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Robert Edward Shervette

A COMPARATIVE STUDY OF THE SULFHYDRYL GROUPS IN THE GASTROCNEMIUS OF THE NORMAL AND X-IRRADIATED PET MOUSE

BY

ROBERT EDWARD SHERVETTE, III

APPROVED:

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ROBERT EDWARD SHERVETTE, III

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ABSTRACT

It has been recognized that hyperpigmentation of the skin is the result of eliminating the -SH inhibition when treatment is by x-irradiation. The results of a study by Reams and Schaeffer indicate a progressive increase of melanocytes in the skin with an increasing dosage of x-irradiation. However, it was also noted that the effect of x-irradiation on the gastrocnemius of the PET mouse resulted in a decrease in the number of intramuscular melanocytes.

The present study was designed to determine whether the changes in the x-irradiated muscle is related to possible changes in -SH concentration. The technique employed is a quantitative measurement of moles -SH by azo-aryl mercaptide coupling in the normal and the x-irradiated muscles.

The data show that the melanocyte population of the right and left legs of the normal mice, and the left control muscle of the experimental animals were essentially identical for the developmental period studied. On the other hand, the melanocyte population of the irradiated muscles showed an ultimate decrease in comparison to the controls. On the basis of the -SH determinations for the normal and the experimental animals, there was no apparent relation between moles -SH and changes in the melanocyte populations.

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Special thanks goes to my wife, Linda, without whom none of this would have been possible.

INTRODUCTION

All the pigment cells of the mammal, except those of the eye, are of neural crest origin (Rawles, '47) and therefore can be assumed to possess the same genetic potential. The extent of their genetic expression, however, is related to the cellular environment in which they come to reside (Markert and Silvers, '56). Most induced modifications of the cellular environment are reflected in the differentiation and behavior of the resident melanocyte.

It is now well recognized that irradiation stimulates hyperpigmentation in the skin. Reams and Schaeffer ('66) found that although increasing dosage of x-irradiation provoked a hyperpigmentation in the skin, there was a progressive decrease in the intramuscular pigment cell population. Similarly, Chase ('49, '50) and Cohen ('63) found that increasing x-irradiation produced a progressive decrease in the melanization of hair.

It has been proposed that hyperpigmentation is the result of elimination of the sulfhydryl (-SH) inhibition in the biosynthesis of melanin (fig. 1). Flesch ('49) and Rothman et al. ('46) have observed that aqueous extracts of human epidermis inhibited the oxidation of l-tyrosine and 3,4-dihydroxyphenylalanine (dopa), thus preventing or interrupting the biosynthetic pathway of melanin. It has been assumed that the inhibition is due to the presence

of -SH groups in the extracts since the effect is counteracted by iodoacetamide, an alkylating agent. A direct relationship was demonstrated between the -SH concentration and the inhibitory power of the extracts. Flesch found that an increase in melanin formation was preceded by a corresponding decrease in the -SH content of the skin. His results indicate that immediately following exposure there is a decrease of from 24% to 83% in the -SH concentration of the skin. This is in contrast to Ogura and Knox ('64) who found that immediately after irradiation there was an increase in -SH concentration; however this was followed by a return to normal in 24 hours. At three days post exposure, the -SH concentration had become lower than normal. By 10 - 15 days following exposure the -SH value again returned to normal. Lerner et al. ('50) found that -SH groups exert most of the inhibitory action by combining with the copper required for enzymatic activity. The inhibition caused by the -SH groups could be reversed by addition of an excess of cupric ion. This suggests that pigment producing stimuli act by eliminating the -SH inhibition, allowing the enzymatic oxidation of pigment precursor to occur (Flesch and Rothman, '48).

If -SH groups regulate the biosynthesis of melanin in the melanocyte, they should affect the copper-containing enzyme tyrosinase (Flesch and Rothman, '48) which is essential for the formation of melanin in mammalian tissues (Flesch,

'49; Seiji et al., '63). Furthermore, -SH containing epidermal extracts neutralize the effect of both tyrosinase and cupric ions in pigment formation (Rothman et al., '46; Flesch, '49). The amount of melanin synthesized by the tyrosine-tyrosinase reaction then should be inversely related to the amount of -SH groups present (Seiji et al., '67). Therefore, it is reasonable to assume that -SH groups are among the controlling factors of melanization in the melanocyte.

The study presented here was undertaken to ascertain if the -SH content could be correlated with the melanocyte population of the gastrocnemius muscle of normal and irradiated PET mice.

The pigment cell terminology used herein conforms to that of Fitzpatrick et al. ('66).

MATERIALS AND METHODS

The mice used in this study were of the PET (Pigmented Extraepidermal Tissue) strain. These mice well serve for this project as melanocytes are found in significant numbers in the leg musculature as well as the dermis (Nichols and Reams, '60).

In order to establish a basis for comparison of the x-irradiated gastrocnemius, ~~curves~~ determinations of the -SH groups and melanocyte counts of the normal right & left gastrocnemius muscles were made ~~were made of 1, 3, 5, 7, 9, 11, 13, and 15-day-old mice~~ mice from 1 to 9 days of age. The determination of the moles -SH per gram dry muscle by the Mercury Orange (1-(4-chloromercuriphenylazo) 4-Napthol[1])[2] method (Bennett and Watts, '58) is through azo-aryl mercaptide-coupling with reference to Mercury Orange uptake of the mercurial by the gastrocnemius muscles immersed in a ~~standard~~ 2×10^{-5} M solution of the reagent. Three muscles were used for each determination. A value for available -SH groups ~~can be derived~~ was computed from spectrophotometric measurements of the solution before and after uptake.

