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BIOCHEMICAL BASIS FOR DEPIGMENTATION OF SKIN BY PHENOLIC GERMICIDES*

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ABSTRACT

Occupational exposure to germicides containing various phenols produces depigmentation of human skin. The chemical basis for this depigmentation may be competitive inhibition of the enzyme tyrosinase. In the tyrosinase reaction tyrosine has a K_m of 2.2×10^{-3} M; *p-tert*-butylphenol has a K_i of 1.95×10^{-4} M. In the dopa oxidase reaction, the estimated K_i for *p-tert*-butylphenol is 2.02×10^{-4} M. It is recommended that chemicals to be included in products that will come into close contact with human skin should be tested for their ability to inhibit tyrosinase.

Cutaneous pigmentation, a prominent phenotypic marker, has major social and economic significance. Spotted or uneven pigmentation of the skin is an ancient stigma, perhaps because both syphilis and leprosy are characterized by spotty depigmentation. Because of the obvious limitations of laboratory experimentation involving human pigmentation, fortuitous environmental influences have assumed greater importance as experimental manipulations.

Recently, Guinter Kahn (1) reported patchy cutaneous depigmentation in twelve hospital employees who used phenolic germicides in their work (Fig. 1). In his study of this problem the intentional application of ptBP (*p-tert*-butylphenol), ptBC (*p-tert*-butylcatechol), and ptAP (*p-tert*-amylphenol), components of several germicides, were found to cause depigmentation.

The experiments reported here were performed to determine the site of interference with pigmentation by these compounds. Melanin pigmentation of the integument can be divided into three separate processes (2) (Fig. 2):

1. melanogenesis
2. intramelanocytic granule transfer
3. pigment granule transfer

Because of the structural similarity of the depigmenting agents reported by Kahn to tyrosine and dopa (Fig. 3), experiments were designed to determine the influence of the compounds on step 1, the enzymic oxidation of tyrosine and dopa to melanin.

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MATERIALS AND METHODS

Dopa oxidation was measured according to the method of Fling, *et al.* (3) in which the appearance of dopachrome, an oxidation product of dopa, is monitored spectrophotometrically at 475 nm.

Tyrosinase was measured by the technique of Pomerantz (4) in which the oxidation of 3′,5′,^{3}H-tyrosine to dopa is accompanied by the generation of ^{3}HOH which is then counted in a liquid scintillation spectrometer.

Tyrosinase was prepared from *Rana pipiens* epidermis as previously described (5). Frog tyrosinase was used because of its availability and high activity. Tyrosinase was activated by trypsin which had been treated with L-(1-*t*osylamido-2-*p*henyl) ethyl *c*hloro*m*ethyl *k*etone to inhibit chymotryptic activity. (TPCK-trypsin, Worthington). Where indicated, tyrosinase was activated with trypsin, Difco 1:250. ptBP, ptAP and ptBC were obtained from Guinter Kahn; other chemicals were obtained commercially.

RESULTS

Dopa oxidation. This function of tyrosinase was inhibited by ptBP (Fig. 4, Table). Complete inhibition could not be achieved because of the limited solubility of ptBP at room temperature. ptBC itself was a substrate for epidermal tyrosinase; that is, a pigmented product was formed when ptBC was mixed with trypsin-activated frog tyrosinase. Frog tyrosinase required tryptic activation in order to catalyze the oxidation of ptBC. The frog epidermal tyrosinase used in the experiments reported here will not oxidize dopa or trypsin until it is activated by trypsin.

ptBP is not oxidized to an absorptive product in the short time of the assay; however, when incubated for longer periods, pigment is formed.

