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Determination of Oxidative Enzymes and their Inhibitors by a Modified Chromatographic Method. (19811)

RUDOLF L. MAYER, MADELON R. GRIMM, AND FREDERICK C. KULL.
From the Microbiological Division, Ciba Pharmaceutical Products, Summit, N. J.

Present-day methods of detecting the action of melanin-forming enzymes and of evaluating the effect of enzyme inhibitors in these systems usually involve the incubation of enzyme-substrate mixtures and subsequent colorimetric determination of the melanin formed. Thus the activity of an anti-metabolite may be expressed as the decrease in melanin formation compared with that exhibited by control enzyme solutions. The sensitivity of this method depends not only upon enzyme concentration but also upon the diluent, the surface-volume ratio, temperature, etc. By this procedure, the anti-melanotic action of thiourea has been estimated at a dilution of approximately 1 x 10^{-4}(1-4). In view of the limitations of this test, a more sensitive method for the study of oxidase and anti-

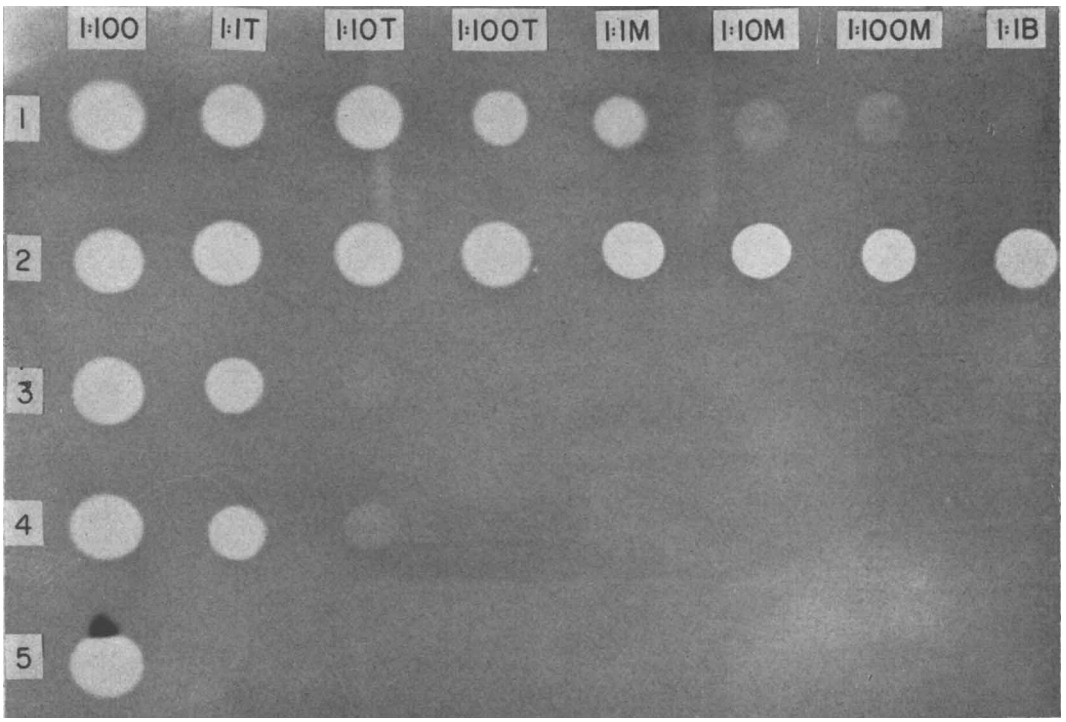

FIG. 1. Inhibition of phenol oxidase by (1) thiourea, (2) phenylthiourea, (3) Itrumil, (4) thiouracil, (5) propylthiouracil.

oxidase activities was developed and is described in this paper.

Experimental. 1. *Detection of enzyme activity.* Tyrosinase (potato phenol oxidase) obtained from fresh potatoes by homogenization under nitrogen was added to an appropriate substrate such as tyrosine, dihydroxyphenyl L-alanine (dopa), or epinephrine and quickly sprayed, under nitrogen pressure, on a large sheet of No. 1 Whatman filter paper. The wet paper was then suspended in a humid chromatographic chamber and observed for color changes. Hallachrome and subsequently melanin developed on the filter paper by oxidation. The shade and rate of filter paper coloration varied with the type of substrate employed. With dopa or epinephrine as melanogens, visible oxidation started immediately. The red color of the ortho-quinone derivatives described by Raper(5) rapidly developed and subsequently changed into the brown and black color of pro-melanin and melanin. With tyrosine as substrate, the oxidation was much slower. At least 30 minutes were required to produce a visible reaction and the color sequence from red-brown to black.

2. *Evaluation of anti-oxidants.* In the manner of a paper chromatogram, drops of serial dilutions of the anti-melanogenic substances to be tested were deposited on filter paper and allowed to dry. The enzyme-substrate solution was then sprayed as described above. After an interval, depending on the substrate used, anti-oxidase activity was manifested as round white spots on a brown or a black background, and the anti-melanotic potency indicated by the highest dilution suppressing complete coloration. Repeated experiments showed that the end points are reproducible and permitted quantitative determination of oxidative enzyme inhibition. The suppression of phenol oxidase by several compounds in shown in Fig. 1.

Results. Employing this method, the extent of anti-oxidase activity of the following compounds was observed:

Phenylthiourea	1×10^{-10}
Thiourea	1×10^{-9}
Thiouracil	1×10^{-4}
Propylthiouracil	1×10^{-3}
5-iodo-2-thiouracil (Itrumil)	1×10^{-4}
Sulfathiazole	1×10^{-3}
Sulfanilamide	1×10^{-3}
p-Aminobenzoic acid (PABA)	1×10^{-3}
p-Aminosalicylic acid (PAS)	1×10^{-4}
p-Acetylaminobenzaldehyde thiosemicarbazone (Tibione)	1×10^{-5}

Phenylthiourea, by this method, exhibited inhibition of phenol oxidase at a dilution greater than 1×10^{-10}. Below 1×10^{-8} concentrations and with tyrosine as substrate the anti-oxidase effect lasted from ½ to 2 hours and subsequently disappeared. In higher concentrations, *i.e.*, greater than 1×10^{-7}, the anti-oxidase activity was complete and permanent.

PABA, PAS and sulfonamides such as sulfanilamide and sulfathiazole suppressed melanin formation in concentrations of 7×10^{-2} and 1×10^{-4}. These substances caused bright yellow and yellow-brown colorations similar to the yellow pigments formed by Mycobacteria in the presence of PABA and PAS(6,7), and from sulfathiazole from Staphylococci(8). Isonicotinhydrazide (Rimifon) also acted as a potent inhibitor and brought about an orange coloration. The yellow and orange compounds are most likely oxidation-products of these various aromatic amines and formed in a sequence of reactions: tyrosine is transformed to the quinoid hallochrome which in turn reacts with PABA, sulfonamides, and Rimifon.

Summary. A simple method for the detection of potato phenol oxidase and the quantitative determination of inhibitors to this and similar systems yielding color variations is described. Melanin is developed on filter paper sheets and anti-melanotic substances tested by a chromatograph, spray-like procedure.

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