The Metabolism of Aromatic Acids by Micro-organisms

A REASSESSMENT OF THE ROLE OF *o*-BENZOQUINONE AS A PRODUCT OF PROTOCATECHUATE METABOLISM BY FUNGI

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1. The addition of aniline to cultures of several yeasts, *Fusarium oxysporum* and *Neurospora crassa* growing with protocatechuate as sole carbon source resulted in the precipitation of dianilino-*o*-benzoquinone (anil). This product was also formed, however, if the medium was uninoculated. 2. The physical presence of yeast cells (living or dead) increased the anil yields in *Debaryomyces subglobosus* cultures. 3. No anil was formed if *p*-hydroxybenzoate was the growth substrate. 4. *o*-Benzoquinone was a strong inhibitor of protocatechuate 3,4-oxygenase and catechol 1,2-oxygenase in these fungi. 5. It was concluded that *o*-benzoquinone formation from protocatechuate is independent of living yeast.

The general metabolic routes for the dissimilation of phenolic compounds in yeasts parallel those found in bacteria (Dagley, Evans & Ribbons, 1960; Ornston & Stanier, 1966), except that all the yeasts and other fungi we have so far examined degrade protocatechuate through β -carboxymuconolactone (Bilton & Cain, 1965; Cain, Bilton & Darrah, 1968) and thus resemble *Neurospora crassa* (Gross, Gafford & Tatum, 1956), whereas the bacterial route involves the corresponding γ -carboxymuconolactone (Ornston & Stanier, 1966; Cain, 1966; Cánovas & Stanier, 1967).

We were therefore puzzled by the report of *o*-benzoquinone as a product of protocatechuate metabolism (Harris & Ricketts, 1962), because our studies have shown (Cain *et al.*, 1968) that our yeasts possessed the full complement of enzymes, and in sufficient amounts, to degrade protocatechuate or catechol to β -oxoadipate very rapidly by the route established for *Neurospora crassa*, which does not involve a quinone. These two substrates (5 μ moles) could be converted quantitatively into β -oxoadipate in 15 min. by extracts (5 mg. of extract protein) of suitably induced micro-organisms such as *Rhodotorula mucilaginosa*, *Sporobolomyces*, *Debaryomyces* or *Fusarium*. Further, though Evans (1947) isolated *o*-benzoquinone as its dianilino derivative (anil) from phenol cultures of *Moraxella calcoacetica* ('Vibrio O1', N.C.I.B. 8250), this quinone has never been implicated as an intermediate in either catechol or protocatechuate breakdown by bacteria in subsequent studies (Hayaishi, 1962). We therefore decided to re-examine the formation of *o*-benzoquinone from catechol and protocatechuate in three of our yeasts and in two other fungi.

MATERIALS AND METHODS

Organisms and cultural conditions. The organisms used were *Rhodotorula mucilaginosa*, *Debaryomyces subglobosus* N.C.Y.C. 459, *Debaryomyces hansenii* IC, *Sporobolomyces* sp. and the two fungi *Neurospora crassa* and *Fusarium oxysporum*. The origin of these micro-organisms was described by Cain *et al.* (1968). The yeasts and *Fusarium* were grown in Wickerham's medium (Difco Yeast Nitrogen Base) containing 0.1% (w/v) of the carbon source and adjusted to pH 5.5 or pH 7.0. The medium was dispensed in 1 l. volumes in 2 l. conical flasks. The carbon sources (*p*-hydroxybenzoate, protocatechuate and catechol), adjusted to the correct pH, were sterilized separately by filtration and added aseptically to the cooled sterile medium just before inoculation. The yeast cultures and *Fusarium* were inoculated with a washed suspension (approx. 0.1 mg. dry wt.) of the appropriate organism derived from a 30 ml. culture on the same medium and were then incubated at 25° on a New Brunswick Gyrotory shaker at 210 rev./min. until the onset of exponential growth, when 1 ml. of sterile freshly distilled aniline was added. Incubation was subsequently continued for 7–10 days.

Neurospora crassa was grown from a spore inoculation (approx. 10^8 spores) in similar flasks on the medium of Ryan, Beadle & Tatum (1943) with glucose (1%, w/v) as the carbon source until heavy mycelial growth had occurred (4 days), at which time the mycelium was harvested with aseptic precautions on a Millipore filter, washed once with 5 vol. of sterile water and then resuspended in 1 l. volumes of sterile Wickerham's medium containing the aromatic carbon source. After incubation of the suspension with shaking for 24 hr., aniline was added as described above. Uninoculated (control) flasks of the same aromatic media were treated with aniline and incubated similarly. Extracts of these micro-organisms were prepared as described by Cain *et al.* (1968).

