A Specific Requirement for Biotin in the Synthesis of Ornithine Carbamoyltransferase by Yeast

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1. Growth of a biotin-requiring strain of *Saccharomyces cerevisiae* in a medium containing a suboptimum concentration of biotin for growth caused a decreased synthesis of ornithine carbamoyltransferase as compared with yeast grown in a medium containing an optimum concentration of biotin. Inclusion of the biotin homologues norbiotin or homobiotin, but not bishomobiotin, in the biotin-deficient medium caused an appreciable increase in ornithine carbamoyltransferase synthesis without affecting growth or synthesis of total RNA and protein. The addition of norbiotin to biotin-deficient medium had no effect on the respiratory activity of the yeast or on the synthesis of aspartate carbamoyltransferase, acid phosphatase, β -fructofuranosidase or malate dehydrogenase. 2. Synthesis of acetylornithine deacetylase and acetylornithine acetyltransferase was slightly diminished by the imposition of biotin deficiency, but the effect was not as great as on ornithine carbamoyltransferase synthesis. Incorporation of norbiotin in the biotin-deficient medium had no marked effect on the synthesis of any other arginine-pathway enzyme except ornithine carbamoyltransferase. 3. L-Ornithine induced synthesis of ornithine carbamoyltransferase in yeast grown in biotin-deficient medium, but in yeast grown in this medium supplemented with norbiotin it repressed synthesis of the enzyme. L-Arginine had no detectable effect on ornithine carbamoyltransferase synthesis by the yeast grown in biotin-deficient medium with or without norbiotin. L-Aspartate repressed synthesis of ornithine carbamoyltransferase in biotin-deficient yeast and completely nullified the stimulatory effect of norbiotin on synthesis of the enzyme in this yeast. 4. There was no increase in ornithine carbamoyltransferase synthesis in biotin-deficient yeast incubated in phosphate buffer, pH 4.5, containing glucose and biotin or norbiotin. In biotin-deficient yeast suspended in complete medium containing an optimum concentration of biotin, there was an increase in ornithine carbamoyltransferase synthesis only after the onset of growth.

The major established metabolic role of the B-group vitamin biotin is as a prosthetic group in certain carboxylases and carboxytransferases (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959; Utter & Keech, 1960; Kaziro, Leone & Ochoa, 1960; Wakil & Gibson, 1960; Wood & Stjernholm, 1961). However, comparisons of the enzymic activities of several biotin-requiring micro-organisms grown in biotin-optimum and biotin-deficient media have shown that other enzymes in addition to those containing biotin show diminished activity in biotin-deficient organisms, and this has led to the suggestion that biotin may be required in the synthesis of certain enzymes. The first intimation of a biotin requirement in enzyme synthesis was by Ochoa *et al.* (1947), who showed that synthesis of L-malate-NADP oxidoreductase (decarboxylating) (EC 1.1.1.40) in pigeon liver was diminished in

biotin-deficient birds, although biotin could not be detected in the purified enzyme. Similar findings were reported by Blanchard, Korkes, Del Campillo & Ochoa (1950) for the inducible L-malate-NAD oxidoreductase (decarboxylating) (EC 1.1.1.38) in *Lactobacillus arabinosus* 17-5. Later studies on this enzyme by Ables, Ravel & Shive (1961) showed that the biotin requirement for synthesis could be replaced by asparagine or, more effectively, by asparagine peptides, though not by aspartate, which inhibited enzyme induction even in the presence of biotin. These workers concluded that the role of biotin in the synthesis of the enzyme was in the biogenesis of a C₄ unit derived from aspartate, but that in bacteria grown in a biotin-deficient medium the mechanisms regulating synthesis of the enzyme were deranged and allowed exogenous aspartate to repress formation of this unit. Duerre & Lichstein

(1961) found, however, that aspartate could partially spare the effect of biotin on the synthesis of the enzyme in *L. arabinosus*.