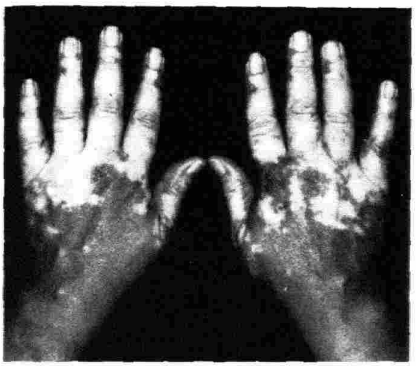

FIG. 1. Depigmentation of the hands of a 47 year Negro laboratory worker who used an antiseptic containing 3% *p-tert*-butylphenol. The spotty depigmentation rapidly developed over an 8 month period and resembles vitiligo. There is no depigmentation elsewhere on the skin.

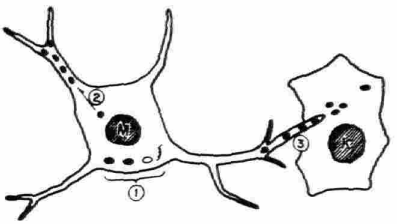

FIG. 2. Mechanism of melanin pigmentation of skin. In this scheme, the process has been divided into three steps:

1. Melanization—tyrosinase is synthesized on the endoplasmic reticulum and transferred to melanosomes where it catalyzes the oxidation of tyrosine to melanin. Progressive melanization of the melanosome results in a pigment granule.
2. Intramelanocytic translocation of pigment granules. This process accounts for rapid color changes in fish, amphibians, and reptiles but has not been demonstrated in mammalian melanocytes.
3. Pigment granule transfer—the tip of the melanocytic dendrite which contains pigment granules enters the keratinocyte (*K*) into which the pigment granules are transferred.

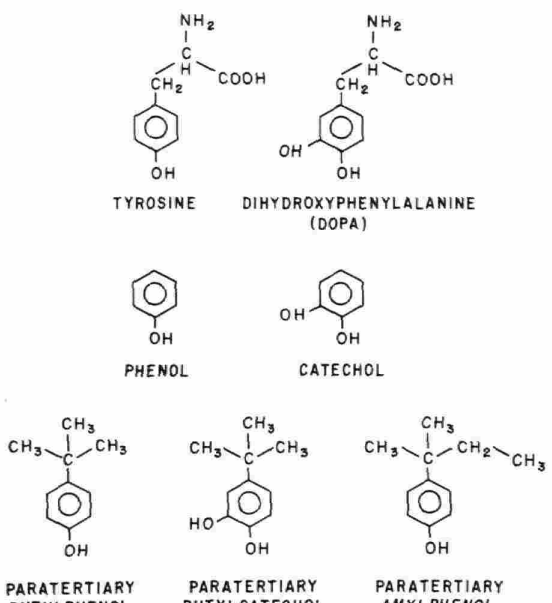

FIG. 3. Structure of melanin precursors showing phenol and catechol structure and the similarity to phenolic germicides.

TABLE

Inhibition of dopa oxidase by p-tert-*butylphenol*

The reaction mixture contained in a total volume of 0.95 ml, 0.9 ml PO_4 buffer 0.05 M, pH 7, 0.8 mg dopa, 50 μg TPCK trypsin, 100 μg crystalline trypsin inhibitor, 10 μl *Rana pipiens* tyrosinase (26 μg protein) and 20 μl dioxane in which the phenol when present was previously dissolved.

The buffer, trypsin and tyrosinase were mixed in a cuvette and after 3 min at room temperature, trypsin inhibitor was added and the contents of the cuvette were again mixed by inversion. Dopa in 0.4 ml buffer and dioxane were added, the contents were mixed and the cuvettes were read in a Gilford recording spectrometer at 475 nm. The enzyme without inhibitor produced an increase in absorbancy at 475 nm of 0.084 O.D. per minute.

