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EFFECTS OF EDTA ON TYROSINASE AND L-AMINO-ACID OXIDASE INDUCTION IN *NEUROSPORA CRASSA*

JOHN C. UREY
Department of Zoology, Pomona College, Claremont, Calif. (U.S.A.)
AND N. H. HOROWITZ
Division of Biology, California Institute of Technology Pasadena, Calif. (U.S.A.)

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SUMMARY

Tyrosinase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) and L-amino-acid oxidase (L-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2), which are normally induced in *Neurospora crassa* during starvation in phosphate buffer (pH 6), do not appear if the mycelium is washed with EDTA before induction. The induction of both enzymes is fully restored by adding calcium, amino acids, and copper or nickel to the induction buffer. The EDTA treatment (1) promoted the loss of amino acids and materials absorbing at 260 mμ from the mycelium, (2) increased the sensitivity of induction to inhibition by actinomycin D, (3) inhibited the uptake of lysine and uridine from the induction buffer and (4) inhibited the synthesis of protein. The inhibition of induction, uptake and protein synthesis appear to be due to removal of calcium from the mycelium and the retention of some EDTA in the mycelium. The EDTA effect was used to confirm that the lag phase of induction is a qualitatively distinct phase of the induction process.

INTRODUCTION

The enzymes tyrosinase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) and L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) are coinduced when a young culture of *Neurospora crassa* is starved in phosphate buffer[1]. Neurospora tyrosinase contains copper[2], but, unlike certain other tyrosinases[3–5], the apo-enzyme, lacking copper, has never been obtained reversibly[2]. Since EDTA chelates copper very strongly[6], it seemed possible that mycelia which had been pretreated with EDTA would form the apo-enzyme during the subsequent starvation in phosphate buffer. When this experiment was performed it was found, as expected, that no tyrosinase activity developed; however, no apo-tyrosinase could be demonstrated. Investigation of this result led to the findings presented in the present paper.

MATERIALS AND METHODS

Wild-type strain 69-1113a has been used in previous studies[1,7]. It was maintained on slants of a complete medium containing VOGEL's basal salts solution[8], 1.5% malt extract (Difco), 0.5% yeast extract (Difco), 0.025% casamino acids (Difco), 2.0% glycerol and 1.5% agar. VOGEL's medium N (ref. 8) containing 2% sucrose was the minimal medium used.

Chemicals

All chemicals were reagent grade or the best grade available except that table sugar was used in growth media when the composition was not critical. Uniformly labelled L-[^{14}C]lysine was purchased from Volk Radiochemical Co. The [^{3}H]uridine was purchased from the New England Nuclear Corp. The actinomycin D was a generous gift of Merck, Sharp and Dohme.

Enzyme induction

The enzymes were induced in two-day-old cultures by the method described by HOROWITZ[1]. After induction, the felts were harvested by suction on Whatman No.1 filter paper on a Büchner funnel. The felt was removed, weighed, wrapped in marked aluminum foil and stored at $-15°$ until assayed.

Treatment with EDTA

Cultures were treated with EDTA by washing twice aseptically, in the same manner used for normal induction, with a solution of 0.5% ethylenediaminetetraacetic acid disodium salt (EDTA) in 0.10 M sodium phosphate (pH 6). Then the cultures were rinsed twice with distilled water and suspended in 5 ml of 0.02 M sodium phosphate (pH 6.0). This buffer was supplemented as indicated in the text with cations, amino acids and/or inhibitors. The mixture of L-amino acids used was 2 mM in each of the following: Ala, Asp, Arg, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Try and Val. The mixture is derived from the amino acid composition of tyrosinase[2] except that tyrosine was omitted to avoid melanization of the extracts.

Enzyme extraction and assay

The enzymes were extracted by grinding the mycelium in a cold mortar with sand and 10 or 20 parts by weight of cold 0.10 M sodium phosphate (pH 6.0). The extracts were centrifuged for 5 min at 10 000 $\times$ g and the supernatant kept for assay. Tyrosinase was determined on 0.01 to 0.50-ml aliquots of the extracts by the photometric method described previously[10], with DL-DOPA as substrate. The results are expressed in Enzyme Commission units[9]. The translation of absorbance readings to Enzyme Commission units has been explained previously[2].