A requirement for biotin in the synthesis of carbamoyl phosphate-L-ornithine carbamoyltransferase (EC 2.1.3.3) (OCT*) was first reported by Estes, Ravel & Shive (1956). They found that extracts of a strain of *Streptococcus lactis* grown in a biotin-deficient medium had much lower OCT activity than extracts of bacteria grown in the presence of an optimum concentration of biotin. The OCT activity of extracts of biotin-deficient bacteria could not be restored by adding biotin to the reaction mixture although it was possible to obtain increased activity by incubating the bacteria for 2 hr. in a medium containing biotin. Shive and his colleagues subsequently attributed the specific role of biotin in the synthesis of OCT by this bacterium to its function in providing C₄ units derived from aspartate, since OCT synthesis was stimulated when biotin-deficient bacteria were incubated in a biotin-free medium containing certain asparagine peptides or, to a smaller extent, aspartate (Ravel, Mollenhauer & Shive, 1961). Ahmad & Rose (1962) reported that the imposition of biotin deficiency in a biotin-requiring strain of *Saccharomyces cerevisiae* resulted in decreased OCT activity in extracts of the yeast. However, the addition of aspartate to the biotin-deficient medium, though it partially restored growth and synthesis of total protein and RNA, caused a further diminution in OCT activity from that recorded in biotin-deficient yeast. This difference in the effect of aspartate on OCT synthesis in these two organisms prompted a further examination of the role of biotin in OCT synthesis by the yeast. During this work, it was found that the biotin homologues norbiotin and homobiotin caused a marked stimulation of OCT synthesis in biotin-deficient yeast without affecting growth (Dixon & Rose, 1964). The present paper describes further experiments on the requirement for biotin in OCT synthesis by the yeast, particularly with regard to the effect of norbiotin, and reports evidence for a very specific role for biotin in the synthesis of this enzyme. A preliminary account of some of these experiments has been published (Dixon & Rose, 1965).

METHODS

Organism. The yeast used was a biotin-requiring strain of *Saccharomyces cerevisiae* Fleischmann (Rose, 1960), obtained from Miss M. T. Clement of the Division of Biosciences, National Research Council of Canada, Ottawa, Canada. It was maintained and stored as described by Dixon & Rose (1964).

* Abbreviation: OCT, ornithine carbamoyltransferase.

Experimental cultures. The chemically defined medium (pH 4.5) of Rose & Nickerson (1956), containing either a suboptimum (0.04 μM) or an optimum (0.80 μM) concentration of D-biotin, was used for all experimental cultures. Medium containing the suboptimum concentration of biotin is referred to as 'biotin-deficient medium' and that containing optimum concentration as 'biotin-optimum medium'. In certain experiments, amino acids or other compounds were added to the medium as solutions (pH 4.5). All supplements were examined for contamination with biotin by examining their ability to stimulate growth of the biotin-requiring yeast in biotin-free medium.

Portions (95 ml.) of medium were dispensed into 350 ml. conical flasks that were sterilized by autoclaving momentarily at 10 lb./in.². Each flask of medium was inoculated with 5 ml. of a suspension containing the equivalent of 1.0 mg. dry wt. of washed biotin-deficient yeast/ml. of 67 mM-KH₂PO₄, pH 4.5. Cultures were incubated statically at 25°. The yeast used as inoculum was prepared by harvesting organisms grown for 120 hr. in 100 ml. cultures of biotin-deficient medium inoculated as described by Rose (1963). Yeast was harvested from experimental cultures by centrifuging at 0° and washed three times in 67 mM-KH₂PO₄ before being analysed. Growth was measured turbidimetrically as described by Rose (1960) and turbidity readings were related to mg. dry wt. of yeast.

Preparation and purification of cell extracts. Extracts of the yeast for use in enzyme assays were prepared with the Mickle (1948) tissue disintegrator. Yeast was harvested by centrifugation at 0° and washed three times in ice-cold water. The cells were resuspended in 5 ml. of ice-cold water and transferred to an ice-cold Mickle cell containing 3 g. of Ballotini beads (grade 12). The yeast was disrupted by shaking the cell at maximum amplitude for a total period of 30 min. To minimize enzyme denaturation during disruption, the disintegrator was operated inside a refrigerator (5°) for alternate periods of 5 min. The suspension of disrupted cells was transferred to a cold tube and centrifuged at approx. 1300g for 30 min. in an International refrigerated centrifuge (model PR-2, no. 259 head). The supernatant was removed and, if not used immediately, stored at -15°. Storage at this temperature was shown not to cause a measurable loss in enzyme activities.