The low solubility of the *p-tert*-butylphenol limits the amount of inhibition; however, it can be estimated from a graph of the above data that the reaction is inhibited 50% by 2.02×10^{-4} M ptBP, a concentration similar to the K_i observed for ptBP and tyrosinase (Fig. 6).

p-tert-butylphenol (M) × 10^{-4}	Activity with inhibitor/ Activity without inhibitor × 100
0	100
0.404	90
0.67	89
1.35	86
2.02	62
2.7	62
4.04	63
6.74	58
10.1	62

Tyrosinase. The oxidation of tyrosine was inhibited by ptBP, ptAP and ptBC (Fig. 5). Low concentrations of ptBC did not inhibit and occasionally stimulated tyrosine oxidation as if the catechol were serving as a cofactor. High concentrations of ptBC inhibited tyrosine oxidation. This biphasic effect resembles the concentration dependent effect of dopa on tyrosinase described by Pomerantz (4)—low concentrations of dopa stimulate tyrosine oxidation and higher concentrations inhibit tyrosine oxidation.

The inhibition of tyrosinase by ptBP is competitive with a K_i of 1.95×10^{-4} M, and the K_m for tyrosine with frog tyrosinase is 2.2×10^{-3} M (Fig. 6). Increasing the concentration of dopa in the reaction mixture reduces the inhibition of tyrosinase by ptBP especially at low concentrations of the latter (Fig. 7). ptBP also inhibits Greene hamster melanoma tyrosinase (Fig. 8) but probably not as well as it inhibits the frog enzyme, because the K_m for tyrosine is smaller with the mammalian enzyme than with frog enzyme.

DISCUSSION

The experimental observations reported here demonstrate that ptBP, ptAP and ptBC inhibit the oxidation of tyrosine. Although in most of these experiments, frog epidermal tyrosinase was used, similar results were obtained in the experiments performed with the Greene hamster melanoma.

Because little is known about intramelanocytic

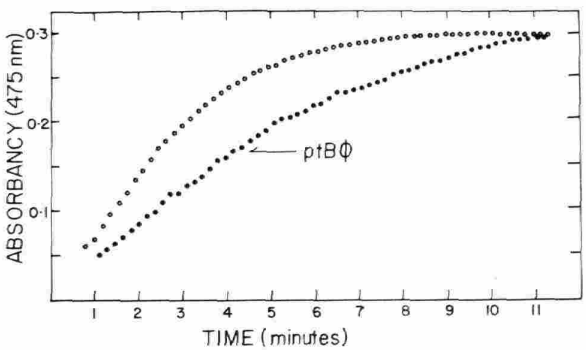

FIG. 4. Inhibition of dopa oxidase by *p-tert*-butylphenol. Each cuvette contained the reaction mixture described in Table and included 20 μl dioxane. The lower curve (closed circles) contained 1 mg ptBP, not all of which was in solution. The initial rate of the uninhibited reaction was 0.07 optical density units per minute. *Rana pipiens* tyrosinase (24 μg protein) was added to each cuvette.

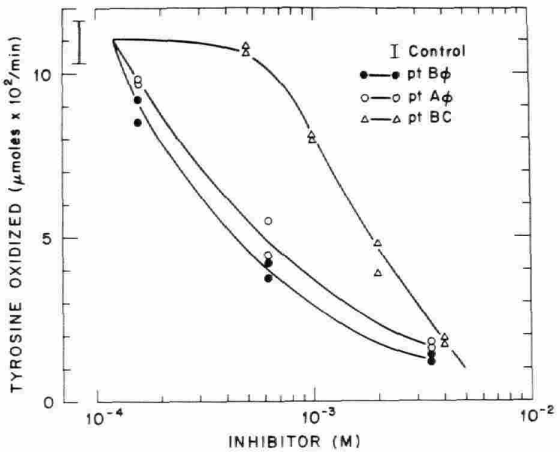

FIG. 5. Inhibition of tyrosinase by *p-tert*-amylphenol, *p-tert*-butylphenol and *p-tert*-butylcatechol. Each reaction mixture contained a total volume of 1.04 ml and was incubated at 37° for 6 min and then inactivated with phosphoric acid. Each reaction mixture contained DOPA 10^{-5} M, Tyrosine 1.98×10^{-3} M, phosphate buffer 0.05 M pH 6.8, and epidermal tyrosinase equivalent to 265 μg protein obtained from *Rana pipiens* and activated with 0.1 mg trypsin. (Difco 1:250). Each reaction mixture contained 71,324 cpm ^{3}H-tyrosine which had been purified by cocrystallization with unlabelled carrier tyrosine. If the reaction had been complete then half of this tritium would be released as ^{3}HOH. The inhibitors were dissolved in 10λ ethanol which had no effect on tyrosinase activity.