L-Amino-acid oxidase was assayed by measuring the rate of formation of phenylpyruvate from L-phenylalanine as described by HOROWITZ[1]. The amount of phenylpyruvate was determined either by the ferric chloride method of JERVIS[11] or the enolborate method of LIN *et al.*[12]. The results are expressed in Enzyme Commission units.

Incorporation of radioactively labelled compounds

The method used was developed by Dr. MARGUERITE FLING of this laboratory.

The mycelial felts, either water-washed or EDTA-treated, were suspended in the induction buffer at the temperature specified in the text. During the time of incorporation, they were shaken continuously. Then the flasks were placed in an ice bath and an excess of the unlabelled compound was added. The felts were harvested by suction on a Büchner funnel and rinsed four times with 10 ml distilled water. The buffer and rinse waters were combined and stored frozen at $-15°$. The felts were also stored at $-15°$.

Extraction and measurement of radioactivity

The mycelium was extracted and fractionated by the method of ROBERTS *et al.*[13] When uridine had been incorporated, the extraction was carried only through the extraction of RNA with hot trichloroacetic acid.

An aliquot of each fraction was added to 10 ml of BRAY's scintillator fluid[14], modified by replacing POPOP by dimethyl POPOP. The samples were then counted in a Packard Tricarb scintillation spectrometer. The amount of quenching was determined by the channel ratios method. All results are reported as counts/min corrected for quenching.

Chromatography

Ascending paper chromatography was performed by the methods outlined by BLOCK, LESTRANGE AND ZWEIG[15]. Whatman No. 1 chromatography paper was used and the mixtures developed with (a) water-saturated phenol, (b) *n*-butanol saturated with 2 M ammonia or (c) *n*-butanol–acetic acid–water (40:10:50, by vol., upper phase). The solutions of unknowns were concentrated by evaporation under vacuum. Amino acids were located by spraying with Nin-Spray (Nutritional Biochemicals Corp.) and developing according to the directions.

RESULTS

The inhibition of induction by EDTA

When two-day-old cultures of wild-type Neurospora are washed free of medium and starved in 0.02 M sodium phosphate (pH 6.0), tyrosinase and L-amino-acid oxidase are coinduced[1]. A brief washing with EDTA prior to the start of starvation inhibits the subsequent induction of the two enzymes, as shown in Table I. When extracts of EDTA-treated cultures were mixed with each enzyme, no inhibition of activity was detected. No tyrosinase activity was detected in extracts after heating for 1.5 min

TABLE I

THE INHIBITION OF INDUCTION BY EDTA

Two-day-old cultures of 69-1113a were washed with water or treated with EDTA and starved for 2 days in 0.02 M sodium phosphate (pH 6.0) and then harvested and extracted.

Extract	*Tyrosinase (units/g)*	L-*Oxidase (units/g)*
Water-washed control	107	4.8
EDTA-treated	0.5	0.13

at 59°, a treatment which activates Neurospora pro-tyrosinase[16,17]. If the inhibition of tyrosinase induction had been due to removal of the copper from the mycelium, apo-tyrosinase might have been made during the starvation in phosphate buffer. However, no tyrosinase activity appeared in extracts of EDTA-treated cultures when they were mixed with either cupric or cuprous ion in the presence or absence of 3 M urea at either pH 5 or pH 6. Furthermore, as shown below, it is possible to restore induction of tyrosinase without adding copper proving that sufficient copper remains in EDTA-treated cultures to form tyrosinase. No L-amino-acid oxidase activity appeared in extracts of EDTA-treated cultures when flavine adenine dinucleotide, the prosthetic group of the enzyme[18], was added to the extracts.

The restoration of induction

Various cations, including Ca^{2+}, Cu^{2+}, Mg^{2+}, Hg^{2+}, Ag^{+}, and Zn^{2+}, were tested to see if they restored the induction of tyrosinase during starvation after the EDTA treatment. Calcium and copper partially restored induction and were investigated further. In combination, these ions cause a restoration much greater than the sum of their individual effects. Other work in this laboratory by Miss HELEN MACLEOD, suggested the addition of amino acids to the starvation buffer. As seen in Table II, Expt. 1, a combination of calcium, copper and L-amino acids completely restores the

TABLE II

THE RESTORATION OF INDUCTION IN EDTA-TREATED CULTURES

Experiment 1. Two-day-old cultures of 69-1113a were washed with water or EDTA and suspended in 5 ml 0.02 M sodium phosphate (pH 6.0) containing L-amino acids at $2 \cdot 10^{-3}$ M each and the other supplements noted. The cultures were harvested after 2 days.

