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STUDIES ON THE GLUCOSE DEHYDROGENASE OF *ASPERGILLUS ORYZAE*.

I. INDUCTION OF ITS SYNTHESIS BY *p*-BENZOQUINONE AND HYDROQUINONE

TCHAN-GI BAK AND RYO SATO
Institute for Protein Research, Osaka University, Osaka (Japan)

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SUMMARY

Glucose dehydrogenase of *Aspergillus oryzae* was not detected in mycelia grown in shake cultures, although the mycelia grown on the surface of standing media contained the enzyme. Hydroquinone and *p*-benzoquinone were found to be specific inducers of the synthesis of glucose dehydrogenase; in their presence marked synthesis of the enzyme was observed even in shake cultures. The induced synthesis of the enzyme in the presence of hydroquinone and *p*-benzoquinone was further accelerated by EDTA and certain other chelating agents, which by themselves showed no inducer activities. These chelating agents caused greatly increased liberation of glucose dehydrogenase into the cultivation medium. The induction of enzyme synthesis was inhibited by metal ions such as Cu^{2+} and Hg^{2+}; the inhibition by Cu^{2+} could be reversed by EDTA. The *p*-benzoquinone-induced formation of glucose dehydrogenase was observed also in resting mycelia.

INTRODUCTION

In 1937 OGURA AND NAGAHISA[1] first reported the presence of a soluble glucose dehydrogenase in *Aspergillus oryzae*. This enzyme catalyzed the oxidation of glucose to gluconic acid by certain redox dyes and quinones; molecular O_2, pyridine nucleotides and flavins were incapable of acting as hydrogen acceptors[2-4]. Xylose, mannose and galactose were also oxidized by this enzyme at slower rates[5,6]. OGURA[2-4] concluded that this enzyme was different from glucose oxidase (notatin) (EC 1.1.3.4) discovered by MÜLLER[7] in *Aspergillus niger* and *Penicillium glaucum*, and studied extensively by many workers[8-12]. It also differs from NAD(P)-linked glucose dehydrogenase (EC 1.1.1.47) detected in mammalian liver[13-15] and certain bacteria[16,17]. KURASAWA AND

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

IGAUE[18–20] have recently studied the *A. oryzae* enzyme in some detail and obtained evidence in favor of OGURA's earlier view that this enzyme possesses no prosthetic group. The occurrence in *A. niger* of a similar glucose dehydrogenase, in addition to glucose oxidase, has been reported by KUSAI[21] who also suggested that the dehydrogenase was not a flavoprotein. However, the *A. oryzae* enzyme has not yet been purified, and much remains to be studied concerning its chemical nature and physiological function.

In the course of a reinvestigation on this enzyme, we noticed that its production by *A. oryzae* was profoundly dependent on cultivation conditions. Further studies showed that its synthesis was specifically and markedly induced by *p*-benzoquinone and by hydroquinone. The inductive effects of these compounds were further increased by the simultaneous presence of EDTA and other chelating agents although chelating agents alone showed no inducer activity. The addition of chelating agents also caused increased liberation of glucose dehydrogenase into the medium. This paper reports the experimental results on these unusual induction phenomena. A preliminary report of this work has already been presented elsewhere[22].

MATERIALS AND METHODS

Microbial strains and cultivation

A strain of *A. oryzae* was kindly supplied by Prof. Y. OGURA and used in most of the experiments. The other organisms were obtained from the Institute for Fermentation, Osaka. The medium used for cultivation of *A. oryzae* contained 5% glucose, 0.2% NH_4NO_3, 0.2% $MgSO_4 \cdot 7H_2O$, 0.3% KH_2PO_4 and 0.2% K_2HPO_4 in tap water. Glucose was autocalved separately and mixed with the sterilized inorganic medium. When necessary, various compounds such as quinones and EDTA were added; quinones were added in solid form to the sterilized medium. The pH of the medium was 6.5. Conidiospores of *A. oryzae* were collected from 3–5-day cultures on nutrient agar plates, suspended in distilled water to an absorbance of 0.5 at 420 mμ (optical path, 9 mm), and used for inoculation. Cultivation was carried out in two different ways, *i.e.* surface and shake methods. For surface cultivation, conidiospores of the organism were sown on the surface of 1.2 l of the above liquid medium (about 2 cm thick). The inoculated vessels were allowed to stand at 28–30° for 42–48 h. The mycelial pads formed were collected and washed with water. For shake cultivation, 50 ml of conidiospore suspension were added to 750 ml of the medium, and the mixture was shaken aerobically in a 3-l erlenmeyer flask at 30–34°, Shaking was continued in a rotary shaker (80 cycles/min) usually for 18 h.

