The role of 3:4-dihydroxytoluene, sulfhydryl groups, and cresolase during melanin formation in a slime mold

During the course of our studies on the biochemical basis of morphogenesis in the slime mold, *Physarum polycephalum*, we have been able to demonstrate the presence of an enzyme in the acellular vegetative, plasmodial form which participates in the production of melanin when this organism forms spores. Although this enzyme appears to be a classical copper-containing oxidase in that its activity is inhibited by cyanide, carbon monoxide (not light-reversible), diethyldithiocarbamate, and other copper complexing agents, several aspects of its action serve to distinguish it from those already reported[1].

The dual nature of tyrosinase (the same homogeneous enzyme can oxidize a variety of monohydric and dihydric phenols) enables one to designate specific monophenolase or cresolase and polyphenolase or catecholase activities; the latter term in each case reflects the wide use of *p*-cresol and catechol as substrates. When *Physarum* extracts[2] were incubated with such monohydric phenols as *p*-cresol, *m*-cresol, phenol, and tyrosine, only *p*-cresol elicited an oxygen uptake; with such dihydric phenols as catechol, 3:4-dihydroxyphenylalanine (DOPA) and 3:4-dihydroxytoluene (3:4-DHT), only 3:4-DHT gave some evidence of an oxygen uptake. Although tyrosinase preparations can be prepared and controlled during purification to give varying ratios of catecholase/cresolase activities, it is rare when cresolase activity appears without catecholase activity as is the case with the *Physarum* enzyme. The argument is entertained that the slight activity of this enzyme to 3:4-DHT can be designated as catecholase activity, but then this becomes an aspect of terminology which perhaps is not valid when an enzyme with such high specificity is encountered.

A characteristic of tyrosinase activity on monophenols is that a period of induction or lag is present before oxygen uptake is observed. This lag can be removed by the addition of small amounts of catechol for the purpose of priming the enzyme for cresolase activity and thus removing the autocatalytic aspects of action[3]. The cresolase activity of *Physarum* extracts exhibits such an induction period (Fig. 1), but as may be expected from the enzymic inactivity toward catechol and other dihydric phenols, the addition of these compounds was ineffective in shortening the lag. It is surprising that 3:4-DHT also failed to decrease this lag period since it would be the most likely primer with such a specific enzyme.

It was subsequently observed that this induction period could be eliminated by the addition to the reaction mixture of such compounds as *p*-chloromercuribenzoate (*p*-CMB), N-ethyl maleimide (NEM), and iodoacetic acid which react with sulfhydryl groups (Fig. 1). The addition of glutathione and cysteine not only reversed the effect of *p*-CMB but extended the lag for longer periods. The results of dialysis experiments were obscure, since undialyzed extracts lost the induction period upon a few hours standing in the cold. These observations do not remove the possibility of the autocatalytic aspects of the induction period, since free sulfhydryls can participate in the oxidation-reduction aspects of dihydric-phenolic primers. It does, however, point to the importance of the presence or absence of sulfhydryls in influencing melanin formation in conjunction with the triggering of cell division in the intact organism. There are at least two reports in the literature of the direct participation of sulfhydryls in mammalian tissue[4] and indirect participation of sulfhydryls in *Neurospora*[5] in decreasing tyrosinase activity and subsequently melanin formation.

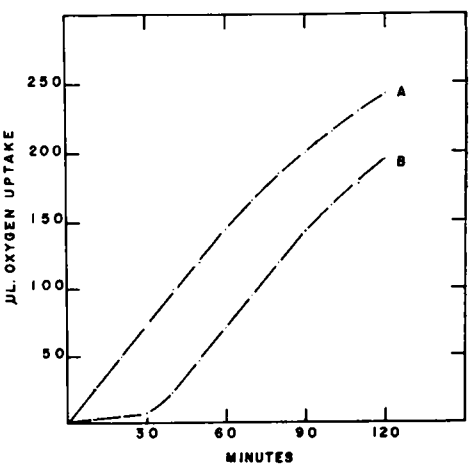

Fig. 1. The effect of *p*-chloromercuribenzoate on the induction period during cresolase activity. Curve A – enzyme + 0.023 *M* *p*-cresol + $2.5 \cdot 10^{-4}$ *M* *p*-CMB. Curve B – enzyme + 0.023 *M* *p*-cresol. 0.025 *M* phosphate buffer, pH 7.6; 2.0 ml reaction volume. *p*-Cresol added from the side arm at zero time.

Since there was no observable enzymic oxidation of dihydric phenols except the slow oxygen uptake observed with 3:4-DHT, it seemed feasible to assume that any dihydroxy intermediate might accumulate in the reaction mixture in spite of possible autoxidation at this pH. To test this possibility, samples were taken from the reaction mixtures at various time intervals corresponding to the oxygen uptake curves in Fig. 1, spotted on strips of Whatman No. 1 filter paper, and chromatographed, using a modified solvent system of benzene–acetic acid and water[6] and phosphomolybdic acid as the developing agent[7]. It can be seen that a spot appeared which corresponds to that of 3:4-DHT on the control strip (Fig. 2). Elution of undeveloped comparable positions on the paper, freeze-drying the eluate, and subjecting the resulting powder to infra-red spectrophotometric analysis, revealed the presence of functional groups on the aromatic nucleus consistent with that of 3:4-DHT. Owing to highly absorbing impurities brought down with elution, a sharply defined absorption spectrum could not be obtained. However, all evidence points to the enzymic insertion of an –OH group on the benzene nucleus *ortho* to the hydroxyl group on *p*-cresol to form 3:4-DHT as an intermediate compound in melanin formation. This is consistent with RAPER's classical mechanism for the oxidation of tyrosine by tyrosinase in melanin formation[8,9]. It is noteworthy that this intermediate is formed unchanged even in the presence of