Treatment	*Supplement to the buffer in addition to amino acids*	*Tyrosinase (units/g)*	*L-Oxidase (units/g)*
Water-washed:	None	161	4.6
		114	3.7
EDTA-treated:	None	1.5	0.2
		0.8	0.4
	$3 \cdot 10^{-4}$ M $CaCl_2$	157	1.3
		122	1.2
	$3 \cdot 10^{-5}$ M $CaCl_2$ *plus* $3 \cdot 10^{-4}$ M $CuSO_4$	114	4.3
		121	4.6

Experiment 2.

Two-day-old cultures of 69-1113a were washed with EDTA and suspended in 5 ml 0.02 M sodium phosphate (pH 6.0) containing $3 \cdot 10^{-5}$ M $CaCl_2$ and L-amino acids $2 \cdot 10^{-3}$ M each and either $CuSO_4$ or $NiCl_2$ at the concentrations noted. The cultures were harvested after 2 days.

Molarity of $CuSO_4$ or $NiCl_2$ added to buffer	*Tyrosinase activity (units/g)*	
	$CuSO_4$	*$NiCl_2$*
0	27	
10^{-5}	18	21
10^{-4}	184	180
10^{-3}	144	144

induction of both tyrosinase and L-amino-acid oxidase following the EDTA treatment. The concentration optima vary broadly around those shown.

Calcium could be replaced only by strontium among the following cations tried: Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, K^{+}, Sr^{2+}, and Zn^{2+}. Magnesium was not required even when the mycelium had been grown on VOGEL's Medium N modified to contain the minimal amount of magnesium supporting optimal growth.

Nickel is interchangeable with copper for the restoration of tyrosinase induction under all the conditions tested as seen in Table II, Expt. 2. In addition, if the calcium concentration is increased to about $3 \cdot 10^{-4}$ M in the presence of amino acids, tyrosinase induction but not L-amino-acid oxidase induction is fully restored in the absence of copper (see Table II, Expt. 1).

Induction in normal cultures was inhibited up to 25% by copper and/or amino acids at restorative concentrations and more than 95% by $CaCl_2$ concentrations exceeding 10^{-4} M. Combinations of copper and calcium gave an inhibition which fell between the individual ions. The anion associated with the metals did not affect the results.

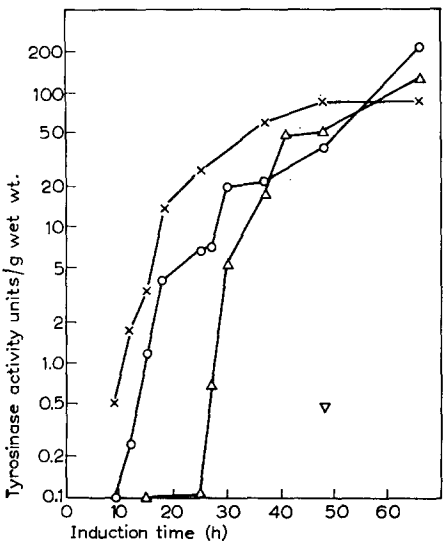
The time course of induction

When calcium, copper and amino acids are present during the entire period of starvation, the time course of induction of tyrosinase in EDTA-treated cultures is normal. As shown in Fig. 1, the lag period that precedes the appearance of tyrosinase activity is less than 10 h. During the second phase, called the rise phase, the activity increases rapidly. In the absence of added cations, the EDTA-treated culture does not complete the lag phase. Thus the cultures treated with EDTA and starved 9 h without any additions to the buffer did not begin to make tyrosinase upon addition of calcium, copper and amino acids until an additional lag period of more than 10 h was completed. In contrast, in the cultures starved for 9 h with $5 \cdot 10^{-5}$ M $CaCl_2$ present, tyrosinase activity appeared within 2 h of the addition of the copper and amino acids. Almost no activity appeared in 48 h if calcium but no copper or amino acids was present during the starvation (see Fig. 1). Since copper alone gives substantial recovery after EDTA, cells can pass through the lag phase in the presence of copper alone. Tyrosinase induction is partially restored by the addition of calcium, copper and amino acids even if they are not added to the buffer until 30 h after the EDTA treatment.

