Enzyme and Permeability Changes During Morphogenesis of *Nocardia corallina*

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(Accepted for publication 8 December 1966)

SUMMARY

Initial growth of *Nocardia corallina* occurred by germination and coenocytic hyphal development which resulted in a large surface-to-volume increase. During this period, endogenous respiration and the ability to oxidize fructose increased rapidly, but the stimulation of oxygen uptake by glucose decreased to zero. This inability to utilize glucose was due to loss of permeability rather than lack of a specific enzyme required for glucose metabolism. This was established by the following data: (1) cell-free extracts of hyphae contained the enzymes for conversion of glucose into fructose-6-phosphate; (2) intact hyphae oxidized fructose; (3) incubation of hyphae with cetyltrimethylammonium bromide increased cell permeability to glucose and oxygen uptake was stimulated. Comparative determinations of enzymes involved in the early steps of glucose metabolism showed quantitative changes associated with the period of impermeability to glucose. The loss of permeability to glucose may represent an initial metabolic change fundamental to morphogenesis in this microbe.

INTRODUCTION

A cyclic series of morphological changes, termed a life cycle by Webb & Clark (1957), has been described for *Nocardia corallina*. The mechanisms which underlie these changes are unknown; however, Brown & Clark (1966*b*) gave evidence that fragmentation was influenced by diffusible metabolic factors which accumulated during growth. One approach toward a better understanding of morphogenesis in this organism is to search for answers at the metabolic level. Evidence for the pentose pathway (Brown & Clark, 1966*a*) and the Krebs cycle (Brown & Clark, 1961) has been reported for this organism, but little is known about metabolic changes potentially correlated with morphogenesis. However, preliminary work showed a loss of ability to utilize glucose which occurred during the growth cycle (Brown, 1964). From the data available, it appeared probable that the block in glucose utilization was the result of a lack of permeability to glucose or a loss of ability to perform one of the component steps in the conversion of glucose to fructose-6-phosphate.

The objectives of this investigation were: (1) to measure throughout the growth cycle, the changes in ability to oxidize glucose; (2) to locate the block in glucose metabolism; (3) to measure the specific activities of those enzymes involved in the early stages of glucose metabolism and to detect changes potentially correlated with the loss of ability to oxidize glucose.

METHODS

Culture. *Nocardia corallina* ATCC 4273 was grown at 29° on nutrient agar + 0.5% glucose. Inocula for experiments were obtained from cultures which had been transferred consecutively at least twice at 48 hr intervals. Such time was sufficient for completion of the growth cycle and physiological and morphological variations were minimized. Cultures, grown as described above, were harvested at various times by alternate suspension and centrifugation for 15 min. at 18,000g and 4° in 0.01 M-potassium phosphate buffer (pH 6.8).

Respiration data. Oxygen uptake was measured by standard Warburg techniques (Umbreit, Burris & Stauffer, 1964). Warburg flasks contained: 0.2 ml. of 10% KOH in the centre well, 0.1 ml. of 0.1 M substrate in a side arm and 2.7 ml. of bacterial suspension in the main compartment. The flasks were equilibrated in air and were incubated at 29° with shaking at 36 excursions/min.

Cetyltrimethylammonium bromide (Cetavlon) a surface-active agent which has been used to increase cell permeability (White, Kell, Suffling & Work, 1964) was tested for ability to induce oxygen uptake by 8 hr hyphae with glucose as substrate. The flasks contained 2.6 ml. of bacterial suspension, 0.1 ml. of 0.1 M-glucose in one side-arm and in a second side-arm, 0.1 ml. of Cetavlon at a concentration of 10, 1, 0.1, 0.01 or 0.001 mM.

The effect of insulin on the permeability to glucose of bacteria from 8 hr cultures was also studied. Insulin for injection (Squibb), 80 and 40 units/ml. and crystalline beef insulin (Lilly) at concentrations of (mg./ml.) 10, 1 and 0.1 were used. The experiments were done as described above except that 0.1 ml of insulin solution was substituted for the Cetavlon.