Cultivation of the other organisms was carried out in the same manner as described for *A. oryzae*.

Preparation of mycelial extract

Mycelia were collected from the culture by filtration under suction, and the filtrate was saved for determination of glucose dehydrogenase liberated into the medium. The mycelia were washed 5 times on the filter with distilled water and then dried as completely as possible by pressing them with filter paper. They were mixed with an equal weight of quartz sand, and the mixture was ground with a mechanically driven pestle at 4° for 20 min. The ground mixture was treated with 0.2 M K_2HPO_4

(about 30 ml/10 g wet mycelia), and centrifuged at 10 000 × *g* for 20 min. The supernatant thus obtained was adjusted to pH 6.5 with NaOH and used as the mycelial extract. All the glucose dehydrogenase activity present in mycelia was recovered in this extract, and no activity was detected in the sedimented debris.

Induction in resting mycelia

The mycelia obtained from a shake culture containing neither inducers nor EDTA were well washed with water, and suspended (10 g wet mycelia/800 ml) in the cultivation medium described above from which glucose had been omitted. The mycelial suspension was then shaken for 18 h at 30° with and without the addition of the inducer. During this period, no detectable growth of the mycelia occurred.

Enzyme assays

All the enzyme assays were performed at room temperature (20–25°) in a Cary model 14 spectrophotometer by following the reduction of hydrogen acceptors at appropriate wavelengths in cuvettes of 1.0-cm optical path. The final volume of the reaction mixture was 1.5 ml. Glucose dehydrogenase was assayed by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 mμ in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.5), 0.1 mM DCIP, 0.2 M glucose, and enzyme. One unit of glucose dehydrogenase was defined as the amount of enzyme causing an absorbance change of 1.00 per min under these conditions, and specific activity was expressed in terms of units per mg protein. NADH-diaphorase (EC 1.6.4.3) was determined also with DCIP as acceptor under the same conditions except that glucose in the reaction mixture was replaced by 0.3 mM NADH. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NADP-linked glucose dehydrogenase (EC 1.1.1.47) and hexokinase[23] (EC 2.7.1.1) were all measured by following the reduction of $NADP^+$ at 340 mμ in 0.1 M Tris–HCl buffer (pH 7.5), containing 6.7 mM KCN and 0.1 M nicotinamide. KCN and nicotinamide were added to prevent powerful NADPH oxidation and destruction of NADP, respectively, caused by the mycelial extract. The reaction mixture for glucose-6-phosphate dehydrogenase assay contained, in addition to enzyme and the aforementioned components, 20 mM glucose 6-phosphate, 0.2 mM $NADP^+$ and 10 mM $MgCl_2$; for NADP-linked glucose dehydrogenase 0.2 M glucose, and 0.2 mM $NADP^+$ were added. For hexokinase assay 0.2 M glucose, 0.5 mM ATP and 10 mM $MgCl_2$ were added to the mixture; sufficient activity of glucose-6-phosphate dehydrogenase was present in mycelial extracts to permit the measurement of the hexokinase activity by this method.

Other procedures

Protein was determined by the biuret method[24]. Ubiquinone in *A. oryzae* was estimated as follows. The neutral lipid fraction was extracted from dried mycelia as described by LESTER AND CRANE[25], and the extract was subjected to silica-gel chromatography as described by ITAGAKI[26]. The eluates containing ubiquinone were combined and evaporated to dryness; the dried material was dissolved in ethanol, and the ubiquinone content of the solution was determined spectrophotometrically according to the procedure of LESTER AND CRANE[25].