EDTA and cell permeability

The EDTA-treated mycelium loses amino acids and material absorbing at 260 mμ to the wash solutions and induction buffer as seen in Fig. 2. No similar material was found in the wash solutions from the untreated controls. The induction buffer of the controls comes to contain such material much later in the course of induction. The second water rinse of EDTA-treated cultures was concentrated and chromatographed on paper. The following ninhydrin-positive spots were tentatively identified: EDTA, Ala, Asp, Asn, Arg, Glu, Gln, Ile, Leu, Lys, Met, Pro and Val.

It was expected that the EDTA treatment would remove detectable amounts of metal ions from the mycelium. However, no differences were detected by flame spectrographic analysis between the ash of cultures washed with 0.10 M sodium phosphate (pH 6) and the ash of cultures washed with 0.5% EDTA in 0.10 M sodium phosphate (pH 6).



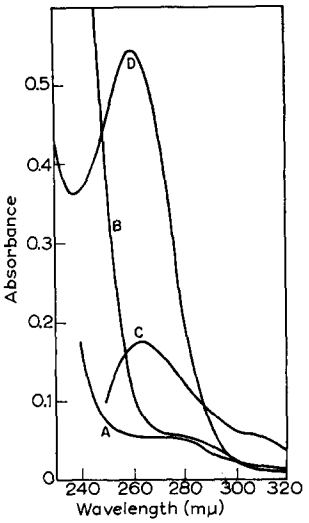


Fig. 1. The time course of tyrosinase induction in EDTA-treated cultures. Two-day-old cultures of 69-1113a were treated with EDTA and placed in 5 ml 0.02 M sodium phosphate (pH 6.0). ×—×, $CaCl_2$, $CuSO_4$ and L-amino acids added at time zero. ○—○, $CaCl_2$ added at time zero, $CuSO_4$ and L-amino acids added at hour 9. △—△, $CaCl_2$, $CuSO_4$ and L-amino acids added at hour 9. ▽, Only $CaCl_2$ added at time zero. Concentrations: $CaCl_2$ $5 \cdot 10^{-5}$ M; $CuSO_4$ $5 \cdot 10^{-4}$ M; L-amino acids $2 \cdot 10^{-3}$ M each.

Fig. 2. Difference spectra of washes of cultures. A. Second water wash of control mycelium *vs.* water: vol., 10 ml; extraction time, 30 min. B. EDTA 0.5% in 0.10 M sodium phosphate (pH 6) *vs.* water. C. First EDTA wash of mycelium *vs.* EDTA: vol., 10 ml; extraction time, 30 min. D. Second water wash of EDTA-treated mycelium *vs.* water: vol., 10 ml; extraction time, 30 min.

According to LEIVE[19,20], washing *Escherichia coli* cells briefly with EDTA changes the permeability of the cells to actinomycin D. Unpublished experiments from this laboratory have shown that actinomycin D inhibits the normal induction of tyrosinase. As seen in Table III, actinomycin D inhibits the induction of tyrosinase

TABLE III

INCREASED SENSITIVITY OF INDUCTION TO ACTINOMYCIN D IN EDTA-TREATED CULTURES

Two-day-old cultures of 69-1113a were washed with water or EDTA. Water-treated cultures were starved in 5 ml 0.02 M sodium phosphate (pH 6.0). EDTA-treated cultures were starved in 5 ml 0.02 M sodium phosphate (pH 6.0) containing $5 \cdot 10^{-5}$ M $CaCl_2$, $5 \cdot 10^{-5}$ M $CuSO_4$ and a mixture of L-amino acids at $2 \cdot 10^{-3}$ M each. All cultures were harvested after 2 days starvation.

Treatment	*Actinomycin D (μMolar)*	*Tyrosinase (units/g)*	*L-Oxidase (units/g)*
Water	0	150	3.9
Water	1.25	75	1.5
EDTA	0	221	4.1
EDTA	1.25	26	0.45
EDTA	0.6	30	0.30
EDTA	0.3	31	0.66

at lower concentrations in EDTA-treated cultures than in water-washed control cultures.