Warburg experiments were devised to determine if glucose oxidation was induced in 8 hr hyphae by extracts of bacteria from a growth cycle stage capable of glucose oxidation. Experimental flasks contained 1.4 ml. of 8 hr hyphal suspension, 1.3 ml. of 48 hr cell-free extract and 0.1 ml. of 0.1 M-glucose. Five separate controls were run: (1) 1.4 ml. of buffer + 1.4 ml. of 8 hr hyphal suspension; (2) 1.4 ml. of 8 hr hyphal suspension + 1.3 ml. of buffer + 0.1 ml. of 0.1 M-glucose; (3) 1.3 ml. of 48 hr cell-free extract + 1.4 ml. of 8 hr hyphal suspension + 0.1 ml. buffer; (4) 1.3 ml. of 48 hr cell-free extract + 1.4 ml. buffer + 0.1 ml. of 0.1 M-glucose; (5) 1.3 ml. of 48 hr cell-free extract + 1.5 ml. buffer. All flasks contained 0.2 ml. of 10% KOH in the centre well and 0.01 M-potassium phosphate (pH 6.8) was used as buffer.

Cell-free extracts. A 20% (wet wt./v) bacterial suspension in 0.01 M-potassium phosphate buffer at pH 6.8, was disrupted at 4° by sonic treatment with a Branson Sonifier (Model S-125). Bacteria were disrupted for 10, 12, 14, 16, 18 and 20 min. to determine the optimum period of sonic treatment. Extracts were centrifuged twice at 18,000g and 4° for 15 min. Approximately 15 ml. supernatant fluid was placed in seamless cellulose tubing (48 Å pore size, 2.54 cm. diameter) and dialysed at 4° against 1 l. of 0.01 M-potassium phosphate buffer (pH 6.8) for 4 hr. The buffer was stirred continuously with a magnetic stirrer and changed for fresh buffer after 2 hr. Protein was determined by the biuret method (Gornell, Bardawill & David, 1949).

Enzyme assays. All extinction measurements were made with a Beckman, Model DU spectrophotometer at 25–26° room temperature using 1 cm, light-path cuvettes. The procedures described by McDonald (1955) were used to measure glucokinase and

fructokinase activities. The complete reaction mixtures were incubated at 30° for 10 min. Samples were removed at 2 min. intervals and changes in extinction were measured at 560 m μ . Under assay conditions, the liberation of 1 μ mole of acid accompanied the phosphorylation of 1 μ mole of sugar and produced a change of 0.032 in extinction. One unit of specific activity is defined as the phosphorylation at 30° of 1 μ mole of sugar/min./mg. protein.

The procedure described by Slein (1955) was used to assay glucosephosphate isomerase activity. The reaction was incubated at 30° and samples were removed at 2 min. intervals for a total of 18 min. The resorcinol procedure (Slein, 1955) was used to measure the fructose-6-phosphate formed by measuring changes in extinction at 540 m μ . One unit of specific activity is defined as a change of 0.001 in extinction/min./mg. protein.

The specific activity of glucose-6-phosphate dehydrogenase was determined as described by DeMoss (1955). Extinction changes were measured at 340 m μ . One unit of specific activity for glucose-6-phosphate dehydrogenase is defined as a change of 0.001 in extinction/min./mg. protein.

RESULTS

Oxygen uptake

Figure 1 shows respiratory data for organisms taken from various stages of growth. During the initial 8 hr period the organisms germinated and developed into coenocytic hyphae with a large increase in surface-to-volume ratio. During this same period, endogenous metabolism and the stimulation of oxygen uptake by fructose and caproate increased. However, the ability to oxidize glucose decreased to zero. The highest values for endogenous metabolism and for fructose and caproate oxidation were obtained with organisms from 8 hr cultures which were not stimulated by glucose. Organisms taken from later stages of growth showed a recovered ability to oxidize glucose.

Enzymes

Table 1 shows that protein concentration in cell-free extracts increased through 20 min. of sonic treatment. The specific activity was relatively constant for 12–20 min. of sonic treatment. A 20 min. period of sonic treatment was used for preparing subsequent cell-free extracts since a higher protein concentration with a higher total activity was obtained.

Table 2 shows comparative specific activities of several enzymes which are involved in initial steps of glucose oxidation via the Embden–Meyerhof and pentose pathways. Comparative data are shown for dialysed and non-dialysed extracts. Cell-free extracts of 8 hr hyphae, which did not oxidize glucose, contained the component enzymes necessary for converting glucose into fructose-6-phosphate. Since these 8 hr hyphae were capable of oxidizing fructose (Fig. 1), the enzymes beyond the fructose-6-phosphate stage were not studied.