RESULTS

Preferential synthesis of glucose dehydrogenase in surface culture

Glucose dehydrogenase of *A. oryzae* has so far been studied only with mycelia grown on the surface of standing media, and little is known of the effect of cultivation conditions on the synthesis of this enzyme. In the present study, it was found that no activity of glucose dehydrogenase (measured with DCIP as acceptor) was present in mycelia grown in submerged or shake cultures (Table I). A definite activity was, however, detectable in mycelia obtained from surface cultures. Despite such an effect on glucose dehydrogenase, the change in cultivation conditions did not significantly affect the levels of hexokinase, glucose-6-phosphate dehydrogenase, or NADH-diaphorase in the mycelial extracts. It was further found that the mycelial extracts

TABLE I

EFFECTS OF CULTIVATION CONDITIONS ON THE PRODUCTION OF SEVERAL ENZYMES

Mycelia of *A. oryzae* were obtained from both shake and surface cultivations, and their extracts were prepared as described in MATERIALS AND METHODS. Enzyme activities in the extracts were determined as described in the text. The activity of glucose dehydrogenase is expressed in terms of units/mg protein, and those of the other enzymes in terms of absorbance increment at 340 mμ (for glucose-6-phosphate dehydrogenase, NADP-linked glucose dehydrogenase and hexokinase) or at 600 mμ (for NADH-diaphorase) per mg protein per min.

Cultivation condition	*Enzyme activity of*				
	Glucose dehydrogenase	*Glucose-6-phosphate dehydrogenase*	*NADP-linked glucose dehydrogenase*	*Hexokinase*	*NADH-diaphorase*
Surface (48 h)	0.35	1.14	0.01	0.15	0.43
Shake (18 h)	0	0.74	0.012	0.25	0.80

obtained from both types of cultures contained low activities of a second glucose dehydrogenase utilizing NADP as acceptor. Its content, in contrast to that of DCIP-linked dehydrogenase, was not altered appreciably by changing the cultivation conditions. Thus, it is clear that the two glucose dehydrogenases are different from each other. In this paper, the DCIP-linked enzyme, which corresponds to the enzyme studied by OGURA[1-6], will be simply called glucose dehydrogenase.

Induction of glucose dehydrogenase synthesis by hydroquinone and p-benzoquinone

In attempts to clarify the reasons for the preferential synthesis in surface cultures, it was found that hydroquinone had a profound effect on the synthesis of glucose dehydrogenase. As shown in Table II, the addition of hydroquinone caused a marked increase in the production of the enzyme in surface cultures. Further, it was found that hydroquinone could induce detectable synthesis of glucose dehydrogenase even in shake cultures, in which no synthesis was observed in the absence of added hydroquinone. The production of NADP-linked glucose dehydrogenase and glucose-6-phosphate dehydrogenase was, however, not significantly affected by hydroquinone. It is suggested that the absence of synthesis of glucose dehydrogenase in shake cultures was due to the lack of an inducer and that hydroquinone or its product could

TABLE II

EFFECTS OF HYDROQUINONE ON THE FORMATION OF SEVERAL ENZYMES IN *A. oryzae*

The organism was grown both under surface and shaking conditions in the medium with or without added hydroquinone, and the activities of three enzymes were measured in the mycelial extracts. The activity of glucose dehydrogenase is expressed in terms of units/mg protein, and those of NADP-linked glucose dehydrogenase and glucose-6-phosphate dehydrogenase are in terms of absorbance increment at 340 mμ/mg protein per min.

Cultivation condition	*Hydroquinone in medium (mM)*	*Enzyme activity of*		
		Glucose dehydrogenase	*Glucose-6-phosphate dehydrogenase*	*NADP-glucose dehydrogenase*
Surface (48 h)	0	0.4	1.7	0.006
	10	11	1.6	0.008
Shake (18 h)	0	0	2.0	0.008
	5	4.2	1.2	0.011

act as the inducer. *p*-Benzoquinone, an oxidation product of hydroquinone, also showed an induction effect on the synthesis of glucose dehydrogenase. This effect of hydroquinone and *p*-benzoquinone was specific; none of the other compounds tested such as phenol, catechol, resorcinol, menadione, α-naphthoquinone, benzoate, thionine, or DCIP was effective as inducer. Since DCIP, thionine, menadione, and α-naphthoquinone are effective acceptors for glucose dehydrogenase[27], the effect of *p*-benzoquinone does not seem to be related to its activity as an acceptor. Although ubiquinone is a derivative of *p*-benzoquinone, this lipophilic quinone did not appear to be effective as an inducer in view of the finding that the mycelia obtained from shake cultures contained nearly the same amount of ubiquinone as those obtained from surface cultures (0.1–0.15 μmole of ubiquinone per g of dried mycelia).

