Tyrosinase Activity in Goldfish Skin.*† (30814)

YU MIN CHEN AND WALTER CHAVIN
Department of Biology, Wayne State University, Detroit, Mich.

A reproducible radiometric assay with high sensitivity and specificity has been developed for assay of tyrosinase activity in goldfish skin(1). Although the tyrosinase activity in the skin of 3 varieties of goldfish has been reported(2) prior to the development of the new assay, reexamination of these results became necessary as the enzyme preparations and experimental conditions have been changed. Using the new method, tyrosinase activity in the skin of 4 varieties of goldfish (white, xanthic, grey and black moor) was determined in the homogenate and its particulate and soluble fractions. In addition, the correlation of degree of pigmentation and of body weight to tyrosinase activity and the distribution of tyrosinase activity in different skin areas also were studied.

Materials and methods. A total of 79 goldfish (*Carassius auratus* L.) obtained from a commercial hatchery, were utilized. The mean weights $\pm$ s.e.$_m$ were 5.56 $\pm$ 0.47, 5.42 $\pm$ 0.28, 5.63 $\pm$ 0.30 and 6.73 $\pm$ 2.33 g for 46 black moor, 12 grey, 13 xanthic and 8 white individuals, respectively. The fish were maintained under constant conditions of temperature (25°C $\pm$ .25°C), light (12 hour photoperiod), and diet. All animals were acclimated to these laboratory conditions for a period of not less than 3 weeks prior to use. The fish were individually captured, immediately decapitated and the body skin, including scales and fins, removed without delay. In the analysis of distribution of tyrosinase in the different areas of the skin, each area was removed separately. Care was taken to avoid contamination of the samples with muscle which is tightly bound to the skin of fish. The skin was immediately placed in a tared chilled beaker, weighed, and frozen (—27°C) for at least five hours. The details of the procedure for radiometric assay of tyrosinase activity have been described(1). All assays were performed in duplicate. The enzymatic activity is expressed in tyrosinase units, in specific activity(1), and in cpm per assay. Tyrosinase activity obtained with uniformly labelled L-tyrosine-C-14 was corrected for decarboxylation and that obtained with DL-tyrosine-2-C-14 was corrected for the optical form(1,3). Calculation of tyrosinase units included previously described factors(1,3). The protein nitrogen determinations utilized the Folin-Ciocalteau procedure(4). Significance of the difference between mean values of groups was determined by analysis of variance and t-tests between groups(5).

Results. Tyrosinase occurred in both the soluble and particulate fractions of the skin homogenate of goldfish (Table I). The total tyrosinase activity increased in both fractions with increasing pigmentation from the white or xanthic variety to the black moor variety. There was no significant statistical difference between the xanthic and white varieties in regard to the homogenate or its fractions. These varieties, therefore, may be considered identical in regard to tyrosinase activity and distribution. The increase in tyrosinase activity of the homogenate was 249% from white and xanthic or 61% from grey to black moor; 575% from white and xanthic or 102% from grey to black moor in the particulate fractions; and 99% from white and xanthic or 16% from grey to black moor in the soluble fractions. The differences in tyrosinase activity of the homogenate, particulate and soluble fractions between white-xanthic, grey and black moor were significant with one exception. The tyrosinase activity of the soluble fractions of the grey and black moor were not significantly different.

Among the 4 varieties utilized, the specific activity was the highest in the homogenate, particulate and soluble fractions of the black moor. Little difference in specific activity occurred between homogenate and fractions of this variety. On the other hand, the specific

* The present investigation was supported by U.S. P.H.S. Research Grant CA-07273-02 GM from Nat. Cancer Inst. and by White Laboratories, Inc.

† Contribution 138, Dept. of Biology.

TABLE I. Comparison of Integumentary Tyrosinase Levels in 4 Color Varieties of the Goldfish, *Carassius auratus* L.

Variety	No. fish	Tyrosinase activity* (cpm/5 mg skin)			Tyrosinase units			Specific activity		
		H	P	S	H	P	S	H	P	S
Black moor	15	474 ± 62	278 ± 46	182 ± 25	805 ± 105	473 ± 78	310 ± 42	84 ± 11	74 ± 12	82 ± 15
Grey	11	295 ± 29	138 ± 16	157 ± 20	502 ± 50	234 ± 27	267 ± 35	52 ± 5	37 ± 4	71 ± 9
Xanthic	10	139 ± 22	38 ± 6	95 ± 15	237 ± 37	64 ± 3	162 ± 26	22 ± 4	10 ± 2	43 ± 7
White	8	133 ± 12	45 ± 5	88 ± 13	227 ± 21	76 ± 25	150 ± 23	24 ± 2	12 ± 1	40 ± 6

* H: homogenate; P: particulate fraction; S: soluble fraction.

activity was particularly high in the soluble fraction and particularly low in the particulate fraction of the white and xanthic varieties, and comparatively high in the soluble fraction and comparatively low in the particulate fraction of the grey variety.

