The Formation of Pigment and Arylamine by Yeasts

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SUMMARY: The growth of several adenine-independent strains of yeasts in defined media containing suboptimal concentrations of biotin and relatively high concentrations of methionine is associated with the formation of a pink pigment in the cells and of an arylamine in the culture fluid. Under similar conditions, pigment and arylamine are not produced by an adenine-requiring strain of *Schizosaccharomyces octosporus*. Culture filtrates containing arylamine promote the growth of *Sch. octosporus* in adenine-free medium, but an adenine-requiring lactobacillus responds only after a considerable lag. The results are interpreted to mean that the arylamine is, or is derived from, a precursor of adenine, whose conversion to adenine is impaired by deficiency of biotin.

This study arose from the observation that the cells of a strain of *Saccharomyces cerevisiae* became pink in a certain nutritional environment. The phenomenon, reported in brief by Cutts & Rainbow (1950*a*) and in greater detail below, may throw light on the biosynthesis of adenine and on the role of biotin in the cell.

METHODS

Experimental cultures. Tube cultures (6 ml.) were carried out as described by Northam & Norris (1951). Cultures of 100 ml. were grown in 250 ml. Erlenmeyer flasks. Incubations were at 25°.

Test organisms and inocula. For most of this work, Yeast 47 (Rainbow, 1948) was used. This yeast requires exogenous *p*-aminobenzoic acid (*p*-AB), but will grow in its absence when provided with relatively high concentrations of methionine, adenine and histidine (Cutts & Rainbow, 1950*b*). Other organisms are listed in the appropriate experimental section. The strain of *Schizosaccharomyces octosporus* was that employed by Northam & Norris (1951). The organisms were maintained, and the inocula for tube tests prepared, as described by Cutts & Rainbow (1950*b*). Proportionately larger inocula were used for the larger test cultures.

The heterofermentative strain of lactobacillus used was maintained in liquid culture in clarified 10 % malt extract supplemented with 5 % of yeast autolysate. This organism, for which we are indebted to Mr W. B. Moore, was isolated from a brewer's pitching yeast and requires an exogenous source of adenine.

Media. The basal medium was that described by Cutts & Rainbow (1950*b*) except that biotin was omitted, 0.10 µg. *p*-AB/ml. added, and the pH adjusted to 4.8. For tests involving *Sch. octosporus*, the basal medium was supplemented with 100 µg./ml. each of DL-methionine and L-histidine HCl.

The medium used to test the growth response of the lactobacillus was

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a modification of that used for the microbiological assay of nicotinic acid (Analytical Methods Committee, 1946) but from which adenine was omitted.

Measurement of growth. The turbidities of 6 ml. tube cultures were measured *in situ* in the Hilger 'Spekker' absorptiometer (Northam & Norris, 1951), using distilled water as reference liquid. For determinations on larger cultures, samples of homogeneous yeast suspension were withdrawn and read in the 1 cm. Spekker cell. Weight of cell dry matter (Fig. 2) was determined from turbidity readings by use of a calibration curve.

Determination of arylamine. Culture filtrates were obtained by centrifuging 6 ml. cultures (or 6 ml. samples of larger cultures). The supernatant was poured off as completely as possible, and arylamine in the filtrate determined by the diazotization and coupling reaction of Bratton & Marshall (1939). To determine 'non-acetylatable' arylamine, the filtrate was treated with one-tenth of a volume of acetic anhydride at room temperature for $\frac{1}{2}$ –1 hr. before diazotization and coupling. Concentration of arylamine was expressed as the absorption of the azo colour in a 1 cm. cell, measured in the Spekker absorptiometer using Ilford filter no. 603 (peak transmission 5000 Å.). The absorption spectra of the azo compounds were determined with the same instrument using a range of filters. Since each filter transmits over a range of wavelengths, such spectra provide only a generalized outline, but which is useful for purposes of differentiating between (qualitatively) different azo compounds.

RESULTS

Pigment and arylamine formation by Yeast 47

Cultures of Yeast 47 were grown in tubes of basal media, each containing suboptimal concentrations of inositol, pantothenate, biotin and *p*-AB, and the effect of added methionine was tested in each case. Pigment was formed only in cultures which were both relatively deficient in biotin and which contained added methionine. Pigmentation of the cells was appreciable in the presence of 50 µg. DL-methionine/ml., but more marked with 500 µg./ml.