The specific activities of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase were 48 and 36% lower, respectively, in extracts of 8 hr hyphae than in extracts of 48 hr coccoids (Table 2). The differences are significant at or below the 0.1% value. The specific activities of glucokinase and fructokinase were 17 and 15% lower in extracts of 8 hr hyphae than in extracts of 48 hr coccoids (Table 2). These differences

are significant at the 2% value. Values in Table 2 are averages of a minimum of four separate determinations for each enzyme, performed on each of two separate 48 hr extracts and two separate 8 hr extracts. The non-dialysed extracts show higher activities than the dialysed extracts (Table 2). However, the values for 8 hr hyphae again were lower than those for 48 hr extracts.

Fig. 1

Fig. 1. Changes in endogenous and exogenous respiration during the growth cycle of *Nocardia corallina*. Endogenous values were subtracted from the data shown for glucose, fructose and caproate.

Fig. 2

Fig. 2. Effects of Cetavlon (0.003 mM) on the oxidation of glucose by 8 hr hyphae of *Nocardia corallina*.

Table 1. Rate of disruption of *Nocardia corallina* by sonic treatment, as measured by release of protein and glucose-6-phosphate activity

Time of sonic treatment (min.)	Protein in non-dialysed cell-free extracts (mg./ml.)	Glucose-6-phosphate dehydrogenase specific activity*
10	2.3	11.3
12	2.8	32.4
14	3.4	30.0
16	4.1	34.6
18	4.3	31.5
20	4.9	32.4

* One unit of specific activity is equivalent to a change of 0.001 extinction unit/min./mg. protein.

Effect of Cetavlon

The effects of Cetavlon upon the ability of 8 hr hyphae to oxidize glucose are shown in Fig. 2. Oxygen intake was not stimulated above the endogenous value by addition of glucose alone. Upon tipping Cetavlon into the system, the oxygen uptake was in-

creased. Separate control experiments, with either glucose or Cetavlon omitted, resulted in no stimulation of endogenous respiration. Cetavlon at concentrations of 0.03 mM or higher, inhibited endogenous respiration.

There was no significant stimulation of oxygen uptake by glucose when 8 hr hyphae were incubated with insulin at various concentrations, as described in Methods.

Stimulation of glucose oxidation by cell-free extracts

Table 3 shows the stimulation of glucose oxidation produced by incubation of 8 hr hyphae with dialysed cell-free extract from 48 hr coccoids. Stimulation of oxygen uptake was not observed in the control systems.

Table 2. *Specific activities of four enzymes in cell-free extracts (supernatant fluid of sonically-treated organisms) of Nocardia corallina*

Enzyme	Specific enzyme activity* Dialysed extract		Specific enzyme activity*. Non-dialysed extract	
	8 hr hyphae	48 hr coccoids	8 hr hyphae	48 hr coccoids
Glucokinase†	1.16 ± 0.06	1.39 ± 0.12	1.26 ± 0.06	1.63 ± 0.04
Fructokinase†	1.29 ± 0.02	1.51 ± 0.07	1.48 ± 0.02	1.78 ± 0.04
Glucosephosphate isomerase‡	15.8 ± 0.66	24.6 ± 0.44	17.9 ± 0.36	27.3 ± 0.10
Glucose-6-phosphate dehydrogenase‡	15.4 ± 0.75	29.4 ± 1.22	29.5 ± 0.65	37.3 ± 0.56

* Mean value ± standard error of the mean based on four determinations of 8 hr extract and four determinations of 48 hr extract. Specific activity of each enzyme is defined in Methods.

† The values for these enzymes in the 8 hr and 48 hr extracts were significantly different, with $P < 0.02$.

‡ The values for these enzymes in the 8 hr and 48 hr extracts were significantly different, with $P < 0.001$.

Table 3. *Stimulation of glucose oxidation by 8 hr hyphae in the presence of 48 hr cell-free extracts of Nocardia corallina*

Contents of Warburg vessels*	O ₂ values†
48 hr cell-free extract	0.0
48 hr cell-free extract + glucose	0.0
8 hr hyphae	9.9
8 hr hyphae + glucose	9.9
8 hr hyphae + 48 hr cell-free extract	10.0
8 hr hyphae + glucose + 48 hr cell-free extract	15.7

* Quantities are shown in Methods section.

† Q_{O₂} = ml. oxygen uptake/hr/mg. dry wt. organism.

