigment cells were revealed fol-
lowing incubation in DOPA, but punctate
dermal melanophores became apparent
(figs. 4a,b). Following treatment at 55°C,
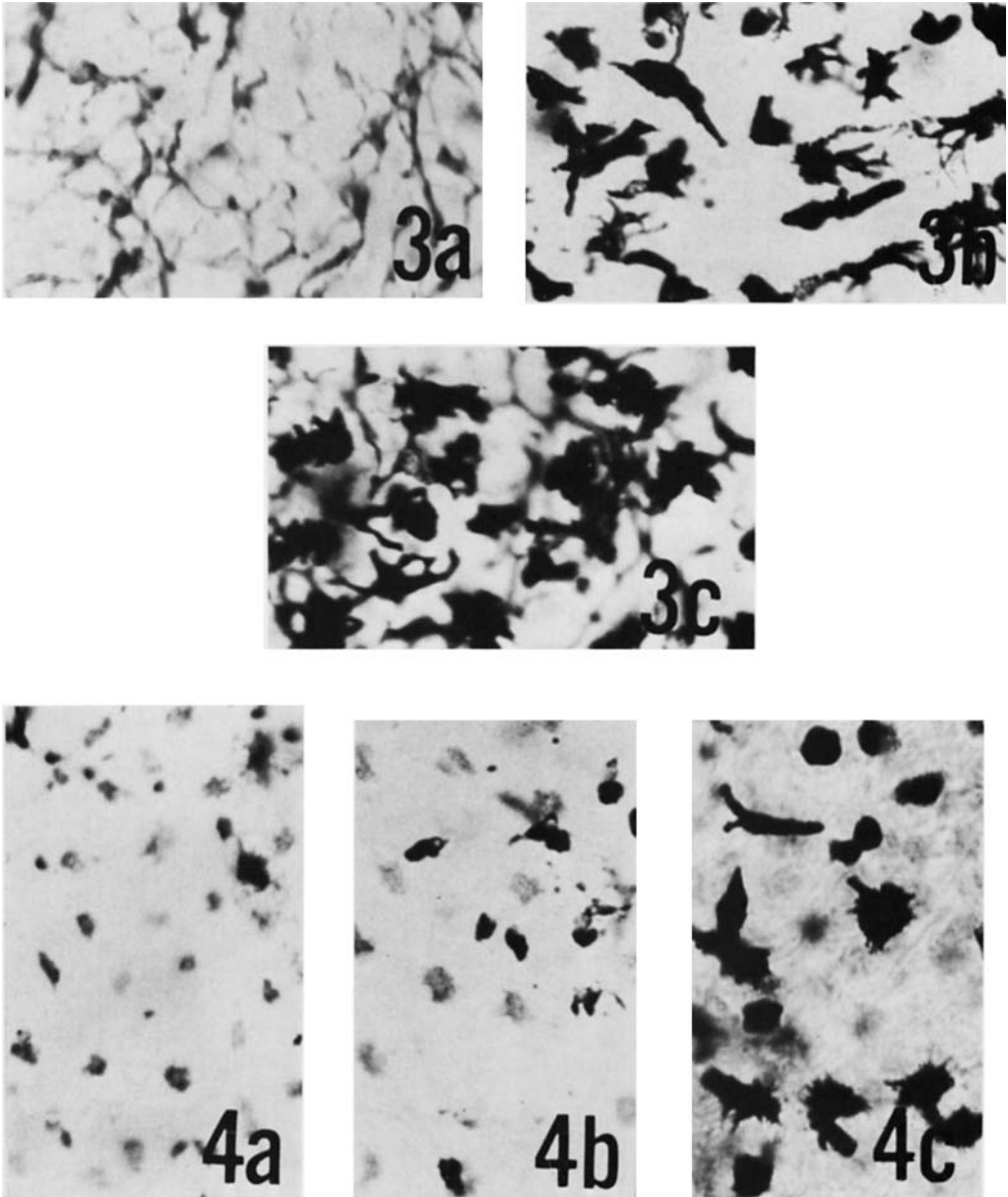
Figs. 3–4   Melanogenesis in Michigan tadpoles, Stage TK–XXIV.
Fig. 3a   "Albino," whole mount after buffer incubation.
Fig. 3b–c   Wild type, whole mount: b, Buffer control; c, L–DOPA. Formaldehyde post-fixation.
Fig. 4a–c   "Albino" dermis: a, Control; b, L–DOPA; c, 55° + L–DOPA. FAA postfixation.

