FLAVIN AND MELANIN IN CAT AND
DOG CHOROIDS

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(Received February 2, 1973)

The association of RF² with melanin from cat and dog choroids was shown by dialysis and spectrophotometric methods. The association obeyed Freundlich's isotherm, and RF was liberated from RF-melanin complex by gel chromatography and by addition of CPZ. The liberation of flavin from cat choroid was not affected by Pronase treatment, though that from kidney was accelerated. It was also shown that flavins in cat choroid are not degraded by illumination. From above findings it was deduced that most of flavin in cat and dog choroids is associated with melanin.

In previous reports we found that the flavin content in cat choroid was related to the presence of melanin (1), and that the extraction of flavin from cat choroid was apparently more difficult than from liver and kidney (2). Similar relation was also reported by Aso et al. (3) in human and animal skin and hair, and it is well known that flavin associates with many aromatic compounds (4). In this report we show that RF associates with melanin and that flavin in the choroids is in associated form with melanin.

EXPERIMENTAL

[2-¹⁴C]RF (specific activity 61mCi/mmole, purity 99.9%) was obtained from the Radiochemical Centre; CPZ hydrochloride was a gift from Yoshitomi Pharmaceutical Industries, Ltd. Pronase (45,000 PUK/g) was the product of Kaken Chemical, Ltd.

Cat melanin was prepared from the residual choroid from which NF was extracted with hot water (5). The residue from about 200 g of cat choroid was

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² Abbreviations used are: RF, riboflavin; CPZ, chlorpromazine; NF, neoflavin; LF, lumiflavin; LC, lumichrome; FAD, flavin adenine dinucleotide.
suspended in 800 ml of water, and the suspension was neutralized with 0.1 M NaOH, and centrifuged at 6,000 × g for 10 min. (All the centrifugation in this paper was done at 6,000 × g) The precipitate was washed twice with 800 ml portions of water. The lipid was removed from the residue by washing twice with 650 ml portions of methanol and twice with methanol-ether (1:3 by volume) successively. The residue was suspended in 500 ml of 1 M KOH and stirred for 4 hr at 37℃. After standing at 30℃ for 40 hr, and acidification to pH 2 with conc. HCl, the suspension was centrifuged. The precipitate was washed with 400 ml of water, and then suspended in 400 ml of 5% aqueous solution of trichloracetic acid and heated at 90℃ for 15 min. After cooling and centrifugation, the precipitate was washed with 400 ml of the same solution of trichloracetic acid and 250 ml of 0.15 M Na₂HPO₄ successively. The residue was suspended in 100 ml of 0.2 M phosphate buffer (pH 7.4), and 90 mg of Pronase and 3 ml of ethanol (to prevent putrefaction) were added to the suspension, which was incubated at 30℃ for 4 days. After centrifugation the precipitate was retreated with Pronase in the same way. The residue was washed three times with 500 ml portions of water, twice with 150 ml portions of absolute ethanol and once with 150 ml of ether (cat melanin I). Yield, 6.4 g.

Three grams of cat melanin I was ground in a mortar and suspended in 100 ml of water. The suspension was centrifuged, and the supernatant was concentrated to about 10 ml in a rotary evaporator and centrifuged again. The dark brown supernatant were added to a column of Sephadex G-50 (medium, 3 × 73 cm) and eluted with water. The dark brown fraction eluted from the bed was collected and concentrated in a rotary evaporator. The concentrate was centrifuged, and the supernatant (cat melanin II) was used in the experiments. Elementary analysis of the dried preparation of cat melanin II; C, 48.57; H, 4.31; N, 11.66; S, 1.49; ash, 12.82%.

Two grams of cat melanin I was treated with 50 ml of conc. HCl for 3 weeks at room temperature. After centrifugation the residue was washed with 0.5 M HCl (twice), water (4 times), ethyl alcohol (twice) and ether (cat melanin III). Yield, 1.5 g. Elementary analysis; C, 54.32; H, 4.66; N, 9.56; S, 1.89; ash, 0.00%.