The individual variance in tyrosinase activity was greatest in black moors. This variety shows a range of skin pigmentation which varies in degree of blackness as judged macroscopically. Therefore, the fish were divided arbitrarily into 3 groups, each of similar weight range: deep velvet-black, black and grey-black. The tyrosinase activity in the skin homogenate of each of these groups was compared (Table II). The tyrosinase activity of the deep velvet-black group was 612% per mg skin or 381% per fish of the grey-black group and 270% per mg skin or 217% per fish of the black group. Further, the tyrosinase activity of the black group was 226% per mg skin or 175% per fish of the grey-black group. All of these differences were statistically significant.

The relation of animal weight to enzymatic activity was studied utilizing deep velvet-black fish skin homogenate. The fish were grouped in a series of weight ranges (Table III) and the tyrosinase activities were compared statistically. No differences in the indicated parameters occurred in the weight range from 2.90-7.68 g. However, the heaviest group (10.18-17.9 g) was significantly different from all the other groups in tyrosinase activity per mg-skin and per gram-weight, but not in total activity per fish. The small number of available animals in the largest size range, may be responsible for the lack of significant difference. The pooled data of the weight range 2.90-7.68 g, indicate that the tyrosinase units are 1,121 ± 124 per mg-skin and $(23 \pm 3) \times 10^3$ per gram-weight. Thus, the smaller animals have a higher tyrosinase level in the skin than the larger animals. This difference is 311% per mg-skin and 475% per gram-weight when compared to the large fish.

The distribution of total tyrosinase activity in different parts of black moor skin was also considered. Tyrosinase activity of the left and the right sides, of the fin and the

TABLE II. Correlation of Degree of Melanin Pigmentation to Tyrosinase Activity in Homogenates of Black Moor Goldfish Skin.

Degree of pigmentation	No. fish*	Wt range, g (mean ± s.e.)	Tyrosinase units: Per mg skin	Tyrosinase units: Per fish (5×10^3)
Deep velvet-black	6 (6)	3.60–5.44 (4.56 ±.30)	1301 ± 183	123 ± 19
Black	15 (13)	3.40–6.00 (4.53 ±.24)	481 ± 29	56 ± 4
Grey-black	5 (3)	3.54–5.89 (4.59 ±.38)	213 ± 48	32 ± 7

* No. of enzyme preparations in parentheses.

TABLE III. Relation of Body Weight to Tyrosinase Activity in Deep Velvet-Black Moor Goldfish.

Wt range, g (mean ± s.e.)	No. fish	Tyrosinase units: Per mg skin	Tyrosinase units: Per g-wt (5×10^3)	Tyrosinase units: Per fish (5×10^3)
2.90– 3.60 (3.21 ± .14)	5	989 ± 95	23 ± 2	74 ± 3
3.90– 4.66 (4.32 ± .22)	3	1351 ± 239	27 ± 6	112 ± 22
5.35– 6.70 (5.83 ± .44)	3	1150 ± 414	23 ± 8	128 ± 40
6.71– 7.68 (7.18 ± .28)	3	1069 ± 409	19 ± 8	131 ± 52
10.18–17.96 (13.41 ±1.52)	5	273 ± 58	4 ± 0	57 ± 13

body skin, and of the dorsal and the ventral skin was determined. No significant difference occurred in the tyrosinase activity of the left and the right sides (Table IV). In comparing skin and fins, the major amount of tyrosinase was in the skin (Table V). The activity of the fin was approximately one-fifth of the total activity in the skin including fins. The difference between mean tyrosinase values was statistically significant. The difference in distribution of tyrosinase activity in the homogenates of the dorsal (above lateral line) and ventral (below lateral line) skin of black moor was pronounced (Table VI). The total tyrosinase activity in the ventral skin was approximately one-fifth of the total skin activity and one-fourth of the total activity in the dorsal skin. When the

TABLE IV. Distribution of Tyrosinase Activity in Left and Right Sides of the Skin of Black Moor Goldfish.

