Phenolic substances can be oxidized by many oxidant as already known in organic chemistry. Then some of the oxidized phenolic substances may polymerize and conceivably form black pigments. Suitable phenolic substances were sought from the following reagents.

Catechol, homocatechol, DOPA, resorcinol, orcin, phloroglucin, p-cresol, m-cresol, guajac gum, eugenol, isoeugenol, veratrol, 2,3-dihydroxynaphthalene, 1,3-dihydroxynaphthalene, 1,4-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, 2,7-dihydroxynaphthalene, β -naphthoquinone, chromotropic acid, alizalin.

Among these substances, catechol was found to be suitable. Catechol was relatively easily oxidized by tissue slices and stained tissue elements in brown color. Various sections of rat and mouse tissues, fresh-frozen, fixed-frozen with formol calcium, and paraffin embedded, were incubated in 1 % catechol solution adjusted to pH 5.0, 7.0 and 8.0 for 2~24 hours. The acidic incubating solution turned to brown color in a several hours. Prominent staining in brown color occurred in the wandering cells, presumably leucocytes and mast cells, in the spleen, intestine, skin etc. The basal portion of hair matrix of the skin was also stained in both rat and mouse. The latter reaction was obviously inhibited by 0.01 M KCN (pretreatment and adding in the incubating medium) and by heating. On the other hand, the former reaction still remained by these treatments by longer incubation, although they were inhibited in some extent. Pretreatment with CuCl and CuCl₂ resulted in increase of staining not only of these cell elements but also of all back ground. These results suggest that the staining of hair matrix might be of somewhat different nature from other wandering cells. The further considerations concerning this staining process were discussed.

Studies on the Optimal Condition of a New Modified Histochemical Method for Cytochrome Oxidase

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“A modified method of histochemical demonstration for cytochrome oxidase” has been reported previously. Further studies on new couplers of pyrazolone compounds and others were performed, and 1-(3'-chloro) phenyl-3-methyl-5-pyrazolone was found to be most useful drug as the coupler for the apparent demonstration of this enzyme.

It was found, however, in this method that the change of color and diffusion of dye were observed in the sections stained, as the pH value of the reaction mixture was changed variously. These phenomena were supposed to ascribe to the condition of the reaction of the reagents.

In the present paper, in order to elucidate the problem, the oxidated reaction products of these reagents by the chemical oxidating agent were measured under the various pH values and concentrations of phosphate buffer by spectrophotometry, and the results from them were compared with histochemical optimum.

Method

Preparation of reagent mixture for histochemical reaction: 10mg of a new coupler, 1-(3'-chloro)phenyl-3-methyl-5-pyrazolone and 10 mg of N-phenyl-p-phenylenediamine were dissolved in 0.5 ml of methanol, followed by addition of 50 ml of phosphate buffer in various conditions as follows:

The phosphate at 8 sorts of concentrations (0.001M-0.15M) and 6 sorts of pH values (pH5.5-8.0). Then 8-12 in thickness of fresh frozen sections from the operated human stomach were incubated for 30-60 minutes at room temperature.

Preparation of media spectrophotometry: By adding 0.5ml of 2% solution of periodic acid, in stead of enzyme preparation, to 5ml of reagent mixture described above, precipitate of dye was obtained and then the precipitate was extracted by 35ml of chloroform for spectrophotometry.

Observations and Results

At the constant concentration of phosphate buffer, it was observed in the histochemical preparations that, according to the shift of pH value from low to high, the excellent localization of enzyme reaction in sections stained in violet-black color, changed to the poor localization with increasing diffusion of reddish-violet dye.

These observations coincided with the results obtained by spectrophotometry, which showed that at the low pH value, only single peak appeared near $420\text{m}\mu$ of wave length (violet-black), while at the high pH value, two peaks appeared; the first one near $420\text{m}\mu$ and the second near $525\text{m}\mu$ of wave length (reddish-violet).

The cause of the poor localization with increasing diffusion of dye, therefore, might be ascribed to the appearance of the 2nd peak in high optical density, because the color demonstrated in the histochemical observation consisted with wave length measured by spectrophotometry.

At the constant pH, the strength of the reaction decreased with increasing the concentration of phosphate buffer, and the enzyme activity showed strongest in the range of 0.03-0.05M of concentration.

In order to get the optimal condition of the reaction, therefore, pH value of the reaction mixture was adjusted so as to show the maximal difference of

optical density between two peaks.

The figure indicated the relation between the optical densities of those two peaks and pH value at various concentrations of phosphate buffer.

The optimal condition of pH value and the concentration of phosphate buffer was almost in the range of pH 6.5-7.0 and 0.03-0.05M in concentration of phosphate buffer.

Under this condition, histochemical findings showed the most satisfactory localization.

Though the pH optimum of cytochrome oxidase itself has been generally considered to be in the wide range from acidic to alkaline, the relatively narrow range of pH optimum of this method was probably due to the characteristics of the reagents.

Accordingly, for the determination of the optimal condition in the histochemical reaction, the reagent specificity should be taken into consideration.

Further studies by using enzyme preparations are now in progress.