concentrations of dopa or tyrosine, the *in vivo* response of tyrosinase to competitive inhibitors such as ptBP cannot be predicted. It is possible, however, to compare the relative affinity of the enzyme for the substrate and the inhibitor and predict what would happen if both were available at similar concentrations. The K_m for tyrosine, (2.2×10^{-3} M) is an order of magnitude larger than the K_i of ptBP (1.95×10^{-4}); accordingly the latter might be predicted to be an effective inhibitor of tyrosinase oxidation. Pomerantz (4) has shown that both tyrosine and dopa, substrate and cofactor respectively of the tyrosinase reaction, are inhibitors of hamster melanoma tyrosinase at certain concentrations. The K_m for dopa as a cofactor is 2×10^{-6} M, the K_m as a substrate is 5×10^{-4} M; the K_i of dopa is 4×10^{-4} M; the K_m of tyrosinase is 3×10^{-4} M (7).

Many chemicals have been evaluated for their ability to depigment black guinea pig skin. In 1968 Bleehan *et al.* (8) surveyed thirty-three such substances and found that most of the compounds that caused depigmentation also produced inflammation; 4-isopropylcatechol (4-IPC) was an exception and at five per cent concentration produced no microscopic inflammation. Five per cent ptBC was a potent depigmenting agent but was irritating. Light microscopic studies of epidermis from guinea pigs treated with 4-IPC revealed reduction of melanin in keratinocytes and a decrease in the number of melanocytes demonstrable by dopa incubation. Many of the remaining melanocytes were abnormal in shape having lost their dendrites. Electron microscopic examinations of 4-IPC treated skin revealed few melanocytes, of which some contained imperfectly melanized melanosomes and some displayed evidence of cell degeneration—i.e. vacuoles, swollen mitochondria. Langerhans cells were not altered in number or morphology by 4-IPC.

Gellin *et al.* (9) have also associated cutaneous depigmentation in four factory workers with exposure to oil containing ptBC. The oil, when applied topically to guinea pigs, caused depigmentation and application of either ptBC or ptBP caused partial to complete loss of epidermal pigment.

Gellin observed that no pigmentary changes occurred in black guinea pigs without antecendent inflammation; however the application of

ptBP or ptBC to the skin of albino guinea pigs caused no "sensitization".

The examples of depigmentation reported by

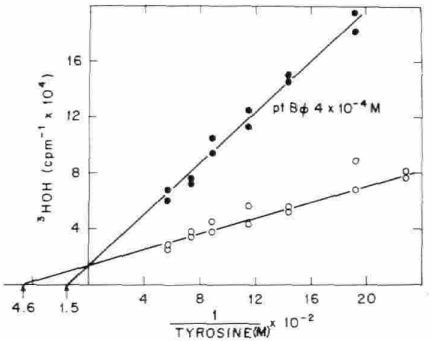

FIG. 6. Competitive inhibition of tyrosinase oxidation by *p-tert-butylphenol*. Lineweaver-Burke plot of reciprocal of velocity vs. reciprocal of substrate concentrations at zero and 4×10^{-4} M concentration of ptBP. Reaction mixtures containing varying concentrations of tyrosine were incubated for 5 min at 37° C. The reactions were stopped with phosphoric acid and ^{3}HOH generation was measured. The volume of each reaction mixture was 1.04 ml and included in addition to tyrosinase: dopa at 10^{-5} M 10λ ethanol, 0.05 M phosphate buffer, pH 6.8, and epidermal tyrosinase from *Rana pipiens* equivalent to 180 μg protein which was activated with 0.1 mg trypsin (Difco 1:250). The specific activity of tyrosine was 470 cpm/mμM.