Low-molecular-weight ninhydrin-positive compounds were removed from cell extracts before they were used in certain enzyme assays. Cell extract (5 ml. containing 500–3000 μg . of protein/ml.) was applied to a column (15 cm. $\times$ 3 cm.) of Sephadex G-25 that had been equilibrated with mM-K₂HPO₄ buffer, pH 7.5. The column was eluted with K₂HPO₄ buffer and fractions (4 ml.) containing the bulk of the protein were pooled and concentrated by using dry Sephadex G-25 (Flodin, Gelotte & Porath, 1960). Cell extracts treated in this way are referred to as 'purified cell extracts'.

Extracts of the yeast for use in the estimation of the contents of intracellular water-soluble ninhydrin-positive compounds, and of individual amino acids in these water extracts, were obtained by heat disruption. A portion of washed yeast containing the equivalent of 10 mg. dry wt. of cells was washed three times in ice-cold water and resuspended in 10 ml. of water. The suspension was placed in a boiling-water bath for 15 min. and, after cooling, the cell debris was removed by centrifuging. The supernatant was removed and, after being supplemented by washings

(2 × 1 ml.) of the cell debris, was made up to 10 ml. with water. The extracts were either used immediately or stored at -15° until required.

Enzyme assays. Enzyme activities in extracts of the yeast were measured with suitable quantities of cell extract and were calculated from initial reaction velocities determined over a period in which plots of the amount of chemical change in the reaction mixture against time were linear. All enzyme activities are expressed as specific activities (μ moles of substrate utilized/mg. of protein/hr.) at the temperature of the assay.

(a) OCT activity was determined by a modification of the method of Estes *et al.* (1956) as described by Ahmad & Rose (1962). Duplicate portions of suitably diluted cell extract containing 5–20 μ g. of protein were added to tubes containing carbamoyl phosphate (dilithium salt) (20 μ moles), DL-ornithine hydrochloride, pH 8.0 (10 μ moles), and tris buffer (10 μ moles) adjusted to pH 8.0 with 0.1 N-HCl in a total volume of 1.0 ml. Tubes containing this reaction mixture were incubated for 2 hr. in a water bath at 30° with gentle shaking after each 30 min. period. The reaction was stopped by the addition of 2.5 ml. of N-HClO₄ to each tube. A reaction mixture containing cell extract that had been heated in a boiling-water bath for 10 min. was used as a blank. The concentration of citrulline in the reaction mixture was then estimated by the method of Archibald (1944). Extinctions were measured in 1 cm. glass cells in the Hilger Spekker absorptiometer (model H760) with the 603 filter (peak absorption at approx. 485 m μ), and readings were related to μ g. of citrulline by using a standard curve prepared with DL-citrulline. There was no detectable effect on the OCT activities of cell extracts when biotin (1.6 μ moles), norbiotin (20 μ moles) or homobiotin (20 μ moles) was included in the reaction mixture.

(b) Acetylglutamate-reductase (*N*-acetylglutamate γ -semialdehyde-NADP oxidoreductase) activity in purified cell extracts was determined by the method of De Deken (1962) at 25°.

(c) Acetylornithine-aminotransferase (α -*N*-acetyl-L-ornithine-2-oxoglutarate aminotransferase; EC 2.6.1.11) activity in purified cell extracts was determined by the method of Albrecht & Vogel (1964) at 30°.

(d) Acetylornithine-deacetylase (α -*N*-acetyl-L-ornithine amidohydrolase; EC 3.5.1.16) activity of purified cell extracts was determined by the method of Vogel & Bonner (1956) at 30°.

(e) Acetylornithine-acetyltransferase (α -*N*-acetyl-L-ornithine-L-glutamate *N*-acetyltransferase) activity in purified cell extracts was determined by the method of Middlehoven (1963) at 30°.