A 2 x 10^{-5} M solution of Mercury Orange in toluene was used as this concentration lies within the range that yields accurate spectrophotometric measurements. Preliminary tests established the volume of Mercury Orange solution necessary to obtain suitable absorbance values.

The hind legs of PET mice were excised at the

thigh, and, following skinning, were placed immediately in 10 ml of 2.5% trichloroacetic acid (Reagent Grade, distilled in vacuo). Fixation was permitted to continue at 4° C for 18 to 24 hours; larger muscles required more time. The tissue was washed 2 to 4 times with glass distilled water to remove the trichloroacetic acid. The tissue was then placed in 70% ethanol and stored at 4° C until used. Prior to -SH determination the legs were placed in 95% ethanol and the gastrocnemius muscles dissected free. The gastrocnemius muscles were then dehydrated successively with absolute ethanol, 50/50 toluene in ethanol, and stored in toluene until use.

The muscles were taken from the toluene, blotted with filter paper to remove excess toluene and placed in the Mercury Orange solution in 25 x 200 mm test tubes that had teflon lined screw caps. The samples were stored at 4° C; however, all readings were made at room temperature. Initial readings were made of the absorbance and recorded. Every 12 hours thereafter, a test aliquot was removed from each test tube and carefully pipetted with an automatic pipet into a 1 cm cuvette and the absorbance at 484 mu was determined. The aliquot was then returned to its original test tube. Great care was used to avoid losses during transfer and measurement. The absorbance (A_1) of the standard solution was determined at the same time. The value of the absorbance at the endpoint (A_2) was taken when a minimum value of

absorbance had been reached. This represented the absorbance of the solution at the time when the sample had taken up a maximum of the Mercury Orange.

The muscles then were removed from the solution, rinsed in toluene, cleared further in methyl salicylate and prepared as wet whole mounts for microscopic examination. Using an ocular reticle and a hand tally counter, counts were made of the number of melanocytes present in the left and right muscles of each mouse. The muscles were then rinsed in toluene, blotted with filter paper, placed in an aluminium envelope, and dried to constant weight in vacuo over phosphorus pentoxide. The sample weight was obtained by the difference between the weights of the empty envelope and the envelope containing the muscles.

The -SH concentration for a sample was calculated according to the following formula:

$$\text{Average Moles -SH per gram dry muscle (uncorrected)} = \frac{\dfrac{(A_1 - A_2) \times V \times M}{A_1 \times 1000 \times W}}{3}$$

Equation 1

where, M = molarity of the Mercury Orange solution; A_1 = absorbance of the standard Mercury Orange solution; A_2 = absorbance of the Mercury Orange solution at the endpoint; V = volume (ml) of Mercury Orange solution used; W = weight in grams of dry muscle sample.

As the weight of the sample (W) was obtained after the muscle had reacted with the Mercury Orange, the value for W used in equation 1 is actually that of the muscle-mercaptide complex. A correction can be made in accordance with the following formula:

$$\text{Average Moles -SH per gram dry muscle (corrected)} = \frac{\text{Average Moles -SH per gram dry muscle (uncorrected)}}{1 - \left[m \times \text{Average Moles -SH per gram dry muscle (uncorrected)} \right]}$$

Equation 2

where m = 483.3, the molecular weight for Mercury Orange.

In order to determine the effects of x-irradiation on the -SH concentration of muscle, the right hind limb of 12 one-day-old mice were irradiated with a single dose of 2000 r. This dosage of x-ray was used as Reams and Schaeffer ('66) found this to give a significant reduction in melanocyte number. To facilitate irradiation, the mouse puppies were mounted on individual polystrene platforms with adhesive strips so that the right limb was extended. A General Electric 1000 KVP Maxitron x-ray machine was used. With a current of three milliamperes, at 1000 KVP, the radiation intensity obtained at 1 cm distance was 500 r per minute, HVL of 3.7mm of lead. An x-ray beam of 16 mm diameter was used. Prior to x-raying, the area to be irradiated was visibly delimited by a light beam for accurate positioning of the limb. The irradiated area included the entire limb except the proximal portion of the thigh. The x-ray machine contained inherent

filtration consisting of a brass-water-brass jacket surrounding the x-ray tube. Additional filters were unncessary because the soft x-rays are unable to pass through the inherent filtration. Only high energy x-rays of practically uniform intensity passed through to the specimen, thus assuring nearly equal radiation intensity in the muscle.

Determinations of the moles -SH and melanocyte numbers for x-irradiated muscle were made for 3, 5, 7, and 9 day-old mice by the same methods employed for the normal muscle. Due to a breakdown of the Maxitron, it was not possible to obtain a full range of irradiated specimens.

Tests of significance between mean values were made using the student t test for correlated data. The various data were also studied graphically. Curves were established from the counts of the melanocytes in the left and right muscles from mice of selected ages from 1 to 15 days. Curves also were established for the moles -SH of the left and right gastrocnemius muscles of various aged normal animals. In similiar manner, curves were made of the melanocyte counts and the moles -SH for the experimental animals. The curves of the melanocyte counts of the left (non-irradiated) and right (irradiated) muscles of the experimental animals were compared with each other and with the curves of the normal animals. Finally the curves of the moles -SH of the left (non-irradiated) and right (irradiated) muscles were compared with each other and with the curve of the normal animals.