EDTA and the incorporation of lysine

As is shown in Table IV, the accumulation of L-[^{14}C]lysine by the mycelium is inhibited in EDTA-treated cultures, which took up only 70% as many counts as did water-washed controls. This inhibition of uptake from the buffer was relieved by the

TABLE IV

THE INCORPORATION OF L-LYSINE BY EDTA-TREATED CULTURES

Two-day-old cultures of 69-1113a were washed with water or EDTA and suspended in 5 ml 0.02 M sodium phosphate (pH 6.0) with the additions noted. After 2 h at room temperature, the flasks were placed on a slowly reciprocating shaker and L-[^{14}C]lysine (780 000 counts/min) was added. The water-washed control was harvested after 15 min. All other cultures were harvested after 1 h and fractionated by the procedure of ROBERTS *et al.*[13].

Treatment and additions to the buffer	*Remaining in buffer*	*Counts/min (in thousands)*				*Total counts recovered*	*Recovered from the mycelium (%)*	*Recovered in protein (%)*
		Cold trichloroacetic acid soluble	*Ethanol soluble*	*Hot trichloroacetic acid soluble*	*NaOH soluble (protein)*			
Water-washed	42	460	50.4	50.8	86	689	94	12.5
control	46	360	10.8	86.9	145	649	93	22.3
EDTA-treated:								
No additions	259	460	2.6	2.1	3.4	727	94	0.5
	234	506	1.7	4.6	11.3	758	68	1.5
$5 \cdot 10^{-5}$ M $CaCl_2$	242	506	1.1	1.7	3.2	754	68	0.4
	218	519	1.9	2.8	4.0	746	71	0.5
$2.5 \cdot 10^{-4}$ M $CaCl_2$	46	482	11.0	51	105	695	93	15.0
	62	579	7.5	30	84	763	92	6.3
$5 \cdot 10^{-4}$ M $CuSO_4$	85	656	5.5	5.3	13.9	765	89	1.8
	110	666	2.9	2.9	4.5	787	86	0.6
$5 \cdot 10^{-5}$ M $CaCl_2$								
plus	104	625	5.2	8.2	21.5	764	86	2.8
$5 \cdot 10^{-4}$ M $CuSO_4$	62	645	22.0	12.9	23.2	765	92	3.0

addition of $2.5 \cdot 10^{-4}$ M $CaCl_2$ or a combination of $5 \cdot 10^{-5}$ M $CaCl_2$ and $5 \cdot 10^{-4}$ M $CuSO_4$.

The incorporation of the radioactivity into protein also was strongly inhibited in the EDTA-treated cultures. They converted only 1.5% of the counts into protein, as compared to 12–22% in the controls. The addition of $2.5 \cdot 10^{-4}$ M $CaCl_2$ overcame this inhibition.

The addition of a mixture of amino acids completely inhibited the uptake of lysine by the water-washed controls even when arginine and lysine were not present in the mixture, probably due to the complex competitive interactions among amino acids for transport found in Neurospora[21].

EDTA and uridine uptake

The EDTA-treatment also inhibited the uptake of [^{3}H]uridine from the induction buffer, as seen in Table V. The number of counts recovered in the fraction soluble

TABLE V

THE INCORPORATION OF URIDINE BY EDTA-TREATED CULTURES

Two-day-old cultures of 69-1113a were washed with water or EDTA and suspended in 5 ml 0.02 M sodium phosphate (pH 6.0) with additions noted. [^{3}H]uridine ($2.36 \cdot 10^6$ counts/min) was added to each flask. The flasks were shaken for 30 min at 80 min on a Dubnoff metabolic shaker at 25°. Then unlabelled uridine was added, the flasks were removed to an ice bath, harvested and fractionated by the procedure of ROBERTS *et al.*[13]

Treatment and additions to the buffer	*Remaining in buffer*	*Counts/min (in thousands)*			*Total counts recovered*	*Recovered from the mycelium (%)*
		Cold trichloroacetic acid soluble	*Ethanol soluble*	*Hot trichloroacetic acid soluble*		
Water-washed control	470	1541	1.4	394	2406	80
	481	1357	14.9	426	2279	79
EDTA-treated: No additions	1472	423	0.8	326	2222	34
	1459	412	0.2	312	2184	33
$5 \cdot 10^{-5}$ M $CaCl_2$ *plus* $5 \cdot 10^{-4}$ $CuSo_4$ *plus* L-amino acids	680	1349	2.6	286	2318	71
	859	1232	—	307	2398	64

in cold trichloroacetic acid of the EDTA-treated cultures was only one-third the number recovered from the water-washed controls. Yet almost the same number of counts were recovered in the RNA-containing fractions of the two cultures. The difference in the cold trichloroacetic acid-soluble counts is probably not an artifact of the extraction procedure since additional washes of the EDTA-treated cells did not remove any additional counts from the mycelium. The presence of calcium, copper and amino acids in the buffer relieved the inhibition of uptake into the cold trichloroacetic acid-soluble pool.