Chemicals. *o*-Benzoquinone was synthesized as described by Willstätter & Pfannenstiel (1904) and a 0.1 M solution of this material made in pure dry acetone. The dianilino

derivative of *o*-benzoquinone (anil) was synthesized by the method of Kehrman & Cordonne (1913) and had m.p. 193°. *p*-Benzoquinone, purified by sublimation and recrystallized from benzene, was also used in some comparative experiments. Melting points are uncorrected.

Chromatography. Paper chromatography of the anil derivatives was carried out on Whatman no. 4 paper by the descending technique in light petroleum (b.p. 80–100°)–diethyl ether–water (9:1:10, by vol.; upper layer used). The reddish-brown anil spot was visible without further treatment.

Enzyme assays. Protocatechuate 3,4-oxygenase (EC 1.13.1.3) and catechol 1,2-oxygenase (EC 1.13.1.1) in cell-free extracts of the fungi were measured manometrically at 30° in air. Double-side-arm Warburg flasks contained in a total volume of 3.0 ml.: substrate, 5 μ moles, in one side arm; Na₂HPO₄–KH₂PO₄ buffer, pH 7.0, 85 μ moles, and extract, 0.1 ml. (protein content varied with the organism, but was in the range 0.3–0.8 mg.) in the main compartment; KOH, 0.2 ml., in the centre well. The quinone inhibitors (3 μ moles in acetone) were added to the second (dry) side arm and the acetone was evaporated off with a slow stream of N₂ before addition of the other flask contents. In this way *o*-benzoquinone, which is soluble, but unstable, in aqueous solution, did not come into contact with water until substrate, enzyme and inhibitor were simultaneously mixed. Protein was measured by the biuret method of Gornall, Bardawill & David (1949).

RESULTS AND DISCUSSION

Isolation of anil from culture media. With *p*-hydroxybenzoate as carbon source no anil derivative was produced in any of the cultures at either pH, though growth was excellent. In protocatechuate cultures, in contrast, a deep-red colour appeared within 24 hr. followed by a precipitate of the anil within 5 days after aniline addition. The anil was isolated and characterized from 3-day-old (stationary-phase) cultures as follows. The turbid cultures were adjusted to pH 7.0 and centrifuged, and the precipitate of cells and anil was extracted three times with benzene. The neutral supernatant was also extracted twice with 0.5 vol. of benzene to recover traces of dissolved anil. The two benzene

fractions were combined, dried over anhydrous sodium sulphate and evaporated to dryness; the oily residue was treated with dilute hydrochloric acid to remove unchanged aniline and then recrystallized from light petroleum and dried, and the yield of anil was weighed. The recrystallized anils from different cultures all melted between 187° and 192° and did not depress the melting point of authentic anil (193°) (Kehrman & Cordonne, 1913) on admixture. Their identity with *o*-benzoquinone anil was confirmed by paper chromatography, when all the isolated samples co-chromatographed with authentic material at *R_F* 0.64.

The anil yield for the five organisms examined is shown in Table 2. The very high yields of anil from protocatechuate at pH 7.0 compared with those at pH 5.5 are almost certainly a result of the considerable autoxidation that this substrate undergoes in neutral or alkaline media subjected to vigorous aeration. It is significant that, though *p*-hydroxybenzoate was metabolized through protocatechuate by these organisms (Bilton & Cain, 1965; Cain *et al.* 1968), no anil was formed in *p*-hydroxybenzoate media (where the substrate is not autoxidizable) at pH 7.0. The yield of anil was also less in a stationary uninoculated incubation of protocatechuate (68.2 mg.) than in a corresponding shaken uninoculated incubation (80.8 mg.), which further suggests an autoxidation origin for the quinone. Protocatechuate was metabolized rapidly to *cis-cis*- β -carboxymuconate by *Rhodotorula* (Cain *et al.* 1968), so that very little was likely to be available for autoxidation to quinone compounds; this probably accounted for the consistently low anil yields found in protocatechuate-grown cultures of this organism (Table 1).

The noteworthy feature of Table 1, however, was the large yield of dianilino-*o*-benzoquinone formed in uninoculated protocatechuate media at pH 7.0. The amount produced was similar to that found in cultures of all the organisms with the exception of *Debaryomyces subglobosus*, and indicated that the

Table 1. *Yields of dianilino-o-benzoquinone produced in 10-day-old cultures of fungi grown with catechol and protocatechuate*

Expt.	Organism	Anil produced (mg./l. of medium)		
		pH 5.5		pH 7.0 Protocatechuate
		Protocatechuate	Catechol	
Expt. 1	<i>Rhodotorula mucilaginosa</i>	5	36	18
	<i>Debaryomyces subglobosus</i>	36	50	255
	<i>Debaryomyces hansenii</i> IC	14	80	167
	Uninoculated medium	5	43	133
Expt. 2	<i>Neurospora crassa</i>	3	—	341
	<i>Fusarium oxysporum</i>	25	—	340
	Uninoculated medium	40	—	328

Table 2. Effect of o- and p-benzoquinone on the oxidation of protocatechuate and catechol by cell-free extracts

Double-side-arm Warburg flasks contained in a total volume of 3.0 ml.: $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 5.6, 200 μmoles ; substrate, 10 μmoles ; inhibitor (in second side arm), 3 μmoles ; fungal extract, 0.5 ml.; 20% (w/v) KOH in the centre well, 0.2 ml. Flasks were incubated at 30°, inhibitor and substrate tipped simultaneously and readings taken at 5 min. intervals.