(f) Argininosuccinate-synthetase [L-citrulline-L-aspartate ligase (AMP); EC 6.3.4.5] activity in cell extracts was determined at 30° by a method described by Ratner (1955a) for use with crude extracts.

(g) Argininosuccinate-lyase (L-argininosuccinate arginine-lyase; EC 4.3.2.1) activity in cell extracts was determined by the method of Ratner (1955b) at 30°.

(h) Malate-dehydrogenase (L-malate-NAD oxidoreductase; EC 1.1.1.37) activity in cell extracts was determined at 25° by a modification of the method of Beaufay, Bendall, Baudhuin & de Duve (1959) as described by Ahmad & Rose (1962).

(i) β -Fructofuranosidase (β -D-fructofuranoside fructohydrolase; EC 3.2.1.26) activity of whole yeast cells was

determined at 37° by the method described by Ahmad & Rose (1962).

(j) Acid-phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2) activity of whole yeast cells was determined at 37° by a modification of the method of Lowry, Roberts, Wu, Hixon & Crawford (1954) with phenyl disodium phosphate as substrate.

(k) Aspartate-carbamoyltransferase (carbamoyl phosphate-L-aspartate carbamoyltransferase; EC 2.1.3.2) activity in cell extracts was determined by the method of Yates & Pardee (1956) at 30°.

Analytical methods. The RNA content of the yeast was estimated by a modification of the method of Schmidt & Thannhauser (1945) described by Ahmad, Rose & Garg (1961). Protein in the residue after acid-soluble u.v.-absorbing compounds, RNA and DNA had been extracted from the yeast was estimated by the micro-Kjeldahl method (Markham, 1942) with an HgO catalyst (Miller & Houghton, 1954). The protein contents of cell extracts used in enzyme assays were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as a standard. Water-soluble ninhydrin-positive compounds in water extracts of the yeast were determined by a modification of the Smith & Agiza (1957) method described by Hagen & Rose (1962). The ornithine content of water extracts of the yeast was determined by the method of Chinard (1952), and the arginine content of these extracts by the procedure of Rosenberg, Ennis & Morrison (1956).

Respiratory activity. Respiratory activity of suspensions of yeast was measured by the conventional Warburg technique (Umbreit, Burris & Stauffer, 1964) with the constant-volume Warburg respirometer (model V85; B. Braun, Melsungen, W. Germany).

Chemicals. All chemicals used were of the highest purity available commercially. When possible A.R. chemicals were used. Vitamins, with the exception of D-biotin, were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., as also were L-asparagine, L-aspartic acid, DL-ornithine and L-ornithine. D-Biotin was purchased from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. Arginase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. D-Homobiotin, D-norbiotin and DL-bishomobiotin were kindly given by Hoffman-La Roche Inc., Nutley, N.J., U.S.A. The purity of these biotin homologues was checked by melting-point determinations and the values obtained compared closely with those reported by Sternbach (1963).

RESULTS

Effect of biotin and biotin homologues on OCT synthesis. Dixon & Rose (1964) reported that growth and OCT synthesis were markedly restricted in yeast grown in biotin-deficient medium as compared with yeast grown in biotin-optimum medium. They also showed that the biotin homologues norbiotin and homobiotin caused a very marked stimulation in OCT synthesis in yeast grown in biotin-deficient medium without affecting growth; these homologues had no detectable effect on OCT synthesis in yeast grown in a medium containing an optimum concentration of biotin. Attempts were

Table 1. OCT activities of extracts of yeast grown in biotin-optimum or biotin-deficient medium in the presence and absence of biotin homologues

Yeast was grown and harvested and OCT activities of cell extracts were determined as described in the Methods section. Homologues were included in media at 10 μM . G denotes growth (mg. dry wt./ml.) and A specific activity (μmoles of ornithine utilized/mg. of protein/hr. at 30°).