Effect of inducer concentrations

Since the induction was observed more clearly in shake cultures, all the sub-

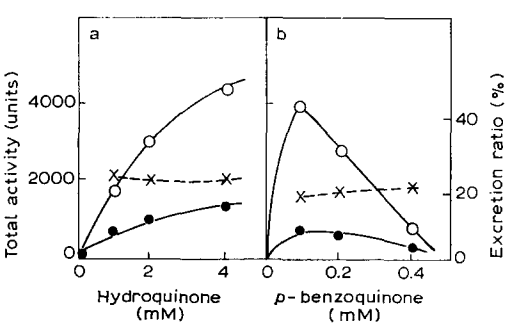

Fig. 1. Effect of concentration of hydroquinone and *p*-benzoquinone on inductive formation of glucose dehydrogenase. Mycelia were grown under shaking in the presence hydroquinone (a) or *p*-benzoquinone (b) at 30° for 18 h. Total activities of glucose dehydrogenase in both mycelial extracts and culture filtrate were determined. ○, total activity in mycelial extracts; ●, total activity in culture filtrate; ×, ratio glucose dehydrogenase in culture filtrate to that in whole culture.

sequent experiments were performed only by the shaking procedure. The glucose dehydrogenase content in mycelia was dependent on the concentration of inducer added to the medium. As shown in Fig. 1a, there was an almost linear increase in the enzyme content as the concentration of hydroquinone was increased up to 4 mM. When *p*-benzoquinone was used as the inducer, on the other hand, maximal synthesis of glucose dehydrogenase was attained at an inducer concentration of about 0.1 mM or less; higher concentrations of *p*-benzoquinone caused a marked decline in the enzyme formation (Fig. 1b). This decline was probably due to the toxic effect of the quinone on the fungal metabolism. The fact that *p*-benzoquinone was effective at much lower concentrations than hydroquinone suggested that the latter exerted its induction effect after its conversion to the former.

In these experiments we became aware that under these cultivation conditions small portions of the glucose dehydrogenase synthesized in mycelia were liberated into the medium. As may be seen from Fig. 1, the amount of enzyme liberated was about 20–25% of the total produced. This ratio was not affected by the inducer concentration.

Acceleration of inductive synthesis by EDTA

As shown in Table III, the induced synthesis of glucose dehydrogenase caused by either hydroquinone or *p*-benzoquinone was further augmented markedly by the simultaneous presence of EDTA in the medium. The addition of EDTA alone in the absence of hydroquinone or *p*-benzoquinone, however, induced no synthesis of the enzyme. Fig. 2a shows the effect of EDTA concentration on the levels of glucose de-

TABLE III

ACCELERATIVE EFFECT OF EDTA ON THE INDUCTIVE FORMATION OF GLUCOSE DEHYDROGENASE BY HYDROQUINONE AND *p*-BENZOQUINONE

Specific activities (units/mg protein) of glucose dehydrogenase were determined in the extracts of mycelia grown at 30° for 18 h in shake cultures. The compounds indicated were added to the cultivation medium in each experiment.

Expt.	*Addition*			*Glucose dehydrogenase (units/mg protein)*
	Hydroquinone (1 mM)	*p-Benzoquinone (0.1 mM)*	*EDTA (0.1 mM)*	
A	−	−	+	0
	+	−	−	2.0
	−	+	−	1.0
	+	−	+	17.0
	−	+	+	4.3
B*	−	+	−	1.1
	−	+	+	2.0

* For Expt. B, NH_4NO_3 in the medium was replaced by 0.2% $NaNO_3$.

hydrogenase in both mycelia and medium attained in the presence of 0.05 mM *p*-benzoquinone. As can be seen, the enzyme content in mycelia was maximal in the presence of 0.1 mM EDTA, whereas that in the medium was highest at about 0.2 mM.

