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# Methaemoglobin-catalysed Formation of Dopa and 6-OH-Dopa from Tyrosine

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Received June 18, 1982

*Abstract.* An extract of bovine retina and choroid with tyrosine hydroxylating and dopa-oxidizing capacity also showed marked formation of 6-OH-dopa on incubation with tyrosine and ascorbic acid. The extract contained appreciable amounts of methaemoglobin as determined spectrophotometrically, and boiled extracts also showed catalytic activity. The effect of methaemoglobin on the oxidation of tyrosine and dopa was therefore investigated. Methaemoglobin catalysed the formation of dopa and 6-OH-dopa in the presence of tyrosine and ascorbic acid. Hydrogen peroxide plays an important role in this reaction: the rates of formation of both dopa and 6-OH-dopa were increased by addition of hydrogen peroxide but diminished by addition of catalase. Methaemoglobin also catalysed the formation of cysteinyldopa from dopa and cysteine.

*Key words:* Methaemoglobin; Dopa; 6-OH-Dopa; 5-OH-Dopa; 5-S-Cysteinyldopa; Catalase; Hydrogen peroxide

The possible role of trihydroxy derivatives of phenylalanine in melanin biosynthesis has been investigated (3, 8, 9, 11–13, 15), and a methylated derivative of 6-hydroxydopa has been identified as a product of the microorganism *Microspira tyrosinatica* by direct comparison with an authentic sample (9). Recent work has demonstrated the formation of another trihydroxy compound, 5-OH-dopa, by mushroom tyrosinase (5, 6), and 5-OH-dopa has also been found to be a substrate of tyrosinase (1).

We now report the formation of 6-OH-dopa on incubation of tyrosine and ascorbic acid with an extract of bovine choroid–retinal pigment epithelium. Bovine retina and choroid were chosen because these melanin-containing tissues are readily available. It turned out however that the dominant catalytic activity of our extracts was not related to tyrosinase, but to haem pigment.

## EXPERIMENTS AND RESULTS

Retina and choroidea from 50 bovine eyes (weight 33 g) obtained from a slaughter-house were dissected and kept on ice. Phosphate buffer, 0.01 M, pH 6.5, was added to 200 ml. The tissue was finely chopped with scissors, and homogenized with a glass homogenizer. The homogenate was kept overnight at −20°C. After thawing, it was treated with ultrasound for 20 min on ice, the temperature being kept below 5°C. Large particles of debris were separated by centrifugating at 200×*g* for 5 minutes, and the supernatant was centrifuged at 30 000×*g* for 10 minutes. Ammonium sulphate was added to the supernatant to 10% saturation, and after 1 hour at 0°C recentrifugation at 30 000×g for 1 hour was performed. There was very little sediment. Ammonium sulphate was added to the supernatant to 60% saturation. The precipitate was suspended in 50 ml 0.5 M phosphate buffer, pH 6.5.

The various sediments and supernatants were examined for dopa-oxidizing capacity. Dopa and cysteine were added to each sample to a final concentration of $10^{-3}$ M

Table 1. *Formation of dopa and 6-OH-dopa (μg/ml) on incubation of tyrosine, $10^{-3}$ M, with methaemoglobin (200 μg/ml) in the presence of ascorbic acid ($10^{-2}$ M) in 1 ml 0.5 M phosphate buffer, pH 6.5, at 37°C for 30 min*

Addition of catalase (10 μg/ml) reduced the rate of hydroxylation, and addition of $H_2O_2$ ($3\times10^{-3}$ M) increased the rate of hydroxylation

| Methaemoglobin | | Methaemoglobin+catalase | | Methaemoglobin+$H_2O_2$ | |
|---|---|---|---|---|---|
| Dopa | 6-OH-Dopa | Dopa | 6-OH-Dopa | Dopa | 6-OH-Dopa |
| 1.5 | 2.2 | 0.35 | 0.86 | 5.3 | 5.0 |
| 1.7 | 2.3 | 0.24 | 0.74 | 5.7 | 5.2 |
| 1.5 | 1.8 | 0.32 | 0.74 | 6.6 | 6.2 |
| 1.7 | 2.2 | 0.45 | 0.82 | 5.5 | 5.2 |

and $10^{-2}$ M, respectively, and were permitted to react at 0°C for 10 min with air bubbling. The formation of 5-S-cysteinyldopa was determined by HPLC and electrochemical detection (5). The precipitate obtained with ammonium sulphate to 60% saturation showed the greatest dopa oxidation capacity. This fraction was also analysed for its capacity to hydroxylate tyrosine to dopa. Tyrosine and ascorbic acid in 0.5 M phosphate buffer were added to final concentrations of $10^{-3}$ M and $2\times10^{-2}$ M, respectively, and were permitted to react for 30 min at 25°C with air bubbling.