RESULTS

The results of this study are presented in tables 1 and 2. The t values represent comparisons between the left and right gastrocnemius muscles of the nomal PET mouse (table 1) and the left (non-irradiated) and the right (irradiated) muscles of the experimental animals (table 2). Table 3 shows the comparison of the left gastrocnemius muscle of the normal and the left (non-irradiated) gastrocnemius muscles of the experimental animals.

The data show that the melanocyte population of the right and left muscles of the normal mice, and the left control muscle of the experimental animals were essentially identical for the developmental period studied. On the other hand, the melanocyte population of the irradiated muscles showed an ultimate decrease in comparison to the controls. On the basis of the -SH determinations for normal (fig. 4) and experimental animals (fig. 5), there was no apparent relation between moles -SH and changes in the melanocyte populations.

DISCUSSION

The pigment cells of the body have long been a source of intriguing study and challenge. Rawles ('53) proved that melanin pigmentation of the epidermis and its derivatives is not autonomous, but is entirely dependent upon one particular type of cell, the melanocyte. The precursor, migatory pigment-forming cells, the melanoblasts, take their origin in the neural crest and spread rapidly throughout the embryo (Rawles, '47). They seem to be directed in their movement by the interface between the ectoderm and the prospective dermis (Rawles, '53). Recent evidence by Weston ('63) shows that the melanoblasts move initially within the ectoderm.

From their origin in the neural crest, melanoblasts in PET mice were found to migrate out and not only colonize the skin, but the leg muscles as well (Mayer and Reams, '62; Reams, '63). The melanoblasts of the gastrocnemius reach their full complement by day 19 of development and are still amelanotic (Nichols and Reams, '60). As development proceeds and conditions become suitable for melanin synthesis, many of the melanoblasts differentiate into melanocytes (Rawles, '53). In the gastrocnemius, unlike the skin, all of the melanoblasts undergo melanogenesis and appear as melanocytes (Reams, '66a, '66b). Melanogenesis (fig. 1) ordinarily depends on the available concentration of three substances:

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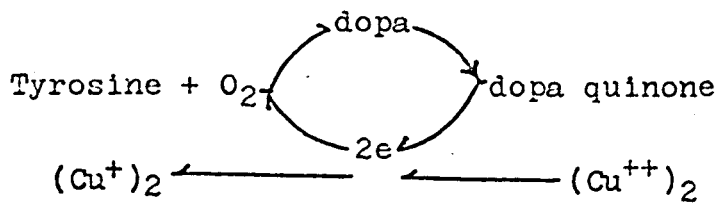
1) the enzyme tyrosinase, a copper-containing oxidase which catalyzes the oxidation of mono and o-dihydricphenols to o-quinones and is attached to ultramicroscopic particles in the cytoplasm of the melanoblast; 2) a suitable substrate, usually tyrosine or dopa; and 3) molecular oxygen (Lerner and Fitzpartick, '50).

Melanin is a protein conjugate formed by the coupling of a quinoid polymer, indol-5,6-quinone, with protein. The polymerization and coupling occur on the surface of a subcellular cytoplasmic particle, the melanosome. The quinoid polymer is derived from the amino acid tyrosine by a chemical reaction catalyzed by an aerobic oxidase, tyrosinase. Since tyrosinase is attached to the melanin granule, melanin synthesis is precisely localized to the cytoplasm of the melanocyte (Fitzpatrick and Szabo, '59).

Mason's ('53, '59) precis on the mechanism of action of the phenolase complex has provided us with a fairly clear picture of the manner in which a single enzyme is able to catalyze the first two reactions. In the presence of dopa, two cupric atoms in tyrosinase are reduced to cuprous atoms. Thus the initial step in the activation is the reduction of the enzyme from cupric to the cuprous state.



(from Lerner and Case, '59)

Dopa, in addition to acting as a substrate, can act as an activator for the reduction of the cupric to the cuprous state (Lerner et al., '50).

As oxidation of tyrosine and dopa is inhibited by extracts of the skin and iodoacetamide interfers with the inhibitory action of the extracts, then the inhibition is assumed to be due to the presence of -SH groups in the epidermis (Rothman et al., '46; Flesch, '49). The hypothesis that melanogenesis does not occur in pigment cells due to the inhibitory action of -SH groups (Rothman et al., '46) gains support in that tyrosinase is a copper-containing enzyme and -SH groups form strong covalent bonds with the copper (Lerner and Case, '59). Further, the inhibition can be reversed by adding excess cupric ion (Lerner and Case, '59).

There are three types of -SH groups: free reacting, sluggish, and masked. The freely reacting and sluggish will react with strong -SH reagents such as Mercury Orange, a mercaptide forming compound. Masked -SH; however, is not available until the molecule has been altered by denaturation causing rupture of the hydrogen bonds (Ogura et al., '62).

Pigmentogenic stimuli such as x-ray act by oxidizing the -SH groups to the inactive disulfide state (Barron et al., '54) whereupon the enzyme tyrosinase can freely act on the substrate resulting in hyperpigmentation. The cause of hyperpigmentation has been attributed to one or more of the

following: an increased rate of melanin formation in existing melanocytes (Fitzpatrick and Szabo, '59; Snell, '63a, 63b; Staricco, '63; Rovee and Reams, '64); an increase in the melanocyte numbers by mitotic activity (Quevedo, '63); the migration of amoeboid melanocytes to the stimulated area (Staricco, '63; Silvers, '58); or, the activation of amelanotic melanocytes (Reynolds, '54; Quevedo and Smith, 63; Rovee and Reams, '64) by release from -SH inhibition (Reams and Schaeffer, '66). Conversely, hypopigmentation may be mediated by the epidermis (Cohen, '63) through the inhibition of melanogenesis, the destruction of the pigment cells (Reams and Schaeffer, '66), or the irreversible inactivation of melanocytes (Chase, '50).