Homogenate	Tyrosinase units*: Left	Tyrosinase units*: Right	% Total tyrosinase activity†: Left	% Total tyrosinase activity†: Right
1	173	172	51.1	48.9
2	1199	1163	52.5	47.5
3	551	479	54.4	45.6
4	214	167	57.2	42.8
5	457	383	50.5	49.5
6	495	537	44.6	55.4
7	445	485	48.8	51.2
8	179	214	42.2	57.8
Mean:	464	450	50.2 ±1.74	49.8 ±1.74

* The fins were divided arbitrarily into 2 parts and each part was combined with the skin of a side.

† % Total activity = $\frac{\text{Total activ. in 1 side of skin}}{\text{Total activity in whole skin}} \times 100$.

TABLE V. Distribution of Tyrosinase Activity in the Skin and Fins of Black Moor Goldfish.

Tyrosinase units: Skin: Per mg	Tyrosinase units: Skin: Total (5×10^3)	Tyrosinase units: Fin: Per mg	Tyrosinase units: Fin: Total (5×10^3)	% total tyrosinase activity: Skin	% total tyrosinase activity: Fin
328	24	275	15	61.5	38.5
573	42	209	12	77.8	22.2
920	64	255	18	78.0	22.0
326	43	177	12	78.2	21.8
442	41	160	9	82.0	18.0
204	29	53	4	87.9	12.1
422	29	156	4	87.9	12.1
Mean: 459	39	184	10	79.0 ± 3.4	21.0 ± 3.4

TABLE VI. Distribution of Tyrosinase Activity in Dorsal and Ventral Body Skin of Black Moor Goldfish.

Tyrosinase units					
Dorsal		Ventral		% total tyrosinase activity	
Per mg	Total (5×10^3)	Per mg	Total (5×10^3)	Dorsal	Ventral
485	18	162	6	75.0	25.0
362	22	92	8	73.3	26.7
653	22	189	6	78.6	21.4
598	32	138	11	74.4	25.6
989	34	122	7	82.9	17.1
1732	52	311	12	81.3	18.8
Mean: 806	30	168	8	77.6 ± 1.6	22.4 ± 1.6

tyrosinase activity was expressed per mg-skin, the activity in the ventral skin was only one-fifth of that in the dorsal part. The difference between mean values was statistically significant.

Discussion. Tyrosinase activity in goldfish is proportional to degree of melanin pigmentation. In addition, as tyrosinase activity may vary with many factors (body weight, age, seasonal change, stress, etc.), valid correlation of any one factor with enzymatic activity is not feasible unless other variables are constant. This may be obviated to some degree as the uniform bilateral distribution of tyrosinase activity in black moor goldfish may be of utility in experimental design. Thus, elimination of individual variation may permit studies not previously possible. In one such case, the effect of freezing upon skin tyrosinase activity has been successfully studied(1).

The increase in tyrosinase activity of the particulate fraction with increasing pigmentation in goldfish skin is greater than that of the soluble fraction. Using differential centrifugation, Lerner *et al*(6) found that tyrosinase activity of Harding-Passey mouse melanoma was associated with the particulate fraction. Seiji *et al*(7) further demonstrated that tyrosinase activity was confined, for the most part, in the large granule fraction of the B-16 mouse melanoma. Thus, the relative percentage of particulate and soluble tyrosinase not only may be an index of the degree of melanin pigmentation in one species, but also characteristic of different species.

The xanthic (containing lipophores) and white (lacking lipophores) varieties of goldfish show no difference in the tyrosinase activity of the homogenate or its fractions. The tyrosinase levels are statistically identical in these two varieties. Previously, on the basis of Masson's "premelanin stain," it has been suggested that the lipophores become melanized(8). As the structure of the melanosome includes tyrosinase(7), it is difficult to rationalize identical skin tyrosinase levels in fish lacking lipophores and those containing lipophores if the hypothesis is correct. However, both varieties have tyrosinase and both varieties may be melanized(9). Therefore, the lipophore is not a melanin synthesizing unit. Indeed, the specificity of Masson's "premelanin stain" has not been demonstrated. This criticism is also true of the other argentaffin methods(10).

Tyrosinase activity in the smaller fish is higher than that in the larger fish. As tyrosinase occurs in the pigment cells in the epidermal-dermal junction of goldfish skin(11), the effect of weight may be due to a surface-volume relationship. Other factors may also be involved as the total activity of the whole skin is also decreased. In part, this may result from increased skin thickness and/or physiological factors.