the amount of DOPA utilized per cell and the number of reacting cells in TK XXIII integument was greatly increased (fig. 4c), whereas the same treatment of wild type integument only slightly increased melanization.

DISCUSSION

The data presented here suggest that the Mexican and Michigan albinos represent two pigment defects which have different metabolic bases. Consequently, if the modified albinism observed in the Michigan frogs has a genetic basis, by implication it must be different from that found for the Mexican albinism. This cannot, however, be evaluated until mating data are available.

The genetic analysis of the Mexican animals indicated a single Mendelian recessive gene underlying their albinism. This agrees with previous reports on the genetic nature of albinism in various species of frogs (Eales, '33; Smallcombe, '49; Tokunaga, '49; Browder, '67, '72; Daito, '68). Unfortunately, Browder's albino strain of South Dakota origin (Browder, '67, '72) has been lost, precluding the mating tests necessary to determine homology with the Mexican albino gene. Since a terminology has not been assigned to this mutant, we propose the designation *am* for the recessive allele responsible for this phenotype. We use this term, which is an abbreviation of amelanotic, because albinism in mammals is attributed to the absence of the enzyme, tyrosinase, which is present in the animals under consideration here. Furthermore, the mechanism of action appears to be different from that of the *a* gene producing albinism in the Mexican axolotl (Humphrey, '67; Benjamin, '70). Experiments designed to determine genekinetochore map distances and possible linkages of *am* are currently in progress (see Nace, Richards and Asher, '70).

The histochemical studies on the *am/am* integument revealed the presence of melanophores in normal numbers with apparently normal morphologies, corroborating the unpublished fine structure studies of J. D. Taylor which revealed the presence of melanophores with unmelanized melanosomes in the integument of albino frogs from this laboratory. Our tyrosine and DOPA incorporation experiments indicated that *am/am* melanophores have both tyrosinase and DOPA oxidase activity, unlike the genes producing albinism in mammals (Searle, '68) or the axolotl (Benjamin, '70). Browder's ('67, '72) studies revealed that under experimental conditions albino melanophores can synthesize small amounts of melanin *in vivo*. The cell-specific nature of the defect, indicated by our thermolability studies, the normal morphology and patterning of the iridophores and xanthophores, and the transplantation studies of Browder ('67, '72) suggest a defect at some level of post-translational control of the melanogenic process. Many examples of similar pigmentary defects have been reported in mammals (rev. Searle, '68). The normal patterning of all the chromatophores also suggests that the process of pattern formation in *R. pipiens* is independent of the intracellular control mechanisms underlying pigment synthesis. Further biochemical and fine structure studies are in progress to fully characterize the *am* gene.

In the Michigan tadpoles, the partial differentiation of melanophores, abnormal patterning of all chromatophore types, and the failure to complete metamorphosis, suggest a general systemic failure, such as hormonal deficiency or anomalies in neural crest development (Smith, '16; Dalton, '50, '53; Dalton and Krassner, '56, '59). The great increase in melanin synthesis following heat treatment may be associated with removal of a thermolabile inhibitor such as that described by Baker ('51) in adult *R. pipiens* skin. Whether the syndrome seen in the Michigan tadpoles was caused by a highly pleiotropic gene, or the teratogenic effect of an environmental agent, cannot at this time be determined.

The defects described here appear to affect the process of melanogenesis at different sites of control. A recent report by McGuire ('70) suggests that *R. pipiens* tyrosinase may exist in a protyrosinase form which must be activated for melanogenesis to occur. The *am* gene and the possible genes responsible for the Michigan "albinism" offer useful tools for investigating the control of pigmentation.

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