The K_m for tyrosine is 2.2×10^{-3} M. the K_i for ptBP was calculated to be 1.95×10^{-4} M. The K apparent for tyrosine in the presence of ptBP was 6.7×10^{-3} M.

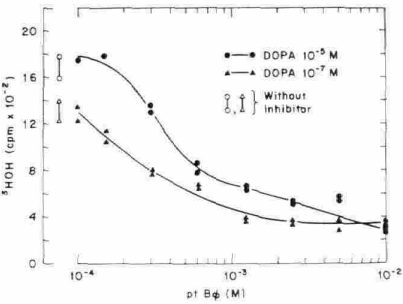

FIG. 7. Inhibition of tyrosinase by *p-tert*-butylphenol at two concentrations of dopa. Reaction volumes of 1.04 ml were incubated at 37° C for 30 min and then inactivated with phosphoric acid. The concentration of ^{3}H-tyrosine in each mixture was 1.56×10^{-3} M containing 33,000 cpm. Epidermal tyrosinase equivalent to 120 μg protein from *Rana pipiens* was activated with 0.1 mg trypsin (Difco 1:250) in each reaction mixture. In addition, each mixture contained 10λ ethanol and sodium phosphate buffer 0.05 M, pH 6.8.

Gellin and Bleehan do not permit identification of the mechanism of color loss. Either interference with melanogenesis or damage to the melanocyte could account for their observations. Kahn observed, by light microscopy, enlarged sparse melanocytes in depigmented human skin. He reported that electronmicroscopic examination revealed reduced melanin but unlike Bleehan *et al.* (8) he observed no structural abnormality in the melanocyte. Three points deserve

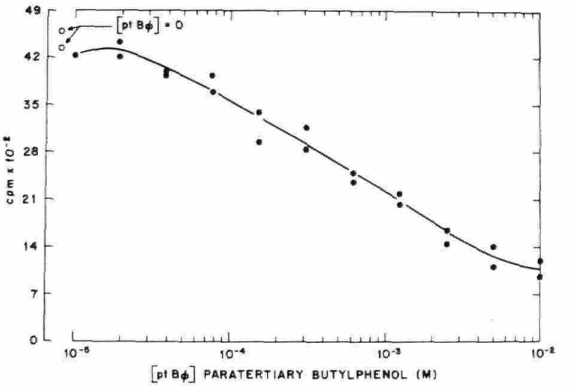

FIG. 8. Inhibition of mammalian tyrosinase by *p-tert*-butylphenol. Each reaction mixture contained tyrosine 1.64×10^{-3} M (69,676 cpm), dopa 10^{-5} M and 0.05 M PO_4 buffer, pH 6.8 in a total volume of 1.02 ml. The mixture was incubated for 60 min at 37°. Each reaction mixture contained 4.25 mg protein of an aqueous extract of an acetone powder of hamster melanoma prepared according to Pomerantz (6).

emphasis: i. Gellin, unlike Kahn and Bleehan, could not dissociate inflammation and depigmentation. ii. Bleehan found significant structural abnormalities in melanocytes located in chemically depigmented areas. iii. Gellin was unable to produce inflammation of albino guinea pig skin with ptBP or ptBC. These observations suggest that the germicidal phenols may not only inhibit melanogenesis but also have a more directly damaging effect on the melanocyte. The observation that ptBP does not produce inflammation in albino guinea pig skin suggests that an oxidation product of the phenol is responsible for the damage to the melanocyte. In the albino skin, the phenol is not oxidized and does not cause inflammation.

The experiments reported here establish the ability of ptBP, and presumably ptAP and ptBC, to inhibit competitively the oxidation of tyrosine.

Our observations do not establish that exposure to compounds which interfere with tyrosinase *in vitro* will necessarily cause cutaneous depigmentation; however, compounds that have been demonstrated to produce depigmentation *in vivo* inhibit tyrosinase *in vitro*. A practical conclusion from these experiments is that chemicals to be included in products that will achieve close contact with human skin should be tested for their ability to inhibit tyrosinase.

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