Dog melanin II corresponding to cat melanin II was also prepared in a similar way. About 200 g of dog choroid were suspended in 800 ml of water and heated at 80℃ for 3 min. The suspension was homogenized in a Waring blender for 10 min. The homogenate, mixed with 20 ml of 80% formic acid, was stirred for 30 min, and centrifuged. After retreatment with 700 ml of 0.4 M formic acid, the residue was treated in the same way as cat choroid. Elementary analysis of the dried dog melanin II; C, 49.22; H, 4.33; N, 10.91; S, 1.96; ash, 13.51%.

Other materials and methods were described in a previous report (7).
1. Pronase treatment of cat choroid and liberation of flavin

The amount of flavin liberated from cat choroid incubated with Pronase was compared to that of the control choroid incubated without Pronase. As a control, the cortices of cat kidney were incubated in the same way as choroids with and without Pronase, and the amounts of liberated flavin were estimated.

Table 1. Liberation of flavin from cat choroid and cortex of kidney by treatment with Pronase.

Paired groups of 5 pieces of cat choroid were prepared from 5 pairs of eyes. One group suspended in 5 ml of water was heated at 80°C for 3 min to denature protein (pretreatment). After cooling, the suspension was centrifuged and the flavin in the supernatant was estimated. The residue was suspended in 5 ml of 0.2 M phosphate buffer (pH 7.4), and 3 mg of Pronase and 0.15 ml of ethanol were added. After incubation at 30°C for 4 days, the liberated flavin in the supernatant was estimated. The residue was treated with Pronase further twice in the same way, and the liberated flavin was estimated. The flavin bound with the last residue was also estimated by extraction with 25 ml of hot water. Another group was treated in the same way without addition of Pronase. Two samples of cortex from a cat kidney were treated in the same way as choroid, i.e. one with Pronase and another without, and the liberated and bound flavin was estimated.

| Tissue        | Weight (mg) | Pronase | Liberated by | Remaining in residue | Total |
|---------------|-------------|---------|--------------|----------------------|-------|
|               |             |         | Pre-treatment (µg) | 1st (µg) | 2nd (µg) | 3rd (µg) |       |
| Choroid       | 249         | +       | 2.9          | 3.0       | 1.9      | 1.9      | 11.6  | 21.3  |
|               | 269         | -       | 4.6          | 2.8       | 1.8      | 1.8      | 10.2  | 21.2  |
| Cortex of kidney | 301    | +       | 2.8          | 19.4     | 5.7      | 2.1      | 1.7   | 31.7  |
|               | 301         | -       | 2.1          | 9.0       | 4.3      | 2.0      | 10.4  | 27.8  |

+ means incubation with Pronase, and - without.

Table 1 shows that nearly the same amount of flavin was liberated from Pronase-treated and untreated choroids, and that about a half of flavin was bound to the residue even after the third treatment. However, more flavin was liberated from Pronase-treated cortex than from untreated, and most was liberated by the first treatment. This means that the flavins in the cortex are associated with protein or some other substance digestable with Pronase, but the flavin in the choroid is associated with other insoluble substance, which may be melanin.

2. Association of RF with melanins from cat and dog choroids

Cat and dog melanin IIIs in cellophane tubes were dialyzed against aqueous solutions of various concentrations of [14C]RF, and after equilibration the amounts
Fig. 1. Association of RF with melanins from cat and dog choroids. One ml of aqueous solution of cat melanin II (4.05 mg) in a Visking cellophane tube (No. 0, diameter 6.4 mm) and that of dog melanin II (3.64 mg) were dialyzed in the same beaker against 50 ml of 0.05 M phosphate buffer (pH 7.0) containing various amount of [14C]RF (21.8 to 174.4 µg or 32 to 256 mCi) at 5°C until the radioactivity of the dialysate became constant (6 days). The difference of the specific radioactivity between the dialysate and the content of cellophane tube was assumed to be the radioactivity of RF associated with melanin. The amount of RF was calculated from the radioactivity which was measured by a gas flow counter, Aloka FC 1E. log (f/m) was plotted against log c, f, amount of RF (µg) associated with melanin; m, amount of melanin (mg); c, concentration of RF in dialysate (µg/ml). The regression lines were obtained by least square method.