DISCUSSION

Glucose metabolism and cell permeability. The loss of ability to oxidize glucose occurred during the first 8 hr of incubation. There was little cell division during this period and the organisms increased in size and in surface-to-volume ratio as they changed from oval coccoids to tube-like hyphae. This was an active metabolic period as evidenced by the increased endogenous metabolism and increased ability to oxidize caproate and fructose. The transient, but complete, loss of ability to oxidize glucose indicated a temporary block either in the transport of glucose or in phosphorylation

or isomerization of glucose. The enzymes beyond the fructose stage were functional since these organisms oxidized fructose.

Cell-free extracts of hyphae, which did not oxidize glucose, contained the necessary enzymes to convert glucose to fructose-6-phosphate. Thus the defect in glucose metabolism appeared to be due to impermeability of the organisms to glucose. This was confirmed by data which showed that 8 hr hyphae oxidized glucose when the organisms were pre-incubated with Cetavlon. This surface-active agent has been used in amino acid decarboxylase assays to increase cell permeability (White *et al.* 1964). When 8 hr hyphae were incubated with 0.003 mM-Cetavlon in the Warburg flasks, endogenous metabolism was maintained but permeability was increased sufficiently to allow glucose to penetrate, and oxygen uptake was stimulated.

The results of Table 3 suggest the presence in 48 hr organisms of transferable factors involved in glucose transport. A study of this transport mechanism and its possible relationship to morphogenesis in *Nocardia corallina* appears to be a fruitful area for future study.

Enzyme changes. Comparison of specific activities of four enzymes involved in initial steps of glucose and fructose metabolism indicated that the concentrations of glucose-6-phosphate dehydrogenase, glucosephosphate isomerase, glucokinase and fructokinase all decreased during the period when glucose was excluded because of the impermeability of the cells. These differences were found in both dialysed and in non-dialysed extracts. The data on non-dialysed extracts were included since such extracts were subjected to a minimum of *in vitro* manipulation which might have resulted in non-uniform inactivation of the enzymes. Since the 8 hr hyphae were several times larger in volume than the 48 hr coccoids, the data suggest that synthesis of the four enzymes occurred during the 8 hr period of germination and coenocytic hyphal growth. If no enzyme synthesis occurred, the specific activities (which are based on total protein concentration) would be decreased by dilution by protein synthesized during this period of increase in cell size.

The site of the block in glucose oxidation in *Nocardia corallina* appears to be similar to the metabolic lesion in the cells of animals with diabetes mellitus. A variety of metabolic sequelae are known to result from the initial lesion in diabetes. The failure of glucose transport in *N. corallina* may also represent a fundamental event preceding other metabolic shifts necessary to accomplish morphogenesis.

We are grateful to Eli Lilly and Company for the generous gift of crystalline zinc insulin.

REFERENCES

- BROWN, O. R. (1964). *Metabolism, morphogenesis and pigmentation in Nocardia corallina*. Ph.D. dissertation, University of Oklahoma, Norman.
- BROWN, O. R. & CLARK, J. B. (1961). The tricarboxylic acid cycle in *Nocardia corallina*. *Proc. Okla. Acad. Sci.* **41**, 94.
- BROWN, O. R., & CLARK, J. B. (1966*a*). Evidence for the pentose cycle in *Nocardia corallina*. *Proc. Soc. exp. Biol. Med.* **122**, 887.
- BROWN, O. R. & CLARK, J. B. (1966*b*). Fragmentation in *Nocardia corallina*. *J. gen. Microbiol.* **45**, 525.
- DEMOSS, R. D. (1955). Glucose-6-phosphate and 6-phosphogluconate dehydrogenase from *Leuconostoc mesenteroides*. *Meth. Enzymol.* **1**, 328.
- GORNELL, A. B., BARDAWILL, C. S. & DAVID, M. M. (1949). Spectrophotometric and turbidimetric methods for measuring proteins. *Meth. Enzymol.* **3**, 450.

- MCDONALD, M. R. (1955). Yeast hexokinase. *Meth. Enzymol.* **3**, 269.
- SLEIN, M. W. (1955). Phosphohexoisomerases from muscle. *Meth. Enzymol.* **1**, 299.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric Techniques*, 4th ed. Minneapolis: Burgess Publishing Co.
- WEBB, R. B. & CLARK, J. B. (1957). Cytogenetic study of *Nocardia corallina*. *J. Bact.* **74**, 31.
- WHITE, P. J., KELL, B., SUFFLING, A. & WORK, E. (1964). Variation of activity of bacterial diaminopimelate decarboxylase under different conditions of growth. *Biochem. J.* **91**, 600.