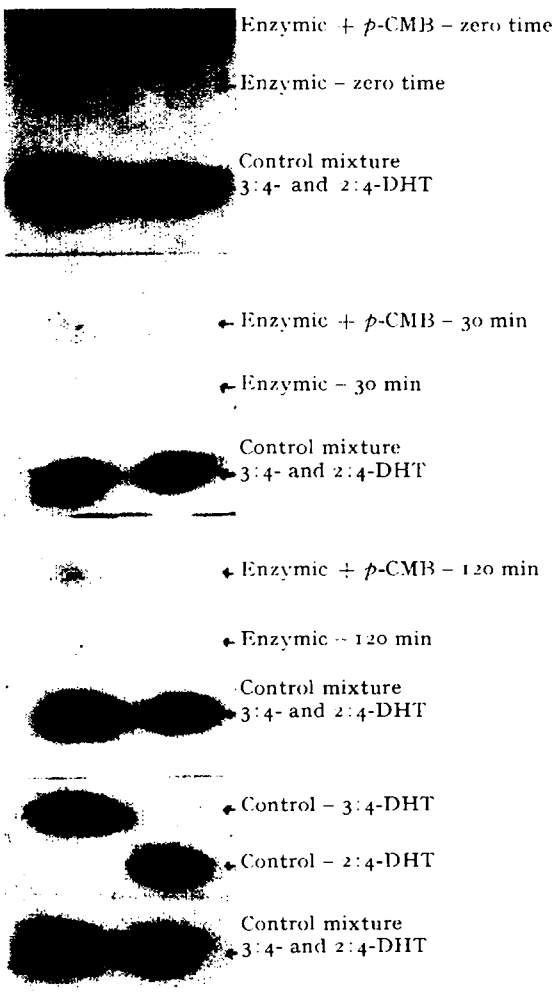

Fig. 2. Chromatographic identification of 3:4-DHT as an intermediate in melanin formation. Reaction mixtures identical with those of Fig. 1. 25 μl of reaction mixtures were spotted at origin. Controls contained 20 μg each of 2:4-DHT and 3:4-DHT.

p-CMB; 3:4-DHT formation is slower in absence of *p*-CMB attesting to the presence of the induction period.

A separate paper describing in more detail the work reported here is now in preparation.

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Regulation of the uptake of glucose by the isolated rat diaphragm

The rate of utilisation of glucose by the isolated rat diaphragm is thought to be limited by the rate at which glucose is transferred from extracellular to intracellular water[1]. The uptake of glucose by the isolated rat diaphragm incubated *in vitro* in a balanced salt solution buffered with bicarbonate has been shown to be greater under anaerobic than aerobic conditions[2]. These observations suggest that the access of glucose to those enzymes in muscle which metabolise it is restricted by a process dependent upon oxidation. Further evidence in support of this view has been obtained by studying the uptake of glucose and release of potassium by isolated rat hemidiaphragms *in vitro* incubated in succession under aerobic and under anaerobic conditions, and also under aerobic conditions in the presence of 2:4-dinitrophenol, sodium arsenite and sodium cyanide.

Hemidiaphragms were obtained from male albino wistar rats of 100–150 g weight which had been fasted for 18–24 h. The conditions of incubation were as described previously[2]. The incubation medium was a balanced salt solution buffered with bicarbonate[3], and gassed either with 95% O_2, 5% CO_2 (aerobic conditions) or with 95% N_2, 5% CO_2 (anaerobic conditions). The uptake of glucose and release of potassium by the tissue during incubation were measured by the changes in the glucose and potassium contents of the medium, glucose being estimated by SOMOGYI's modification[4] of Nelson's method, and potassium by flame photometry. Glucose uptake was calculated as mg glucose disappearing from the medium/g of wet diaphragm/h of incubation and potassium release as μequiv. potassium appearing in the medium/g of wet diaphragm/h of incubation.

When diaphragms were incubated anaerobically under these conditions, both glucose uptake and release of potassium were greater than under aerobic conditions (Table I). This confirms earlier observations for both glucose[2] and potassium[5]. If diaphragms were incubated anaerobically for 20 min and then aerobically for a further 40 min, or incubated aerobically for 20 min and then anaerobically for a further 40 min, the overall uptake of glucose and release of potassium in either instance were both significantly less than the values for diaphragms incubated anaerobically for 60 min and significantly greater than those for diaphragms incubated aerobically for 60 min (Table I). In further experiments it was found that the enhanced uptake of glucose and release of potassium under anaerobic conditions could be readily discerned after only 5 min of incubation. Furthermore, when the conditions of incubation were changed after 20 min of incubation from anaerobic to aerobic or from aerobic to anaerobic the rates of uptake of glucose and release of potassium decreased in the former instance and increased in the latter within 10 min of changing the gas phase. Thus the changes which occur in the diaphragm under anaerobic conditions and which lead to increased rates of glucose uptake and potassium release can be reversed by restoration of aerobic conditions.

When diaphragms were incubated aerobically but in the presence of 2:4-dinitrophenol ($5 \cdot 10^{-5} M$ or $2.5 \cdot 10^{-4} M$), sodium arsenite ($10^{-3} M$) or sodium cyanide ($10^{-3} M$) the rates of uptake of glucose and release of potassium were very greatly increased (Table I).