Fig. 1 shows that log (f/m) is linear to log c, that is, Freundlich's isotherm (6) is applicable to the association of RF with melanin;

\[ \log (f/m) = a + n \log c \]

or

\[ f/m = kc^n \]

where k and n are constants and log k = a. In the case of cat and dog melanin, a is 0.635 and 0.557, k is 4.32 and 3.60, n is 0.716 and 0.739, respectively.

If it is assumed that in choroids flavin is associated only with melanin and has the same affinity as in the above experiment, we can calculate the percentages of associated flavin from the contents of melanin, water, and flavin (I) in the choroids. Previously (7) we reported the contents of melanin (corresponding
ing to melanin I) and water in cat and dog choroids: the former, 5.3 and 5.0\%, and the latter, 74.1 and 78.0\% in fresh tissues. Assuming that melanin Is of both animals have the same affinity for RF as melanin IIIs, the percentages of associated flavin were calculated as 99.6 and 99.8\% at least in cat and dog choroids, respectively, i.e. almost all of flavin is in associated form. The concentrations of dissociated flavin in cat choroid was also calculated as about 20 times of that in dog. This may suggest an occurrence of mechanism concentrating RF in cat choroid. The high content of flavin in cat choroid may be not always explained only by the affinity of RF for melanin.

The cat and dog melanin IIIs contain impurities such as ash and ninhydrin reactive substance which are released by conc. HCl-treatment. In order to decide whether such impurities contribute to association of cat melanin II with RF, the affinities of cat melanin II and III for RF were compared by dialysis method similar to that described in Fig. 1.

### Table 2. Association of RF with HCl-treated cat melanin.

A solution of cat melanin II (4.58 mg in 1 ml of 0.05 M phosphate buffer, pH 7.0) and a suspension of III (5.73 mg in 1 ml of the buffer) in cellophane tubes were dialyzed in a beaker against 200 ml of the buffer containing [14C]RF (1 μCi or 0.56 mg) at 5°C until the radioactivity of the dialysate became constant (for 8 days). See Fig. 1 for other conditions.

| Radioactivity of RF in dialysate (cpm/ml) | Radioactivity of RF associated with melanin II (cpm/mg) | Radioactivity of RF associated with melanin III (cpm/mg) |
|-------------------------------------------|--------------------------------------------------------|-------------------------------------------------------|
| 3,586                                     | 11,425                                                 | 13,346                                                 |

Table 2 shows that the ratio of affinities of cat melanin II and III for RF (affinity is defined as amount of RF associated with melanin/weight of melanin) is 1:1.17. This may suggest that melanin II contains 17\% of impurities which don’t contribute to association with RF, and that RF associates with melanin itself.

3. **Absorption spectra of cat melanin, RF, and their mixture**

The absorption spectra of cat melanin II, RF, in 0.05 M phosphate buffer (pH 7.0) and their mixture, in the same concentration as in each solution, were measured by a Hitachi recording spectrophotometer EPS–2U (Fig. 2). Though the concentrations of each component in the mixture were equal to those in solutions of each, the absorbance of RF-melanin mixture was unequal to the sum of the absorbances of both components, i.e. the difference of absorbance between the mixture and the sum of the components was not zero. This demonstrates the complex formation between RF and melanin.
4. Dissociation of RF-melanin complex by gel chromatography

The aqueous solution of cat melanin II equilibrated with solution of [14C]RF was chromatographed on a column of Sephadex G–10, and melanin was separated from RF (originally unassociated and dissociated by chromatography) (Fig. 3). The melanin fraction was rechromatographed, and dissociated flavin was separated from melanin. The percentages of RF, free and associated with melanin, before and after chromatography, were calculated (Table 3).

Figure 3 and Table 3 show that 95.6% of the associated RF with melanin were dissociated by the first gel chromatography, and that small part of RF (about 2% of added) was still associated with melanin after the second chromatography. Association of RF with melanin would be essentially reversible and small amount of RF, tightly associated with melanin, would dissociate slowly.