Concn. of biotin in medium	Age of culture (hr.)	Homologue included in medium							
		None		D-Norbiotin		D-Homobiotin		DL-Bishomobiotin	
		G	A	G	A	A	A	G	A
0.04 μM (deficient)	16.5	0.12	22.2	0.10	69.6	0.13	56.2	0.09	21.5
	65	0.22	13.0	0.25	102.0	0.25	35.5	0.21	13.9
0.80 μM (optimum)	18	0.37	38.6	0.39	37.5	0.34	38.5	0.40	38.5
	42	0.80	25.5	0.84	24.3	0.82	25.9	0.79	24.5

Table 2. Specific activities of arginine-pathway enzymes in extracts of yeast grown in biotin-optimum medium and in biotin-deficient medium with and without norbiotin

Yeast was grown and harvested and enzyme activities in cell extracts were determined as described in the Methods section. All enzyme activities are expressed as specific activities (μmoles of substrate utilized/mg. of protein/hr.) at the temperature of the assay.

Medium	Age of culture (hr.)	Enzyme activity						
		Acetyl- glutamate reductase	Acetyl- ornithine amino- transferase	Acetyl- ornithine deacetylase	Acetyl- ornithine acetyl- transferase	Ornithine carbamoyl- transferase	Arginino- succinate synthetase	Arginino- succinate lyase
Biotin- optimum	12	1.50	0.95	0.26	1.32	30.5	0.55	1.02
	24	1.20	0.82	0.23	1.50	43.7	0.71	0.98
	36	1.12	0.79	0.24	1.45	27.0	1.01	1.40
Biotin- deficient	12	1.25	1.02	0.16	1.11	19.5	1.54	2.50
	24	1.40	0.63	0.22	1.15	21.8	1.44	0.80
	48	1.17	0.23	0.18	1.29	13.0	1.32	1.18
Biotin- deficient + norbiotin (10 μM)	12	1.24	1.01	0.17	1.12	54.5	1.56	2.57
	24	1.30	0.58	0.18	1.14	59.8	1.44	0.81
	48	1.20	0.24	0.22	1.20	92.5	1.22	1.20

made to obtain other members of the homologous series in order to examine their effect on OCT synthesis in the yeast. However, it was possible to obtain only one other homologue, bishomobiotin, and, as shown in Table 1, this compound had no effect on the growth of, or OCT synthesis by, the yeast grown in biotin-deficient or biotin-optimum medium.

Localization of the effect of biotin homologues on yeast grown in biotin-deficient medium. No detectable differences were found in the contents of RNA, total protein and water-soluble ninhydrin-positive compounds in yeast grown in biotin-deficient medium supplemented with norbiotin or homobiotin (10.0 μM) as compared with yeast grown in unsupplemented biotin-deficient medium. Nor could differences be detected in the respiratory

activities of yeast grown in these media. Ahmad & Rose (1962) showed that the imposition of biotin deficiency on this strain of yeast caused changes in the specific activities of acid phosphatase, β -fructofuranosidase and malate dehydrogenase in cell extracts in addition to the changes in OCT activity already reported. However, the incorporation of norbiotin or homobiotin (10.0 μM) in biotin-deficient medium had no detectable effect on the activities of these enzymes in cell extracts.

Aspartate carbamoyltransferase has a similar type of catalytic activity to that of OCT, and it was decided to examine the effect of biotin deficiency on the former activity in extracts of the yeast. Ravel, Grona, Humphreys & Shive (1958) showed that extracts of a biotin-requiring strain of *L. arabinosus* had lower OCT and aspartate-carbamoyltransferase

activities when the bacterium was grown in a biotin-deficient medium than when grown in a medium containing an optimum concentration of biotin. Inclusion of biotin (10.0 μM), norbiotin (10.0 μM), homobiotin (10.0 μM) or L-aspartate (2.0 mM) in the biotin-deficient or biotin-optimum medium had no marked effect on the aspartate-carbamoyl-transferase activities of the extracts. Nor did the inclusion of L-aspartate in biotin-deficient medium affect the synthesis of aspartate carbamoyltransferase by the yeast.