Culture filtrates from pink cells, but not those from normal white cells, gave an arylamine reaction much too intense to be ascribed to the *p*-AB content of the medium. The reactive compound differed from *p*-AB with respect to the absorption spectrum of the azo colour (Fig. 1) and in that it was non-acetylatable.

Tests in which L- and D-methionine were substituted for the DL-compound showed that the substance active in promoting pigment and arylamine formation was the L-methionine, the D-isomer being inactive.

Other conditions being suitable, cultures were substantially free from pigment and arylamine when any of the following four sets of conditions were observed: (a) the concentration of D-biotin was raised to 0.08 µg./ml. (Fig. 2); (b) *p*-AB was omitted from the medium and replaced by 100 µg./ml. each of adenine and L-histidine HCl (Table 1); (c) 100 µg. adenine/ml. was included in the basal medium (Table 1); (d) DL-methionine was absent or replaced by an equal concentration of any of the following amino-acids: DL-alanine, DL- α -aminobutyric acid, L-arginine, L-aspartic acid, L-cysteine,

L-cystine, L-histidine, DL-homocystine, L-hydroxyproline, L-glutamic acid, glycine, L-leucine, L-lysine, DL-norleucine, DL-norvaline, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, DL-valine. DL-Tryptophan also failed to induce pigment formation, but in this case arylamine was not tested for, since the amino-acid itself gave a coloured compound on diazotization and coupling.

Table 1. *Arylamine formation by Yeast 47 in the presence and absence of adenine*

Six ml. cultures in basal medium containing 0.02 m μ g. D-biotin/ml. Series A contained 10 m μ g. *p*-AB/ml.; series B contained no *p*-AB, but 100 μ g./ml. each of adenine and L-histidine HCl; series C contained 10 m μ g. *p*-AB and 100 μ g. adenine/ml. Incubation time 90 hr.

	DL-methionine (μ g./ml.)	Growth (Spekker reading)	Colour of cells	Non-acetylable arylamine (absorption units)*
Series A	0	0.53	White	0.052
	125	0.52	Pink	0.227
	250	0.50	Pink	0.281
	500	0.48	Pink	0.279
Series B	0	0.32	White	0.018
	125	0.49	White	0.018
	250	0.50	White	0.019
	500	0.52	White	0.018
Series C	0	0.44	White	0.019
	125	0.44	White	0.031
	250	0.45	Sl. pink	0.070
	500	0.44	Sl. pink	0.080

* Absorption units = concentration of arylamine expressed as the absorption of the azo colour in a 1 cm. cell, measured in Spekker absorptiometer using Ilford filter no. 603 (peak transmission 5000 Å.).

Fig. 2, in which the ratio arylamine reading/dry weight of cells is plotted against concentration of biotin, shows that arylamine formation per unit weight of cell substance increased as biotin deficiency became more marked and as methionine concentration increased. Maximum arylamine formation occurred in cultures containing approximately 500 μ g. DL-methionine and 0.02 m μ g. D-biotin/ml. The maximum was reached in 96 hr., arylamine concentration diminishing thereafter with time (Table 2).

Table 2. *Progress of arylamine formation by Yeast 47*

Cultures in basal medium containing 0.01 m μ g. D-biotin and 500 μ g. DL-methionine/ml.

Incubation (days)	Growth (Spekker reading)	Arylamine absorption units*
2	0.14	0.065
3	0.21	0.173
4	0.25	0.240
5	0.26	0.218
6	0.30	0.178
9	0.36	0.105
11	0.39	0.083

* See footnote to Table 1.

Arylamine and pigment formation by other yeasts

The following strains of biotin-dependent yeasts and yeast-like organisms, maintained in this laboratory, produced pigment and arylamine when grown for periods up to 150 hr. in defined medium partially deficient in biotin and containing 500 μg . DL-methionine/ml. namely: *S. carlsbergensis* 4228, five

Fig. 1

Fig. 2

Fig. 1. Absorption spectra of diazotized and coupled *p*-AB and arylamine.

Fig. 2. Relationship of arylamine formation by Yeast 47 to concentration of biotin and methionine. The figures above the curves are concentrations of DL-methionine in μg /ml.

single-cell isolates of brewer's top fermentation yeasts (*S. cerevisiae*) and a strain of *Brettanomyces*. Pigment was also formed by *Saccharomycodes ludwigii* and *Sch. pombe*, but these culture filtrates gave a relatively feeble reaction for arylamine. *Kloeckera brevis* and *K. apiculata* formed little and no pigment respectively and little arylamine.