5. Liberation of RF from RF-melanin complex by CPZ

The mixture of dog melanin II and [14C]RF in a cellophane tube was dialyzed
Fig. 3. Elution pattern of cat melanin equilibrated with RF from a column of Sephadex G–10. The aqueous solution of cat melanin preparation (4.06 mg in 2 ml) in a cellophane tube was dialyzed against 50 ml of aqueous solution of [14C]RF (0.4 μCi, 24.6 μg) at 5°C until the radioactivity of the dialysate became constant (9 days). An aliquot (0.58 ml) of the content of the cellophane tube was charged to a column of Sephadex G–10 (medium, 0.9 × 26 cm) and eluted with water at a rate of 0.5 ml/hr. Fractions (1 ml) were collected, and mixed with 2 ml portions of water. The absorbances at 450 mμ and the radioactivities in 1 ml aliquots of the diluted fractions were measured. ———, absorbance; ———, radioactivity.

Table 3. Percentage of free and associated RF with melanin before and after gel chromatography.

|                        | RF associated with melanin (%) | Free RF (%) |
|------------------------|-------------------------------|------------|
| Before the 1st gel chromatography<sup>a</sup> | 91.5                          | 8.5        |
| After the 1st gel chromatography<sup>b</sup>  | 3.7                           | 96.3       |
| After the 2nd gel chromatography<sup>c</sup> | 61.5                          | 38.5       |

<sup>a</sup> The equilibrated solution of cat melanin and [14C]RF in Fig. 3. The amount of associated RF was calculated as described in Fig. 1.

<sup>b</sup> Calculated from radioactivities of melanin and RF fractions in Fig. 3.

<sup>c</sup> The melanin fractions in Fig. 3 (tube No. 5–9) were combined and concentrated to about 1 ml in a rotary evaporator. The concentrate was rechromatographed on the same column of Sephadex, and the absorbance and radioactivity were measured as described in Fig. 3.

against phosphate buffer. When an equilibrium was reached, CPZ hydrochloride was added to the dialysate, and the change of the concentration of RF
Fig. 4. Dissociation of RF from RF-melanin complex by CPZ. The mixture of aqueous solution of dog melanin II (3.82 mg in 1.06 ml) and of [¹⁴C]RF (6.56 μmole or 0.4 μCi in 0.04 ml) were stood at 5°C for 5 hr. 1.04 ml of the mixture in a cellophane tube were dialyzed at 5°C against 50 ml of 0.05M phosphate buffer (pH 7.0) which was stirred by a magnetic stirrer. The radioactivity of 0.5 ml aliquot of the dialysate was measured every day. When the radioactivity became constant, 0.5 ml of aqueous solution of CPZ hydrochloride (5.1 μmoles) was added to the dialysate (1). When the radioactivity of the dialysate became constant again, further 1 ml of the solution of CPZ was added (2), and the radioactivity was followed for a week.

in the dialysate was followed. Figure 4 shows that RF associated with melanin was liberated by the addition of CPZ.

At the first equilibrium where CPZ was not added, 48.3% of RF added were associated with melanin. At the second and third equilibrium where 5.1 and 15.3 μmoles of CPZ were added, 17.5 and 6.9% of RF added were associated with melanin, respectively. Essentially the same result was obtained in the preliminary experiment using cat melanin II (2).

Two mechanisms are probable for this phenomenon. One is that CPZ replace RF associated with melanin. Another is that CPZ associates with RF and shifts the equilibrium between RF and melanin. The former mechanism is supported by the studies by many investigators. BLOIS (8), BLOIS and TASKOVICH (9), and PORTS (10) showed that CPZ associates with melanin. YAGI et al. (11) demonstrated that CPZ associates with D-amino acid oxidase and inhibits its activity competitively. From these reports it will be probable that CPZ and RF compete in association with melanin. Of course, more quantitative investigation is necessary to decide whether it is competitive or not. The latter mechanism is also probable from the report of YAGI et al. (12), who demonstrated that CPZ
associates with isoalloxazine ring of FAD, and calculated that the dissociation constant is $1 \times 10^{-3}$ mole/liter. Assuming that RF-CPZ complex has the same dissociation constant as FAD-CPZ complex, when 5.1 $\mu$moles of CPZ is added to the dialysate of the first equilibrium, it is calculated that about 9% of RF in the dialysate will form complex with CPZ. Therefore, our finding would be explained by combination of both mechanisms.