In this study, the morphogenetic development of the melanocyte population in the normal (table 1; fig 2) proceeds in a near linear fashion with an increase in age until the full complement of pigment cells is expressed. This confirms earlier work done by Nichols and Reams. ('60). At day nine, the rate of melanogenic activity decreases as the pigment cell population reaches the maximum which the muscle is capable of supporting (Reams, '66b). Also, the right and left hind legs of the normal mice appear very similar in their melanocyte development in the gastrocnemius muscles with no significant difference at the .05 level of confidence (table 1).

In comparison, the non-irradiated left gastrocnemius of the experimental animal follows the same pattern of development as the normal mouse (tables 1 and 2; figs. 2 and 3).

It was found that, with the exception of the three-day-old, there was no significant difference at the .05 level of confidence between the left (non-irradiated) and the left normal gastrocnemius muscles (table 3).

Having ascertained that the developmental patterns are similar, a comparison of the right (irradiated) with the left (non-irradiated) muscles shows a significant difference for days 5, 7, and 9. This indicates that the x-irradiation did result in reduced melanogenesis but not necessarily in changes in the pigment cell population.

At day three, there is no significant difference between the left (non-irradiated) and the right (irradiated) muscles which indicates that there is a "lag period" for the effect of x-irradiation to show itself (table 2). This is in agreement with Chase and Hunt ('59) who found that in a growing hair follicle there is an immediate increase in pigmentation over three to four days and then a decrease. Further, it is proposed that this "lag period" is the result of carry-over from melanoblasts already in the process of depositing melanin and is supported by the relative constancy of melanocyte numbers over the experimental ages used. This is in agreement with Reams and Schaeffer ('66).

In turning to the moles -SH, it is seen to be very irregular (tables 1 and 2; figs. 4 and 5). In contrast to Flesch and Rothman ('48), the present data indicate that as the melanocyte number increases, the -SH concentration increases

also. Possibly this is due to the mitotically active cells, as Brachet ('60) indicates that an increase in -SH concentration precedes mitotic activity and occurs in a cyclic type of change. This seems to prevail in both the normal and the experimental.

In the experimental animals, the moles -SH for the left (non-irradiated) muscle increases as the melanocyte population increases (fig. 5). In comparison of the curves for the left and right muscles, there is a definite difference in the -SH content except for day three. Thus x-irradiation apparently does have an effect on the -SH content but no correlation can be made for its effect on the melanogenic activity in the muscle. This leads to the presumption that in the muscle it is not the -SH groups but some other factor which inhibits melanogenesis.

In summary, the evidence available from this study demonstrates that for the gastrocnemius of the PET mouse. the behavior of the pigment cells in both normal and irradiated animals is independent of the -SH concentration.

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Table 1. Melanocyte counts and the -SH determination for the normal gastrocnemius muscles.

Age in days	Muscle	Mean number melanocytes	t values	Average Moles -SH per gram dry muscle (corrected) (x 10^{-5})
1	L	347	.470	.193
	R	337		.153
3	L	588	1.307	.323
	R	538		.272
5	L	827	.135	.268
	R	820		.263
7	L	981	.690	.526
	R	930		.474
9	L	1039	-.233	.676
	R	1059		.681
11	L	1087	-.303	.529
	R	1110		.609
13	L	1050	.046	.499
	R	1047		.397
15	L	1166	.749	.662
	R	1113		.685

df = 10; t = 2.22 at the .05 level of significance

Table 2. Melanocyte counts and the -SH determination for the experimental mice.

Age in days	Muscle	Mean number melanocytes	t values	Average Moles -SH per gram dry muscle (corrected) (x 10^{-5})
3	Ln	650	.927	.203
	Rx	684		.183
5	Ln	854	10.452	.707
	Rx	523		.421
7	Ln	1147	2.801	.842
	Rx	678		.589
9	Ln	1031	2.851	.878
	Rx	639		.787

df = 4; t = 2.78 at the .05 level of significance
Ln = left (non-irradiated); Rx = right (irradiated)

Table 3. Comparison of the normal left (L) with the experimental left (Ln) non-irradiated gastrocnemius muscles.

Age in days	Muscles	Mean number melanocytes	t values	Average Moles -SH per gram dry muscle (corrected) (x 10^{-5})
3	L	588	2.550	.323
	Ln	650		.203
5	L	827	.523	.268
	Ln	854		.707
7	L	981	2.365	.526
	Ln	147		.842
9	L	1039	.123	.676
	Ln	1031		.878

df = 7; t = 2.37 at the .05 level of significance

Figure 1. Biosynthesis of melanin.