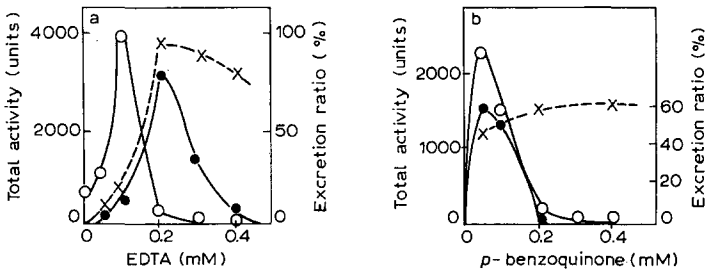

Fig. 2. Effects of concentrations of EDTA and *p*-benzoquinone on the inductive formation of glucose dehydrogenase. Mycelia were grown at 30° for 18 h in the medium containing *p*-benzoquinone and EDTA. (a) The medium contained 0.05 mM *p*-benzoquinone and the EDTA concentration was varied. (b) The medium contained 0.1 mM EDTA and the *p*-benzoquinone concentration varied. ○, total activity in mycelia; ●, total activity in culture filtrate; ×, ratio of glucose dehydrogenase in culture filtrate to that in whole culture.

Moreover, it is evident from this value that the presence of increasing concentrations of EDTA not only accelerated the induced synthesis of the enzyme but also increased the liberation of the enzyme into the medium. In the presence of 0.2 mM EDTA almost all of the enzyme activity was recovered in the medium. When the concentration of EDTA was fixed at 0.1 mM and that of *p*-benzoquinone was varied, the enzyme contents in both mycelia and medium were maximal at 0.05 mM benzoquinone (Fig. 2b). The patterns obtained were somewhat similar to those obtained in the absence of EDTA, but the ratio of liberated enzyme to the total activity synthesized was increased to 45–60%, as compared with an original ratio of 20–25%. This again indicated that EDTA promoted the liberation of the enzyme. When EDTA was replaced by another chelating agent, *o*-phenanthroline (0.05 mM), a similar acceleration was observed.

Time course of inductive synthesis

Fig. 3 shows the time courses of glucose dehydrogenase production induced by *p*-benzoquinone in the absence and presence of EDTA. The total enzyme activity detected in mycelia growing in the presence of 0.05 mM benzoquinone alone increased with cultivation time, and reached a maximal level after 48 h of cultivation. On fur-

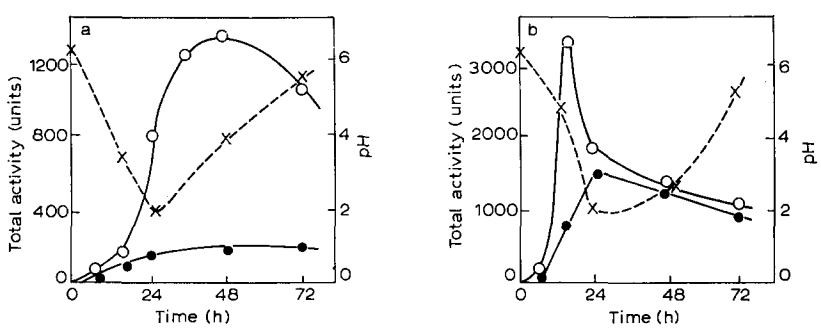

Fig. 3. Time courses of formation of glucose dehydrogenase in shake cultures containing *p*-benzoquinone or *p*-benzoquinone *plus* EDTA. Mycelia were grown at 30°. (a) 0.05 mM *p*-benzoquinone. (b) 0.05 mM *p*-benzoquinone *plus* 0.1 mM EDTA. ○, total activity in mycelial extract; ●, total activity in culture filtrate; ×, pH of culture filtrate.