A control using a boiled sample was run simultaneously. HPLC analysis was done as described earlier (5). A LKB 2091 mass spectrometer equipped with a glass column packed with 3% OV 17 was used for the mass spectrometric analysis of the pentafluor-acetylated methyl ester of 6-OH-dopa (5).

The hydroxylation was initially small, and did not differ from that in the control. The rate of dopa formation was 3 μmoles/h/g protein, whereas the rate of dopa oxidation determined as 5-S-cysteinyldopa formation was 1 mole/h/g protein. A considerable quantity of a substance with the properties of 6-OH-dopa was formed in the incubate containing tyrosine and ascorbic acid. The compound had the same retention time as 6-OH-dopa in several systems on high-pressure chromatography. The quantity present was about the same as the quantity of dopa. For further identification, ion-exchange chromatography with subsequent gas-chromatography mass-spectroscopy was used (5). The derivative of the substance formed in our incubate had the same gas-chromatographic properties as 6-OH-dopa. The fragmentation pattern obtained on mass spectroscopy was the same as that of authentic 6-OH-dopa with a molecular ion at $^m/_e$= 811 and the base peak at $^m/_e$=485. The substance was formed in similar amounts in the boiled sample.

The UV spectrum of the incubate with the greatest dopa-oxidizing capacity showed a strong absorption at 407 nm and weak bands at 536, 576, and 630 nm; after reduction a strong band was found at 430 and a weak one at 555 nm. These bands suggest that a haem-containing compound was responsible for the oxidative reactions, and the bovine eye extract was therefore replaced by purified methaemoglobin for the incubation of tyrosine and ascorbic acid. The HPLC analysis of the incubate showed a marked ability of methaemoglobin to catalyse the formation of both dopa and 6-OH-dopa from tyrosine, in analogy to the bovine-eye extract.

Addition of hydrogen peroxide to the methaemoglobin incubate increased the formation of both dopa and 6-OH-dopa. 5-OH-dopa was also found under these conditions in amounts about 10% of the 6-OH-dopa value. The importance of hydrogen peroxide for the hydroxylation reaction was further shown by addition of catalase to the incubate, which led to inhibition of both the hydroxylating steps (Table 1).

The catalysis of dopaquinone formation by methaemoglobin was studied in a system where 193 μg L-dopa and 1.21 mg L-cysteine were incubated with 200 μg methaemoglobin in 1 ml 0.5 M phosphate buffer, pH 6.5, at 37°C. Under these conditions 4.5 μg 5-S-cysteinyldopa could be demonstrated after 10 minutes. Because 5-S-cysteinyldopa is only one of the cysteinyldopas formed, and because further oxidation of cysteinyldopa occurs, the quantity of 5-S-cystinyldopa found is a minimum figure for dopaquinone formation.

## DISCUSSION

The oxidative reactions described may have biological implications and must be considered in investigations of melanin precursors. Other haem-containing compounds may have similar functions.

The formation of 6-OH-dopa by incubation of tyrosine with the tissue extract in the presence of ascorbic acid was demonstrated by several different chromatographic methods and finally by comparison with authentic material in gas-chromatography mass-spectroscopy.

The extract forming 6-OH-dopa also had a marked capacity to hydroxylate tyrosine and oxidize dopa. It is obvious that the 6-OH-dopa production was not caused by tyrosinase activity in our extract, because boiling the extract slightly increased the production of 6-OH-dopa. Udenfriend

et al. (16) demonstrated the hydroxylating power of ferrous and ferric ions in the presence of ascorbic acid and EDTA: ascorbic acid could be replaced by other compounds with an ene-diol or a diketo structure.

Ito et al. (7) studied the oxidizing properties of Fenton's reagent at neutral pH. They found marked formation of cysteinyldopas when dopa and cysteine were oxidized by $FeSO_4$ and $H_2O_2$ in the presence of EDTA. The hydroxyl radical was proposed to mediate the dopa oxidation. The reported yields of cysteinyldopas and the recovered dopa accounted for almost all dopa originally present, and no formation of hydroxylated compounds such as 6-OH-dopa was reported.

Although the hydroxylating properties of the ferrous–ascorbate complex have been examined in several studies, the ultimate hydroxylating species has not been settled (4). The addition of superoxide ion to an ortho-semi-quinone followed by an intramolecular reaction forming a cyclic peroxide has been proposed: the cyclic peroxide opens to form a hydroxylated quinone, a compund compatible with the reaction products found (10). In a recent kinetic study on the mechanism of oxidation of a substituted *o*-benzoquinone with $H_2O_2$, a nucleophilic 1,2-additon reaction of the $H_2O^-$-ion to one of the carbonyl groups was assumed. A hydroxylated *o*-quinone was established as one of the reaction products, and the mechanism for its formation was proposed to incorporate an epoxide as an intermediate (14).