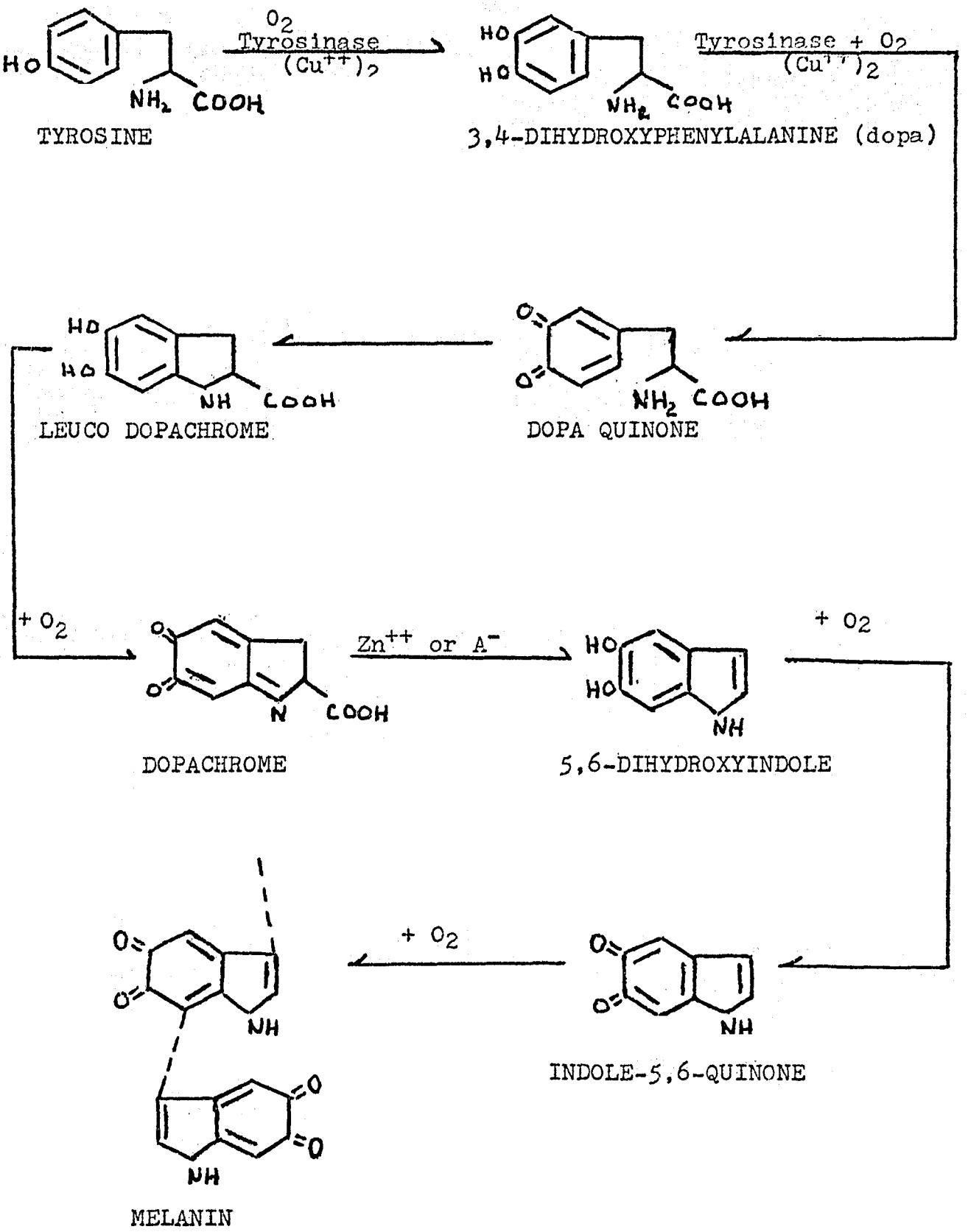

HO
NH2 COOH
O2
Tyrosinase
(Cu++)2
TYROSINE
HO
HO
NH2 COOH
Tyrosinase + O2
(Cu++)2
3,4-DIHYDROXYPHENYLALANINE (dopa)
O
O
NH2 COOH
DOPA QUINONE
HO
HO
NH COOH
LEUCO DOPACHROME
+ O2
O
O
N COOH
DOPACHROME
Zn++ or A-
HO
HO
NH
5,6-DIHYDROXYINDOLE
+ O2
+ O2
O
O
NH
INDOLE-5,6-QUINONE
O
O
NH
O
O
NH
MELANIN

Figure 2. Number of melanocytes of the left and right gastrocnemius of the normal PET mouse.