Organism	Substrate	Inhibitor	Rate of oxidation of substrate ($\mu\text{l.}/\text{hr.}/\text{mg. of protein}$)	Inhibition (%)
<i>Neurospora crassa</i>	Protocatechuate	None	33	—
		o-Benzoquinone	4	88
		p-Benzoquinone	1	97
<i>Fusarium oxysporum</i>	Protocatechuate	None	1280	—
		o-Benzoquinone	43	96
<i>Rhodotorula mucilaginosa</i>	Protocatechuate	None	134	—
		o-Benzoquinone	64	52
		p-Benzoquinone	26	81
<i>Debaryomyces subglobosus</i>	Catechol	None	81	—
		o-Benzoquinone	1	99
		p-Benzoquinone	0	100

Fig. 1. Spectrophotometric demonstration of the inhibition of protocatechuate 3,4-oxygenase in *Sporobolomyces* extracts by o-benzoquinone. Cuvettes contained in a total volume of 3 ml.: $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7.2, 250 μmoles ; extract, 0.1 ml. (0.32 mg. of protein); protocatechuate, 0.4 μmole ; and ●, no inhibitor, ○, 0.5 μmole , or △, 1 μmole of o-benzoquinone dissolved in acetone and added immediately after substrate. The amount of acetone added was adjusted to 0.1 ml. in all cuvettes including the control. Substrate disappearance was followed by the decrease in extinction at 290 m μ (ϵ 3890).

decarboxylation and oxidation of protocatechuate to a C_8 o-quinone was not necessarily enzymic. There was no evidence that catechol, a possible

precursor of the o-quinone, was formed in cultures of *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, *Fusarium oxysporum* or *Neurospora crassa* utilizing protocatechuate (Gross *et al.* 1956; Cain *et al.* 1968).

Anil formation in Debaryomyces cultures. To examine whether the simple physical presence of the yeast cells was favouring the increased anil formation observed in *Debaryomyces subglobosus* cultures (Table 1), six similar cultures (triplicate pairs) of this organism were grown on protocatechuate until the stationary phase (7 days), with aniline added to three of them. In these three cultures the anil yield was measured as described above. The remaining (untreated) three were each harvested, the cells were resuspended in water and boiled (30 min.) and the killed cells (checked by plating a loopful) were inoculated into triplicate flasks of fresh protocatechuate media, where incubation for 7 days was carried out after aniline addition. The viable cultures gave a mean anil yield of 83.8 ($\pm$ 12.5) mg.; the killed cultures (with the same final cell density as the viable cultures) gave a mean yield of 87.9 ($\pm$ 7.2) mg.; an uninoculated control yielded 48.3 mg. The appearance of an o-quinone in *Debaryomyces subglobosus* cultures was thus not a result of enzymic oxidation of the diphenol under these conditions, though the physical presence of cells, either living or dead, increased the anil yield.

The position of o-benzoquinone as a biologically formed intermediate in protocatechuate and catechol metabolism by these fungi is thus highly suspect. The failure of p-hydroxybenzoate-grown cultures to form an anil though they use an identical metabolic pathway, and anil production in both growing and dead cultures of *Debaryomyces subglobosus* as well as uninoculated incubations,

strongly suggest that its formation is independent of the living yeast.

Effect of benzoquinones on protocatechuate 3,4-oxygenase and catechol 1,2-oxygenase. With the exception of *Debaryomyces subglobosus*, all the organisms examined rapidly converted protocatechuate into β -carboxymuconate, a reaction catalysed by protocatechuate 3,4-oxygenase (Cain *et al.* 1968). This enzyme was severely inhibited by both *o*- and *p*-benzoquinone (Table 2 and Fig. 1); the inhibition could not be reversed by increasing the substrate concentration. *Debaryomyces subglobosus*, after growth with *p*-hydroxybenzoate, oxidized catechol to *cis-cis*-muconate (Cain *et al.* 1968). The catechol 1,2-oxygenase effecting this oxidation was almost completely inhibited by the quinones at 1 mM concentrations. This inhibition of the fungal enzymes catalysing the oxidation of the only two substrates from which it might be formed also makes it most unlikely that *o*-benzoquinone is an intermediate in protocatechuate metabolism.

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