Effect of biotin and norbiotin on the synthesis of arginine-pathway enzymes. In view of the marked specificity shown by biotin and norbiotin in OCT synthesis in biotin-deficient yeast, an examination was made of the effect of these compounds on the synthesis of other arginine-pathway enzymes in the yeast. Table 2 shows the activities of seven arginine-pathway enzymes in extracts of the yeast grown in biotin-optimum medium or in biotin-deficient medium with or without norbiotin. No reliable method is available for assaying *N*-acetylglutamate synthetase. Two enzymes are thought to catalyse the conversion of *N*-acetylglutamate into *N*-acetylglutamic γ -semialdehyde, the conversion proceeding through *N*-acetylglutamate phosphate (Vogel, Bacon & Baich, 1963). However, in this study, the overall ability of cell extracts to produce *N*-acetylglutamic γ -semialdehyde from *N*-acetylglutamate is expressed as acetylglutamate-reductase activity. The specific activities of each of the enzymes in extracts of biotin-optimum yeast were of the same order as reported for yeasts by other workers (Middlehoven, 1963; De Deken, 1963). Two enzymes catalysing the conversion of *N*-acetylornithine into ornithine were detected in cell extracts, namely acetylornithine deacetylase and

acetylornithine acetyltransferase, the specific activity of the acetyltransferase in extracts of biotin-optimum yeast being about four times that of the deacetylase. Extracts of yeast grown in biotin-deficient medium had higher activities of argininosuccinate synthetase and argininosuccinate lyase as compared with extracts of the biotin-optimum yeast, whereas the activities of OCT and, to a smaller extent, acetylornithine deacetylase and acetylornithine acetyltransferase were lower. Incorporation of norbiotin in biotin-deficient medium had no marked effect on the synthesis by the yeast of any of the enzymes except OCT. The specific activities of OCT in extracts of biotin-optimum and biotin-deficient yeast were much greater than the activities of the other arginine-pathway enzymes examined.

Effect of norbiotin on the induction and repression of OCT synthesis. L-Arginine (100 $\mu\text{g./ml.}$) does not repress the synthesis of OCT in biotin-deficient or biotin-optimum yeast (Dixon & Rose, 1964). Arginine at this concentration had no marked effect on the synthesis of other arginine-pathway enzymes by biotin-deficient or biotin-optimum yeast. Moreover, the inclusion of norbiotin (10.0 μM) in arginine-containing biotin-deficient or biotin-optimum medium had no appreciable effect on the synthesis of any of these enzymes. OCT synthesis in biotin-deficient and biotin-optimum yeast is, however, induced by ornithine (100 $\mu\text{g./ml.}$) (Dixon & Rose, 1964), but the synthesis of other arginine-pathway enzymes was not detectably affected by the presence of ornithine in the medium. There was, however, no induction of OCT synthesis by ornithine in yeast grown in biotin-deficient medium containing norbiotin; indeed, ornithine repressed OCT synthesis by yeast grown in this medium (Table 3). L-Aspartate, which represses synthesis of OCT in

Table 3. *Effect of norbiotin on OCT synthesis by yeast grown in biotin-deficient medium supplemented with L-ornithine or L-aspartate or both*

Yeast was grown and harvested and OCT activities of cell extracts were determined as described in the Methods section. OCT activity is expressed as μmoles of ornithine utilized/mg. of extract protein/hr. at 30°.

Supplements in biotin-deficient medium	Age of culture (hr.)	OCT activity in extracts of yeast grown in medium	
		Lacking norbiotin	Containing norbiotin (10.0 μM)
None	16.5	22.2	69.6
	65	13.2	102.0
L-Aspartate (2.0 mM)	12	7.9	9.9
	22	8.3	7.2
L-Ornithine (100 $\mu\text{g./ml.}$)	16	23.3	54.0
	65	22.4	89.4
L-Aspartate (2.0 mM) + L-ornithine (100 $\mu\text{g./ml.}$)	10	24.5	26.0
	22	21.5	22.0

biotin-deficient yeast (Dixon & Rose, 1964), completely nullified the stimulatory effect of norbiotin on OCT synthesis in biotin-deficient yeast (Table 3). However, the activities of OCT in extracts of yeast grown in biotin-deficient medium containing ornithine and norbiotin were two or three times as great as when aspartate was also included in the medium (Table 3).