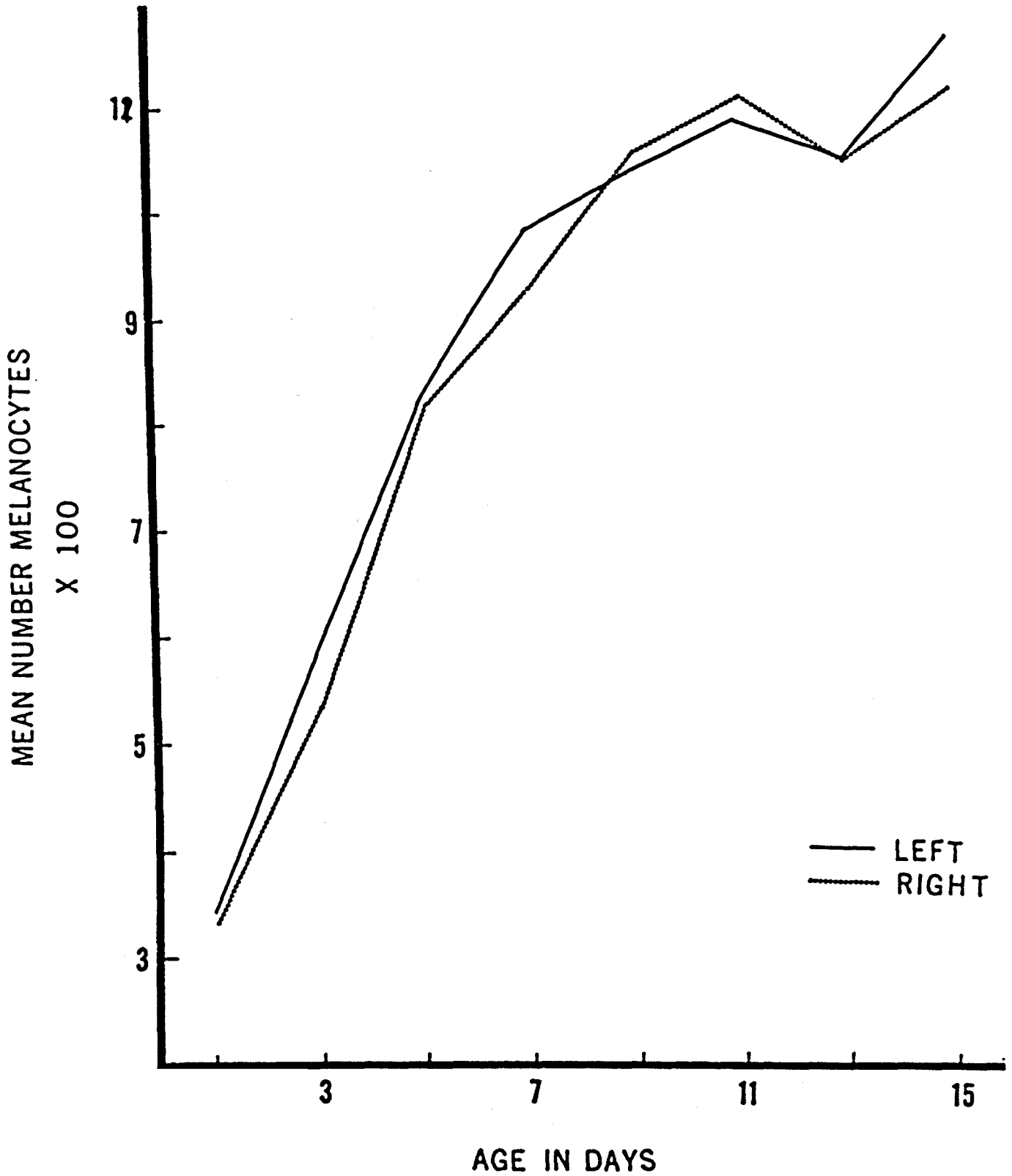

MEAN NUMBER MELANOCYTES
X 100
12
9
7
5
3
3
7
11
15
AGE IN DAYS
LEFT
RIGHT

Figure 3. Number of melanocytes of the left (non-irradiated) and right (irradiated) gastrocnemius of the experimental PET mouse.