Neither pigment nor arylamine was produced in cultures of the adenine-dependent strain of *Sch. octosporus* (Northam & Norris, 1951).

Growth-promoting properties of culture filtrates containing arylamine

Neither *Sch. octosporus*, which requires exogenous adenine, nor Yeast 47 when grown in media from which *p*-AB is omitted (so that it is unable to synthesize adenine) forms appreciable arylamine. This suggests that when arylamine accumulates in cultures independent of exogenous adenine, it may do so as a result of a block in adenine synthesis. Arylamine, or a substance from which arylamine is derived, may be therefore a precursor of purine.

Culture filtrates containing arylamine were therefore tested for ability to replace adenine in the nutrition of *Sch. octosporus* and the heterofermentative lactobacillus, both of which require exogenous adenine for growth.

Cultures (100 ml.) of Yeast 47 were grown in basal medium supplemented (1 ml.) as follows: medium I, 2 m μ g. D-biotin; medium II, 0.01 m μ g. D-biotin; medium III, 0.01 m μ g. D-biotin and 500 μ g. DL-methionine. After 96 hr., the

Fig. 3

Fig. 4

Fig. 3. Response of *Sch. octosporus* in adenine-free medium to culture filtrates containing arylamine. Filtrates were added at the rate of 0.3 ml. (expressed as original filtrate)/ml. of medium. Arylamine contents, in terms of the original filtrates, are in brackets above the appropriate curves.

Fig. 4. Response of an adenine-requiring lactobacillus in adenine-free medium to a culture filtrate containing arylamine. Curve A, no added filtrate; curve B, +1 ml. filtrate/ml.; curve C, as B, but 10 μ g. adenine/ml. added ($\downarrow$) after 91 hr.; curve D, +10 μ g. adenine/ml. The filtrate was identical with filtrate III (Fig. 3).

cultures were centrifuged, the supernatant fluids filtered through Whatman no. 44 paper and the filtrates concentrated *in vacuo* below 40° to one-sixth volume. After withdrawing a sample for arylamine determination the concentrates were tested for growth-promoting activity for *Sch. octosporus* and the lactobacillus in adenine-free media. Culture filtrate III (which contained arylamine) was much superior to filtrates I and II (which contained little or no arylamine) as a substituent for adenine in the nutrition of *Sch. octosporus* (Fig. 3). For the lactobacillus, filtrate III replaced adenine after a considerable delay (148 hr.), whereas the response to added adenine was almost immediate (Fig. 4). This delay was not caused by inhibitors added with the filtrate, since addition of adenine after 91 hr. evoked an immediate response. The delayed response of the lactobacillus to filtrate III thus indicates that the active substance in the filtrate is not adenine itself.

DISCUSSION

In this work, the formation of pigment by cells was invariably associated with the presence of arylamine in the culture filtrate. However, pigment and arylamine are not identical, since filtrates containing relatively high con-

centrations of the latter are usually (but not invariably) colourless. Pigment may result from the interaction of the arylamine with some cell constituent.

Our pigment may be identical with, or related to, that reported by Lindegren & Lindegren (1947) who associated the formation of a pink pigment by certain mutants of *S. cerevisiae* with a block in adenine synthesis, coupled with the presence of excess methionine and with other substances. Assuming such a relationship, the following scheme might represent the data:

The Lindegrens suggested that the accumulation of an intermediate by their mutants caused pigment formation. Provided there is no further synthetic disability between the intermediate and adenine, the former (or an arylamine derived from it) might be expected to replace adenine in the nutrition of adenine-dependent micro-organisms, such as we have demonstrated. That arylamine did not accumulate in cultures containing more nearly optimal quantities of biotin suggests that, in Yeast 47, biotin may function in converting the intermediate to adenine.

Stetten & Fox (1945) observed the accumulation of diazotizable amine in cultures of *Escherichia coli* under conditions of sulphonamide bacteriostasis. This amine, 5(4)-amino-4(5)-imidazolecarboxamide, was regarded by Shive, Ackermann, Gordon, Getzendaner & Eakin (1947) as an intermediate in purine biosynthesis and may be related to our amine, although the latter appears distinct with respect to the absorption maximum of the azo compound and in being relatively insensitive to oxidation by a current of air passed through solutions overnight.

That pigment and arylamine formation took place in other species and genera of biotin-dependent yeasts suggests that these phenomena reflect some abnormality of fundamental metabolic processes common to several yeasts and yeast-like organisms.

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