DISCUSSION

Under our experimental conditions, EDTA has at least 5 effects on Neurospora: (1) The induction of tyrosinase and L-amino-acid oxidase is inhibited. (2) The ability of the cells to retain amino acids and materials absorbing at 260 mμ is reduced, allowing these substances to pass into the medium. (3) The sensitivity of enzyme induction to inhibition by actinomycin D is increased. (4) The uptake and concentration of lysine and uridine by the mycelium is inhibited. (5) The incorporation of lysine into protein is inhibited.

These experiments were undertaken in the hope of obtaining the apo-enzyme of tyrosinase. No evidence of its being formed in EDTA-treated cultures was obtained. The severe inhibition of protein synthesis in EDTA-treated cultures makes it improbable that any apo-tyrosinase could have been made. Furthermore, it is clear from the results that the EDTA treatment does not remove sufficient copper from the mycelium to limit subsequent synthesis of tyrosinase.

We propose two primary effects of the EDTA treatment: (1) Some essential calcium is removed from the cells. This is shown by the fact that calcium is required for full restoration of induction. Of the ions tested, only strontium could replace calcium for this purpose. (2) It appears that some EDTA remains in the mycelium following EDTA treatment, where it binds ions (probably calcium) essential for protein synthesis. This conclusion follows from the observation that part of the calcium required for full restoration of tyrosinase induction can be replaced by nickel or copper. Since nickel has no known biological function, but, like copper, shows a very high binding constant with EDTA, its effectiveness—as well as that of copper—can best be accounted for by assuming that it combines with residual EDTA in the mycelium.

SUSSMAN[22] has found that germination of *Neurospora tetrasperma* ascospores is inhibited by treatment with EDTA. He also concludes that loss of calcium was the cause of the inhibition.

Since tyrosinase and probably also L-amino-acid oxidase is synthesized *de novo* during normal induction[1,7], it follows that any treatment relieving inhibition by EDTA must restore protein synthesis. The finding that $CaCl_2$ or a combination of $CaCl_2$ and $CuSO_4$ added to EDTA-treated cultures significantly increased the incorporation of lysine into protein is consistent with this inference.

The role of the L-amino-acid mixture is probably to oppose the loss to the medium of amino acids from the internal pools. In this regard one should note that the list of amino acids we detected corresponds closely to the list of amino acids for which FUERST AND WAGNER[23] found measurable pools inside the mycelium.

In Table III, the amount of tyrosinase made by the EDTA-treated culture upon restoration of induction was greater than the amount made by the normally induced control. The same result occurs with the L-amino-acid oxidase. This effect was observed frequently enough to suggest that the EDTA removes some inhibitor of induction, probably a heavy metal ion.

The loss of amino acids and material absorbing at 260 mμ is probably due to a change in cell permeability. It is not reversed by the cation additions. Similar materials appear in the induction buffer of normal controls, but much later in the course of induction. Thus the EDTA treatment may only accelerate a normal increase in permeability of the mycelium which occurs during starvation.

LEIVE[19,20] showed that EDTA causes a great increase in the permeability of *E. coli* to several compounds including actinomycin D. The increased sensitivity to actinomycin D inhibition which we observed following EDTA treatment may be due to a similar effect. However, other possible explanations have not been excluded.

The treatment with EDTA allows us to separate the lag phase of induction from the subsequent synthesis of tyrosinase. As shown in Fig. 2, EDTA-treated cultures can complete the lag phase in the presence of $CaCl_2$ alone. Almost no tyrisonase is made by such cultures. However, upon addition of copper and amino acids, tyrosinase activity quickly appeared. These results indicate that the lag period is a functionally distinct phase of induction.

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