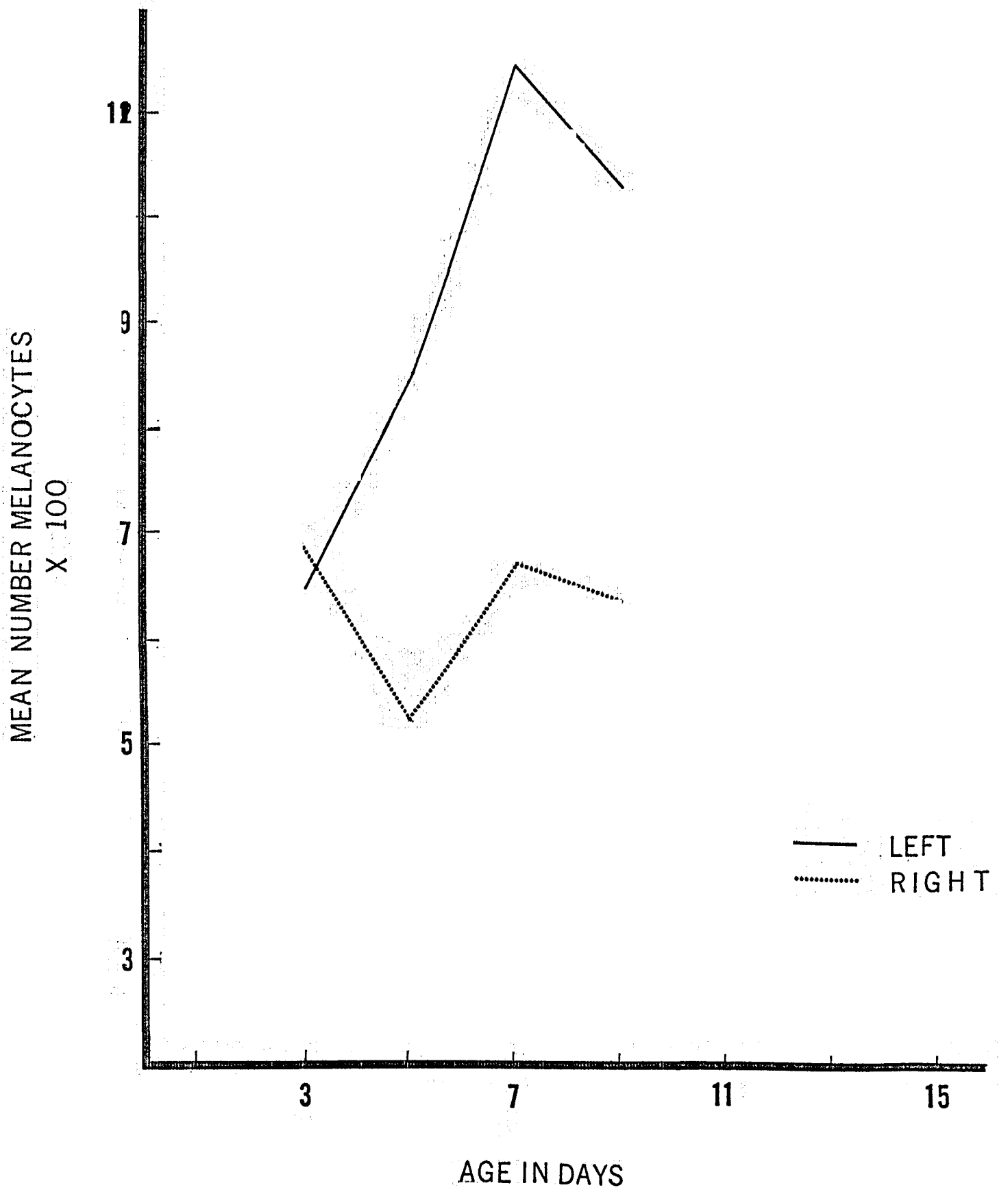

MEAN NUMBER MELANOCYTES
X 100
12
9
7
5
3
3
7
11
15
AGE IN DAYS
LEFT
RIGHT

Figure 4. Moles -SH per gram dry muscle for the left and right gastrocnemius of the normal PET mouse.