ther cultivation, there was a gradual decrease in the enzyme content. The enzyme activity in the growth medium, on the other hand, increased slowly during 24 h of cultivation, and thereafter remained at a constant level; the ratio of liberated enzyme to the total activity was about 13% at this stage (Fig. 3a). When 0.1 mM EDTA was present in the medium in addition to 0.05 mM *p*-benzoquinone, the time course of enzyme formation was considerably different. The total content of glucose dehydrogenase in mycelia increased rapidly, and a maximal level was reached after 16 h of cultivation. Thereafter, it decreased rapidly to about half the maximal level in about 8 h, followed by a much slower decline. As mentioned above, a much larger amount of the enzyme was liberated into the medium from mycelia under these conditions. The amount of the enzyme recoverable in the medium reached a maximum level after 24 h of cultivation, and then decreased gradually. About half the total enzyme was present in the medium after 24 h (Fig. 3b). The results shown in Fig. 3 also confirm the above conclusion that EDTA accelerates not only the inductive synthesis but also the liberation of glucose dehydrogenase. As can be seen from Fig. 3, there was a tendency for the pH of the medium to decrease when there was active formation of the enzyme. It might be suggested that the enzyme formation is in some way related to the decrease in pH. When $NaNO_3$ was used as the nitrogen source in place of NH_4NO_3, the pH of the medium was not decreased so pronouncedly. As shown in Table III, the enzyme formation in this medium was not so extensive.

Effects of metal ions

In view of the finding that the inductive formation of glucose dehydrogenase can be accelerated by EDTA, it might be supposed that EDTA removes certain metal ions which inhibit the inductive enzyme formation by *p*-benzoquinone. To test this possibility the effects on the enzyme formation of various metal ions added to the cultivation medium containing *p*-benzoquinone were examined. As recorded in Table

TABLE IV

EFFECTS OF METAL IONS ON THE INDUCTIVE FORMATION OF GLUCOSE DEHYDROGENASE

Mycelia were grown in shake cultures for 20 h at 30° in the medium containing 0.05 mM *p*-benzoquinone and the indicated metal salts. The activities of glucose dehydrogenase in mycelial extracts as well as in culture filtrates (medium) were measured. Total activity of glucose dehydrogenase determined in the extract of mycelia grown in the absence of added salts was taken as 100%.

Metal salts added	*Concn. (mM)*	*Glucose dehydrogenase (relative total activity)*	
		Mycelial extract	*Culture filtrate*
None	—	100	41
$CuSO_4$	0.01	100	41
$CuSO_4$	0.05	25	12
$CuSO_4$	0.1	5	3
$HgCl_2$	0.3	0	0
$CaCl_2$	0.4	50	20
$MnCl_2$	0.4	40	17
$FeSO_4$	0.5	64	20
$ZnSO_4$	0.5	91	41

IV, the inductive enzyme formation by 0.05 mM *p*-benzoquinone was considerably inhibited in the presence of certain metal ions; Cd^{2+}, Cu^{2+} and Hg^{2+} were especially effective. The inhibition of the enzyme formation by 0.05 mM Cu^{2+} was 75%. It was also found, though not shown in Table IV, that this inhibition could be completely eliminated by the addition of 0.1 mM EDTA to the medium. Consequently, it seemed likely that one of the effects of EDTA is to trap the inhibitory metal ions present in the medium, although much is still to be explored before any conclusion can be drawn. Since metal ions such as Cu^{2+} have been shown to inhibit the activity of glucose dehydrogenase itself[19,27], it was possible that apparently decreased synthesis observed in the presence of metal ions was merely due to the masking of the activity and that EDTA acted as an unmasking agent. However, this possibility could be ruled out, since the glucose dehydrogenase activity in mycelial extracts was not activated even by dialysis against 0.1 M phosphate buffer containing 8-hydroxyquinoline, EDTA, *o*-phenanthroline, or KCN.