Effect of biotin and norbiotin on the synthesis of OCT in yeast-cell suspensions. Since biotin (or norbiotin) appeared to have a very specific role in the synthesis of OCT by the yeast, an attempt was made to discover how the vitamin acts in the synthesis of the enzyme. One possibility was that biotin-deficient yeast can synthesize an inactive form of the OCT molecule and that the conversion of this into a catalytically active form involves one or more reactions that require biotin or norbiotin. To test this hypothesis, biotin-deficient yeast was suspended in phosphate buffer, pH 4.5, with and without biotin and norbiotin, and the OCT activity of extracts of the yeast was measured after the suspension had been incubated for different periods of time at 25°. The OCT activities of extracts of yeast that had been suspended in buffer containing biotin or norbiotin declined slightly over a period of 6 hr. Similar results were obtained when the yeast was suspended in buffer containing biotin and glucose. When the yeast was suspended in medium containing an optimum concentration of biotin for growth (0.8 μM), there was an increase in the OCT activity of extracts of the yeast, but only after 7–10 hr. incubation, at approximately the same time as the commencement of growth (Table 4).

Table 4. OCT activities of extracts of yeast suspended in biotin-containing medium

Biotin-deficient yeast was harvested from cultures grown for 120 hr. in biotin-deficient medium as described in the Methods section. The yeast was washed and resuspended to a density of about 0.5 mg. dry wt. equiv./ml. of 67 mM-KH₂PO₄, pH 4.5, in medium containing biotin (0.8 μM). The suspension was incubated at 25° and shaken every 15 min. Portions of the suspension were removed at intervals and the OCT activity in extracts of the yeast was determined as described in the Methods section. OCT activities are expressed as μmoles of ornithine utilized/mg. of extract protein/hr. at 30°. The content of yeast in the suspension was measured as described in the Methods section.

Age of suspension (hr.)	OCT activity of extract	Content of yeast in suspension (mg. dry wt. equiv./ml.)
0	7.4	0.51
5	8.3	0.51
7	9.4	0.53
10	19.8	0.68

DISCUSSION

The discovery that the biotin homologues norbiotin and homobiotin stimulated OCT synthesis in biotin-deficient yeast, but were without effect on growth or synthesis of total RNA and protein, suggested that the homologues acted by substituting for biotin in one or more of the biotin-requiring reactions involved in OCT synthesis but were unable to replace biotin in other biotin-requiring reactions that are essential for growth. If one assumes that the biotin homologues act in this way, our data can be taken to indicate that biotin has a very specific role in OCT synthesis in yeast. This conclusion agrees with that reported by Dixon & Rose (1964). It is, however, at variance with the explanation given by Ravel *et al.* (1961) to account for the role of biotin in OCT synthesis by *Strep. lactis*. These workers suggested that biotin functions in OCT synthesis in this bacterium by providing C₄ units (such as aspartate and asparagine) for synthesis of the enzyme. This function is difficult to reconcile with a specific role for biotin in OCT synthesis in the yeast since, on general biochemical grounds, it is reasonable to assume that these C₄ units are required in the synthesis of all enzymes. Our data also differ from those reported by Ravel *et al.* (1961) with regard to the effect of homobiotin and aspartate on OCT synthesis by biotin-deficient cells.

As there was no effect on the synthesis of any of the arginine-pathway enzymes examined, except OCT, when norbiotin, homobiotin or ornithine was included in biotin-deficient medium, it is clear that the control of OCT synthesis in the yeast is independent of the synthesis of other enzymes on the pathway. A similar finding was reported by Gorini, Gunderson & Burger (1961) for certain strains of *Escherichia coli*. The specific activities of OCT in extracts of the yeast were, under all of the conditions studied, greater than those of any of the other arginine-pathway enzymes, and it would seem that the reaction catalysed by OCT in the yeast is not rate-limiting on the pathway to arginine synthesis. Davis (1962) reported a similar finding for a mutant strain of *Neurospora crassa*. It was also shown (B. Dixon & A. H. Rose, unpublished results) that, in yeast grown in biotin-deficient medium containing ornithine, there was a marked increase in the accumulation of intracellular citrulline as compared with yeast grown in unsupplemented biotin-deficient medium.