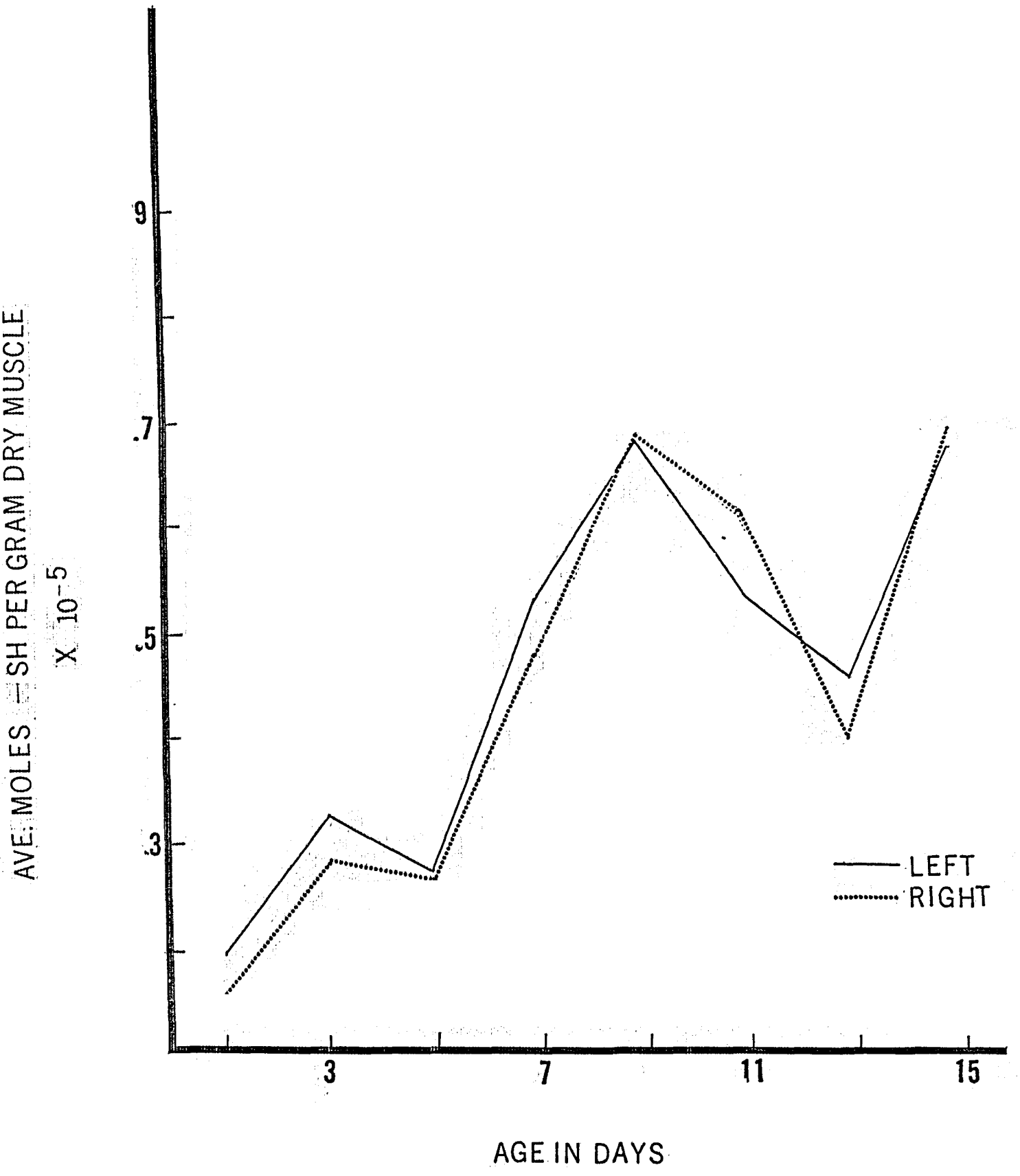
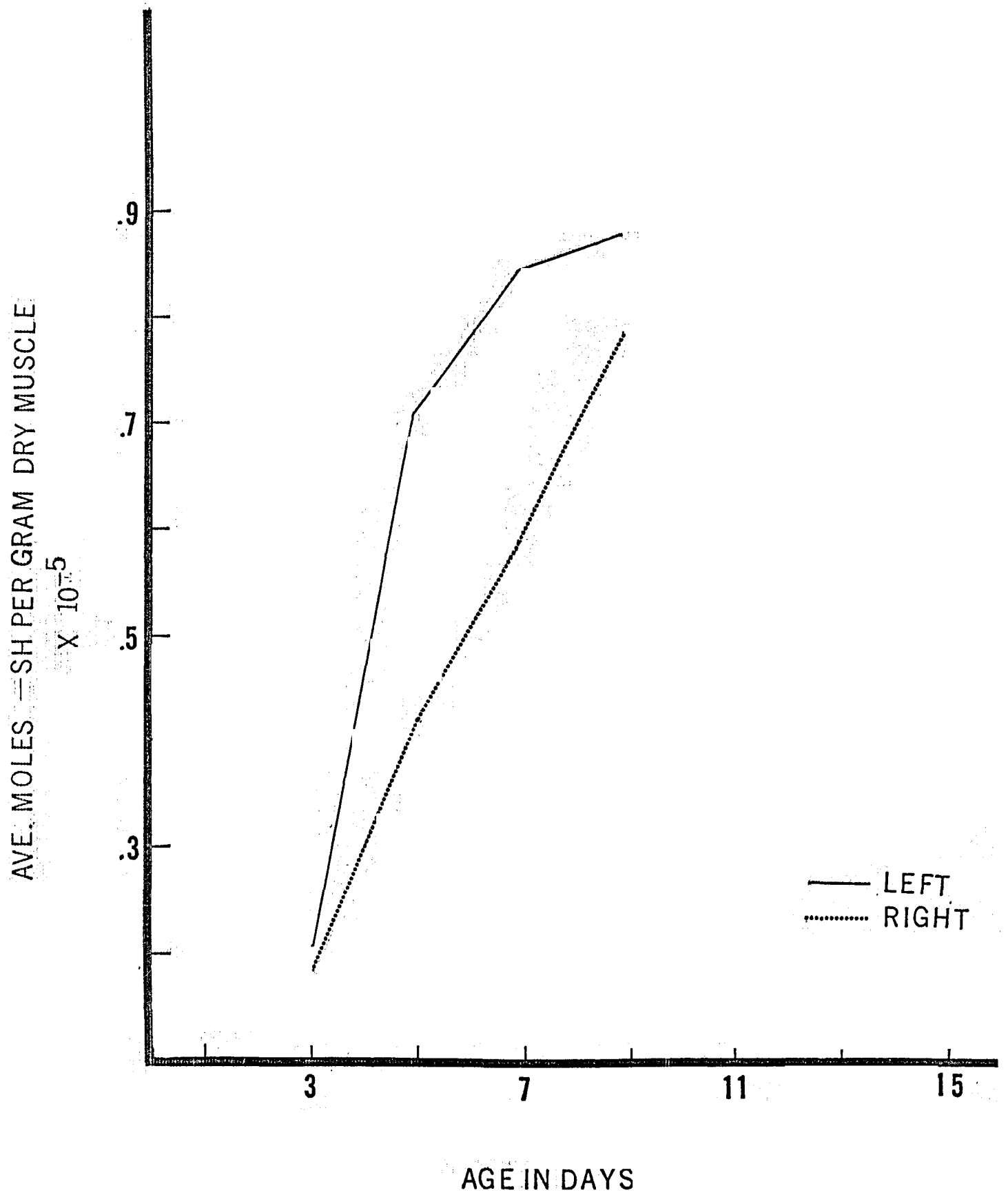

AVE. MOLES ⁻SH PER GRAM DRY MUSCLE
X 10⁻⁵
.9
.7
.5
.3
3
7
11
15
AGE IN DAYS
LEFT
RIGHT

Figure 5. Moles -SH per gram dry muscle for the left (non-irradiated) and right (irradiated) gastrocnemius of the experimental PET mouse.


AVE. MOLES —SH PER GRAM DRY MUSCLE
X 10^{-5}
.9
.7
.5
.3
3
7
11
15
LEFT
RIGHT
AGE IN DAYS

VITA

Robert Edward Shervette, III was born in Enfield, North Carolina on 13 October 1937. He attended elementary school in Enfield and was graduated from Enfield High School in May 1956. He entered Wake Forest College in September 1956. He transferred to the University of Richmond in September 1958 where he received the B. A. degree in Psychology in August 1961.

In October 1961, he entered the United States Marine Corps and served on active duty till January 1965. At present, he is a Captain in the United States Marine Corps Reserve.

In January 1965, he continued his education at the University of Richmond studying for the Master's degree in Developmental Biology. During this time, he assisted in the General Biology and Comparative Anatomy laboratories. He became a member of Beta Beta Beta, the American Society of Zoologists and the Virginia Academy of Science.

He graduated with the M. S. in Biology in August 1967. He received a teaching assistantship in the Department of Biology, University of Virginia where he plans to continue his academic career studying toward the doctoral degree.

He is married to the former Linda C. McKinney.