In a comparison of the results of the present study with those previously reported(2), the tyrosinase activity in 3 different varieties of goldfish has been found to be different. In the previously utilized assay, 72 μg DL-tyrosine-2-C-14 and 3.94 μg DL-dopa were used in the 1 ml incubation mixture rather than 40 μg uniformly labelled L-tyrosine-C-14 (or DL-tyrosine-2-C-14) and 4 μg DL-dopa (or 2 μg L-dopa) of the new radiometric

assay. In the 2 assay procedures, the degree of substrate saturation levels can be assumed to be similar except for the specific activity of the radio-tyrosine used. The probable reasons for the previously reported lower tyrosinase activities are: (a) incomplete extraction of enzyme, (b) loss of enzymatic activity by use of low speed centrifugation to obtain a clear homogenate, and (c) incomplete collection of the reaction product (melanin).

Summary. The tyrosinase activity in 4 varieties of goldfish (*Carassius auratus* L.), white, xanthic, grey and black moor, was determined. Tyrosinase activity occurred in both the soluble and particulate fractions of skin homogenates. Total tyrosinase activity was increased in both fractions with increasing melanin pigmentation. The activity increase, however, occurred, for the most part, in the particulate fraction. White and xanthic goldfish were identical in tyrosinase content and distribution. Tyrosinase activity in black moor skin is: skin to fin ratio, 4:1; dorsal skin to ventral skin ratio, 4:1; right side to left side ratio, 1:1. Further, smaller fish have a higher skin tyrosinase level than larger fish.

1. Chen, Y. M., Chavin, W., Anal. Biochem., 1965, v13, 234.

2. Kim, K. H., Tchen, T. T., Chavin, W., Biochem. Biophys. Acta, 1962, v59, 577.

3. Chen, Y. M., Chavin, W., Nature, 1965, in press.

4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.

5. Bailey, N. T. J., Statistical Methods in Biology, Wiley & Sons, N. Y., 1959.

6. Lerner, A. B., Fitzpatrick, T. B., Calkins, E., Summerson, W. H., J. Biol. Chem., 1949, v178, 185.

7. Seiji, M., Shimao, K., Birbeck, M. S. C., Fitzpatrick, T. B., Ann. N. Y. Acad. Sci., 1963, v100, 497.

8. Mishima, Y., Loud, A. V., J. Cell Biol., 1963, v18, 181.

9. Matsumoto, J., Jap. J. Zool., 1965, v14, 45.

10. Pearse, A. G. E., Histochemistry, Theorectical and Applied, Little, Brown & Co., Boston, 1960.

11. Chavin, W., J. Exp. Zool., 1965, v133, 1.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Effects of Glucagon and Epinephrine in Fasted Dogs.* (30815)

J. DE PLAEN† AND G. GALANSINO

Department of Physiology, The Chicago Medical School, Chicago, Ill.

Glucagon and epinephrine have a major common effect *in vivo* and *in vitro*: liver glycogenolysis as evidenced by hyperglycemia. Another common effect of both hormones has been noted *in vitro*: lypolysis as measured by FFA release(1,2). This action of epinephrine has received innumerable confirmations with *in vivo* preparations(3,4), but a lipolytic action of glycagon in similar conditions has not been observed(5,6).

In vivo experiments may introduce several variables: length of fasting, diminishing the effectiveness of insulin(7); consciousness or type and duration of anesthesia, affecting the level of circulating cathecholamines(8). Since catecholamines are dominant factors controlling lipolysis and insulin is essential for lipogenesis(9), these variables may influence the basal state of the animal and need to be evaluated.

The purpose of this paper is to assess the effect of glucagon on plasma FFA *in vivo* and to compare it with that of epinephrine, taking into consideration the variables just mentioned.

Methods. Healthy mongrel dogs of both sexes, weighing 9-15 kg, were kept in metabolic cages on a regular diet for at least 2 weeks prior to the first experiment and thereafter. An interval of 2 weeks was allowed between experiments performed on the same dog. Experiments were carried out in anesthetized (pentobarbital sodium I.V. 35 mg/kg) and in unanesthetized animals. Two periods of fasting (24-hr and 48-hr) were stud-

* Aided by Grant TI AM 5102 from Nat. Inst. of Arthritis & Metab. Dis., U.S.P.H.S.

† Post-doctoral Trainee.