Induction in resting mycelia

To elucidate the induction mechanism, it was desirable to use resting mycelia. Washed mycelia were therefore obtained from shake cultures which had been incubated in the absence of *p*-benzoquinone, and suspended aseptically in the growth medium from which glucose had been removed. Neither detectable growth nor glucose dehydrogenase production occurred when this suspension was shaken at 30° for 18 h. As shown in Table V, however, a clear induction was observed when *p*-benzoquinone or the quinone *plus* EDTA were included in the shaking medium. The addition of EDTA alone was not effective. The accelerative effect of EDTA on the induction under

TABLE V

EFFECTS OF CHELATING AGENTS AND SOME INHIBITORS ON THE *p*-BENZOQUINONE-INDUCED FORMATION OF GLUCOSE DEHYDROGENASE IN RESTING MYCELIA

Mycelia (10 g) obtained from a shake culture containing no inducers were suspended in 800 ml of the glucose-free medium, and the mycelial suspension was shaken for 18 h at 30° in the presence of *p*-benzoquinone and/or other compounds indicated. The total activities of glucose dehydrogenase in both mycelial extracts and culture filtrates (medium) were then measured.

Addition	*Glucose dehydrogenase (total activity, units per culture)*	
	Mycelial extract	*Culture filtrate*
None	0	0
0.1 mM 8-hydroxyquinoline	0	0
0.1 mM EDTA	0	0
0.1 mM *o*-phenanthroline	0	0
0.05 mM *p*-benzoquinone	189	96
0.05 mM *p*-benzoquinone + 0.1 mM EDTA	227	48
0.05 mM *p*-benzoquinone + 0.1 mM 8-hydroxyquinoline	720	111
0.05 mM *p*-benzoquinone + 0.1 mM *o*-phenanthroline	1060	576
0.05 mM *p*-benzoquinone	189	96
0.05 mM *p*-benzoquinone + 1 mM NaN_3	25	0
0.05 mM *p*-benzoquinone + 1 mM KCN	72	23
0.05 mM *p*-benzoquinone + 0.1 mM dinitrophenol	378	326

these conditions was clearly different from the observed in the cultivation experiments. In resting mycelia, EDTA had no significant effect on the induction by *p*-benzoquinone or on the liberation of enzyme. On the other hand, when the other chelating agents such as *o*-phenanthroline and 8-hydroxyquinoline were added, both the formation and liberation of the enzyme were accelerated, although these compounds, like EDTA, induced no synthesis of glucose dehydrogenase when added in the absence of *p*-benzoquinone. As shown in Table V, the induction of the enzyme formation in resting mycelia was inhibited by KCN and NaN_3, suggesting that energy production was required for the induction. However, the addition of 2,4-dinitrophenol together with *p*-benzoquinone markedly increased the total yield of the enzyme; more than 40% of the enzyme thus produced was recovered in the medium. It seems that the nitro groups of the dinitrophenol were reduced to the amino stage, and this product exerted a high inductive effect.

Induction of glucose dehydrogenase in other organisms

It seemed of interest to ascertain whether the induction of glucose dehydrogenase formation by *p*-benzoquinone could also be observed in other organisms. As shown in Table VI, such an induction phenomenon was observed most clearly in *A. oryzae* and was slightly detectable in *Aspergillus cinnanomeus*, *Penicillium chrysogenum* and *Penicillium notatum*. In the other organisms tested such as *Aspergillus niger*, *Aspergillus wentii*, *Mucor mucedo*, *Rhizopus formosensis*, *Geotrichum candidum*, *Fusarium oxysporum* f, *Nematospora coryli*, *Trichoderma viride*, *Pichia membranaefaciens*, *Eremothecium ashbyii*, *Pseudomonas fluorescens* and *Pseudomonas mildenbergii*, no glucose dehydrogenase was formed in the presence of *p*-benzoquinone or the quinone *plus* EDTA.

TABLE VI

DISTRIBUTION OF INDUCTIVE ACTIVITIES OF GLUCOSE DEHYDROGENASE BY *p*-BENZOQUINONE IN SEVERAL MOLDS

Mycelia grown in shake culture for 20 h at 30° in the medium containing benzoquinone and EDTA. Mycelial extracts of moulds were obtained in the same manner as from *A. oryzae*.