The possibility that OCT is a biotin enzyme was rejected by Ravel *et al.* (1958) when they reported that the purified enzyme from *Strep. lactis* did not contain biotin as assayed microbiologically, and that its activity was not inhibited by avidin. We have also shown that the OCT activity in extracts of the yeast was not affected measurably when avidin

was added to the reaction mixture (unpublished work). Biotin may, however, be bound to OCT in such a way that it does not combine with avidin and is not released by acid hydrolysis in a form detectable by microbiological assay. The possibility that biotin may be involved in the synthesis of a non-protein prosthetic group on the OCT molecule is discounted as there has been no report of the existence of such a prosthetic group attached to OCT. Moreover, the inability of biotin-deficient yeast to synthesize increased amounts of OCT when suspended in buffer containing an optimum concentration of biotin, with or without glucose, suggests that the vitamin does not act by converting an inactive form into a catalytically active form of OCT. This finding is relevant in view of the report by Rogers (1965), who demonstrated an increase in OCT activity in reaction mixtures containing mechanically disrupted spheroplasts of *E. coli*, and attributed the increase not to synthesis of the enzyme but to the 'completion' of polypeptide chains to give catalytically active proteins.

Several of our data suggest that biotin may function in OCT synthesis through mechanisms that regulate induction and repression of enzyme synthesis. The marked increase in OCT synthesis in yeast that had been incubated in biotin-containing medium, once growth had started, is very suggestive of the operation of a de-repression process. Also, ornithine induced OCT synthesis in biotin-deficient and biotin-optimum yeast (Dixon & Rose, 1964) but not in yeast grown in biotin-deficient medium supplemented with norbiotin. As the content of ornithine in the intracellular amino acid pool was not increased by growing biotin-deficient yeast in the presence of norbiotin, it seems likely that the biotin homologue caused a de-repression of OCT synthesis by some mechanism other than increasing the concentration of intracellular inducer. However, it is not clear how norbiotin brings about this de-repression unless it is assumed that it is involved in reactions that lead to an alteration in the effector-binding capacity of the repressor protein. The repression of OCT synthesis caused by aspartate in biotin-deficient yeast is even more difficult to explain. The possibility that aspartate acted indirectly on OCT synthesis by inducing the synthesis of aspartate carbamoyltransferase, and that the enhanced activity of this enzyme caused a deficiency of carbamoyl phosphate, which might induce OCT synthesis, is ruled out by the finding that aspartate did not induce aspartate carbamoyltransferase synthesis. Possibly, therefore, aspartate acts directly on the mechanisms that regulate OCT synthesis in the yeast.

The relative effects of norbiotin, homobiotin and bishomobiotin on the stimulation of OCT synthesis in biotin-deficient yeast show that the length of the

side chain in the molecule is critical for the participation of the vitamin as a prosthetic group in the enzymes involved in the synthesis, and that a chain length of more than five carbon atoms would seem either to prevent the homologue from combining with the apoenzyme or to preclude the holoenzyme from functioning catalytically. Little is known about the ability of biotin homologues to replace biotin as the prosthetic group in biotin enzymes. It has been shown, however, that neither norbiotin nor homobiotin can replace biotin as the substrate in the reaction in which biotin is incorporated into methylmalonyl-CoA carboxylase by the synthetase from *Propionibacterium shermanii* (Lane, Young & Lynen, 1964), or in the reaction in which the vitamin is incorporated into propionyl-CoA carboxylase by the synthetase from rat liver (Kosow, Huang & Lane, 1962). Lynen (1961) reported that norbiotin and homobiotin were carboxylated by methylcrotonyl-CoA carboxylase from a mycobacterium, although the reaction occurred at a lower rate than with biotin. It is conceivable therefore that the biotin-requiring reactions involved in OCT synthesis by the yeast differ from any of those already described.

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