The results presented in this paper can contribute to the understanding of the mechanism of aromatic hydroxylation. The formation of 6-OH-dopa shows that the ferric–ascorbate complex can conduct two consecutive hydroxylating steps. The second hydroxylating step introduces the hydroxyl group predominantly in the meta-position to the closest hydroxyl substituent.

The mechanism of the formation of 6-OH-dopa demonstrated here is shown to be quite different from that of the formation of 5-OH-dopa by tyrosinase (1).

Many metal ions are bound to melanin, and the conditions for the reaction of the methaemoglobin-type may be present also in vivo in melanin-containing cells. It may be speculated that the preferential toxicity of ascorbic acid to malignant melanoma cells in culture is related to the formation of cytotoxic products such as 6-OH-dopa (2, 17).

## ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Cancer Society (project 626-B82-10XA), the Swedish Medical Research Council, the Thure Carlsson Foundation for Scientific Research, the Walter, Ellen, and Lennart Hesselman Foundation for Scientific Research, the Edvard Welander Foundation for Scientific Research, the donation funds of the University Hospital at Lund, and the donation funds of the Faculty of Medicine, University of Lund, and the Finsen Foundation for scientific Research.

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## Skin Surface and Open Comedone pH in Acne Patients

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Received July 8, 1982

*Abstract.* To assess the variability of surface and comedone pH in one and the same individual and between individuals, pH measurements were made on extracted single comedone material and on the adjacent skin surface, at face and back sites on 56 acne patients. The pH values were normally distributed. The mean values were: comedone pH 5.52 and surface pH, 5.26. The pH ranges were similar: comedones 4.01 to 6.85 and surface, 4.10 to 6.81. There was no correlation between comedone pH and adjacent skin surface pH.On each person and at one site there was variability of surface pH and comedone pH. Variability of pH from person to person was greater than within an individual at the same site.

*Key words:* pH measurement; Skin surface; Open comedone

Acne is a pathological condition of the pilosebaceous system and it has been suggested (1) that if environmental changes occur within individual follicles this will lead to a change in the physiology of the resident microflora which would be the initial step in triggering the production of non-inflamed and inflamed lesions. The pH of a follicle is a variable which would affect the physiology of the bacteria.

The contributory factors influencing the pH of both the skin surface and follicles are water-soluble components from transepidermal water loss, cellular excretions and sebum. Such components would be lactic acid, glutamic and aspartic acids, other amino acids, proteins and free fatty acids. The variation in pH from site to site on the same person and from follicle to follicle at the same site will be influenced by the proportions of these components.

The aim of this investigation was firstly to determine the pH of individual extracted open comedone material and to assess the variability, if any, from comedone to comedone on the same individual and from person to person. Secondly, to compare the pH of comedones with the pH of normal surface skin either adjacent to or in the close vicinity of the open comedones.

### MATERIALS AND METHODS

*Subjects.* Fifty-six acne patients were studied, 29 females and 27 males. Their ages ranged from 13 to 32 years (mean 18.5 years).

Oral and topical treatment for acne had been stopped at least 6 weeks before the investigation commenced. The patient's skin was free of cosmetics and had not been washed for at least 3 hours before sampling.

Measurements were made on the back and face of each individual before mid-day, in a non air conditioned room. The patient had rested for 15–30 min before measurements were made.

*Assessment of pH.* A Beckman 3500 digital pH meter was used in conjunction with a Beckman pH flat-head combined reference and glass electrode. The electrode/meter was calibrated using two standard Beckman buffers, pH 4.01 and pH 6.88, and the pH was measured in a manner identical with the comedone material.

Table 1. *Synopsis of comedone and skin surface pH values*

| Site | Number of readings | pH range | Mean pH | Standard error |
|---|---|---|---|---|
| *Comedone* | | | | |
| Female face | 38 | 4.22–6.39 | 5.66 | 0.09 |
| Female back | 51 | 4.01–6.85 | 5.28 | 0.08 |
| Male face | 47 | 4.72–6.59 | 5.73 | 0.06 |
| Male back | 63 | 4.06–6.58 | 5.39 | 0.07 |
| *Surface* | | | | |
| Female face | 45 | 4.42–5.93 | 5.20 | 0.06 |
| Female back | 80 | 4.10–5.86 | 5.07 | 0.04 |
| Male face | 42 | 4.22–6.80 | 5.58 | 0.08 |
| Male back | 104 | 4.16–6.81 | 5.20 | 0.04 |

Mean pH contactant = 5.75.