Mould	*Addition to growing medium*	*None*		*0.05 mM p-benzoquinone*		*0.05 mM p-benzoquinone + 0.1 mM EDTA*	
		Glucose dehydrogenase (specific activity, units/mg protein)					
		Mycelial extract	*Culture filtrate*	*Mycelial extract*	*Culture filtrate*	*Mycelial extract*	*Culture filtrate*
A. oryzae 1002		0	0	0.57	2.5	1.2	8.3
A. cinnanomeus		0.01	0	0.09	0.83	0.11	0.42
P. chrysogenum		0.01	0.35	0.06	0.36	0.17	0.72
P. notatum		0	0	0.1	0	0.06	1.6

DISCUSSION

The results reported in this paper indicate that glucose dehydrogenase is formed by *A. oryzae* when grown in surface cultures but is not synthesized in shake cultures.

It is reported further that the synthesis of this enzyme in surface cultures is markedly and specifically increased by the presence of hydroquinone or *p*-benzoquinone. The presence of these compounds also induces a marked synthesis of the enzyme even in shake cultures. It is therefore likely that these substances act as specific inducers, and that the failure of the organism to produce glucose dehydrogenase in shake cultures is due to the lack of such inducers under shaking conditions. However, it is not yet known whether the natural inducer operative in surface cultures is identical with either of these two compounds. Although *A. oryzae* contains ubiquinone, this compound cannot be an inducer as already discussed.

Of the two effective compounds, *p*-benzoquinone at low concentrations is much more active in inducing the enzyme synthesis than is hydroquinone. This and other observations suggest that hydroquinone exerts its action only after its conversion to *p*-benzoquinone. Preliminary evidence has in fact been obtained that mycelial extracts of *A. oryzae* catalyze a slow oxidation of hydroquinone by oxygen. This suggestion is of particular interest in view of the finding that *p*-benzoquinone acts as an effective hydrogen acceptor for glucose dehydrogenase[2]. However, the other effective acceptors[27] such as menadione, α-naphthoquinone, thionine, and DCIP possess no inducer activities, indicating that the induction phenomenon observed here is somewhat different from the usual induction of enzyme formation by substrates or their analog.

Inductive synthesis of glucose dehydrogenase by *p*-benzoquinone and hydroquinone is greatly accelerated by the simultaneous presence of EDTA and some other chelating agents, which themselves have no inducing actions. The addition of these chelating agents also causes increased liberation of glucose dehydrogenase into the medium. It is further reported that the inductive formation of the enzyme is strongly inhibited by metal ions such as Cu^{2+} and Hg^{2+}. The inhibition by Cu^{2+} can be reversed by EDTA. These findings suggest that the induction-accelerating action of EDTA is caused by the removal of these inhibitory metal ions, although it is doubtful whether the medium employed contained sufficient concentrations of these metal ions to inhibit the enzyme synthesis. It seems that the increased liberation of glucose dehydrogenase in the presence of chelating agents is due to alterations in the permeability of the mycelia. It has been reported that treatments of *Escherichia coli* cells with EDTA cause an increase in permeability of the membrane toward various compounds such as actinomycin[28]. In the present study no efforts were made to clarify the nature of the changes induced by EDTA in the envelope structures of the organism.

The *p*-benzoquinone-induced formation of glucose dehydrogenase can also be observed in resting mycelia of *A. oryzae*. The induction under these conditions is accelerated by chelating agents such as *o*-phenanthroline which also causes increased liberation of the enzyme. However, EDTA shows no induction-accelerating action, the reason for which is not yet clear. The induction in resting mycelia is inhibited by cyanide and azide, suggesting the requirement of energy for the synthesis of the enzyme.

Since our main objective in this study was to establish optimal growth conditions for maximal production of glucose dehydrogenase, the mechanism involved in this rather unusual induction has not been studied to any extent. However, it seems that investigations along this line are of importance for our understanding of the regulation of enzyme synthesis.

The mycelial extracts of *A. oryzae* contain, in addition to the glucose dehydro-

genase discussed above, a second enzyme catalyzing the oxidation of glucose by NADP. The level of this latter glucose dehydrogenase is not affected by cultivation conditions or by *p*-benzoquinone and hydroquinone. Although the significance of the occurrence of two different glucose dehydrogenases in the same organism is not yet known, it is of interest that only one of them is subject of profound change by cultivation conditions.

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