6. **Photostability of flavin in cat choroid**

One group of cat choroid from pairs of eyes was illuminated and the other was not. The flavin and LF contents of the groups were measured. Table 4 demonstrates that there was no significant difference of flavin content between illuminated and control choroids, and that LF was detected in neither illuminated nor control choroids.

Table 4. Amount of flavins in illuminated cat choroid.

| Choroid     | Weight (mg) | LF ($\mu$g) | Flavin ($\mu$g) |
|-------------|-------------|-------------|-----------------|
| Illuminated | 332         | 0.0         | 26.3            |
| Control     | 293         | 0.0         | 24.6            |

The thin layer chromatogram of extracts from illuminated and unilluminated choroids is shown in Fig. 5. That of illuminated solution of RF is also shown for comparison. Figure 5 demonstrates that none of the fluorescent photodegradation products of RF was detected in the extracts of illuminated and control choroids by thin layer chromatography.

The above experiments show that flavin in cat choroid is stable in light. Of course, the melanin in the choroid will shield the light and protect the flavin from photodecomposition, but it might be unable to protect the flavin in tapetum. It was deduced that cat tapetum, at least, has some substance or substances to protect flavin from photodecomposition. In frog’s skin a similar phenomenon was reported by YAGI, who showed that a pteridine compound plays a role (13). The photostability of flavin in cat choroid may be, at least partially, explained by association of flavin with melanin and rodlet in tapetum (14) (see Discussion), because it is known that melanin is a phenolic compound and that phenol protect RF from photodecomposition (15, 16).
DISCUSSION

The experiments described above show that most of flavin in cat and dog choroids is associated with melanosins of both animals and not with proteins, and that flavin in cat choroid is stabilized against light by association with melanin. However, the purity of melanin preparations used in experiments must be considered. For preparation of "pure" melanin, conc. HCl-treatment is used generally, though some part of the structure may be destroyed (17). As Table 2 shows that HCl-treated cat melanin III has higher amity for RF than cat melanin II. Therefore, it is highly probable that RF-binding property is that of melanin itself and not of impurities, and that the results obtained by using melanin II would, at least qualitatively, represent the properties of melanin III, "pure" melanin. But cat melanin I is contaminated, II and III may be also, with some impurity from cat tapetum. The amount of this impurity is calculated to be about 8% from the weight percentage of tapetum and the content of melanin in the choroid (7). This impurity might bind flavin; because the white choroid of cat having tapetum contains more flavin than that having no tapetum and than dog choroid (1); and Elliot and Futterman (18) described that they could observe the binding of flavin with rodlets in cat tapetum. Though rodlets have never been characterized chemically, Bernstein and Pease (14) supposed that it might be a derivative of melanin granul on the basis of electron microscopy findings. Thus, this impurity might contribute partially to flavin binding capacity of cat melanin preparation. However, the melanin preparation from dog choroid is not con-
taminated with this kind of impurity, because dog tapetum does not contain rodlets, though it contains a zinc-cystein complex which is solubilized and removed by dilute formic acid (19). At any rate it is certain that melanin associates with RF.

The nature of interaction between RF and melanin must be considered. An acid-base interaction is improbable because RF is neutral at pH 4–7.5 and is too weak as acid or base to interact with melanin at physiological pH; pKₐ, 10.2 and pKₐ, 1.7 (20). The interaction may be of the charge transfer or hydrogen bond type.

The liberation of RF from RF-melanin complex by CPZ is very interesting from the standpoint of ophthalmology. It is known that phenothiazines are concentrated in uvea of eyes (10, 21, 22), and that a long-term administration of phenothiazines (23, 24) or chloroquine (25) injures uvea and retina. One of the mechanisms of the injury is considered to be the association of these drugs with melanin in eyes (10, 25). It is probable that the deprivation of RF bound to melanin with these drugs is a mechanism of the injury.

We thank Prof. K. Uehara of Osaka University for his kind discussion, Yoshitomi Pharmaceutical Industries, Ltd. for a gift of CPZ hydrochloride, and Dr. H. Morimoto of Takeda Chemical Laboratories, Takeda Chemical Industries, Ltd. for elementary analyses of melanins.

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