ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. XXXIII, 47 SECTIO D 1978

Zakład Biochemii. Instytut Chemii Podstawowych. Akademia Medyczna w Lublinie
Kierownik: prof. dr med. Marian Szymona

Tadeusz SZUMIŁO, Marian SZYMONA

Pyruvate Formation as a Test for the Induction of some Enzymes in *Mycobacterium* sp. 279

Tworzenie pirogronianu jako test na indukcję niektórych enzymów u *Mycobacterium* sp. 279

Образование пирувата — тест на индукцию некоторых энзимов у *Mycobacterium* sp. 279

Inducible synthesis of an enzyme can be observed on the basis of an increase in its specific activity. If the enzyme is functionally connected with cell respiration; and many catabolic enzymes are, it may be possible to reveal induction by stating the continuously increasing oxygen uptake in a Warburg apparatus. This indirect method proved valuable in a screen for inducible enzymes in mycobacteria, especially so when the cells were starved prior to manometric determinations (4).

The aim of this work has been to use pyruvate formation as another criterion of inducible enzyme synthesis. It was expected that increased amount of an enzyme engaged in carbohydrate metabolism would be manifested by an increased accumulation of pyruvate, provided the test is carried out in the presence of an inhibitor of the Krebs cycle. Xylitol: NAD 2-oxidoreductase (EC 1.1.1.9, xylitol dehydrogense) served as the model enzyme since it was shown to yield pyruvate via the following reaction sequence: xylitol→D-xylulose-5-P→→D-fructose-6-P→ →pyruvate (5).

MATERIALS AND METHODS

The organism employed was a D-xylose mutant of *Mycobacterium* sp. 279, isolated in this Laboratory from the parent strain (5). It was grown on the surface of a glucose-glutamate-citrate-salts medium (2) at 37°C for 3 days. After incubation, cells were harvested and washed with water by centrifugation. The procedures of starvation, induction and disintegration of cells were as described earlier (4, 5). Oxygen uptake of cell suspension was measured with the conventional Warburg method (6). Protein content in cell-free extracts was estimated by the Lowry et al. method (3) using bovine serum albumin as a standard.

Xylitol dehydrogenase activity was determined in a Zeiss spectrophotometer VSU-2G at room temperature with the use of 1-cm light path cuvettes. The reaction mixture contained: 63 mM glycine-NaOH buffer, *pH* 10.7; 150 mM xylitol; 0.4 mM NAD; and extract (80--250 μg protein) in a total volume of 1.65 ml. The rate was followed at 340 nm for several minutes.

The pyruvate accumulation test was performed using a mixture which contained: 100 mM phosphate buffer, *pH* 7; 100 mM xylitol; 10 mM sodium arsenite; and bacteria (equivalent to about 8 mg dry cell weight) in a total volume of 2.5 ml. The cell suspension was incubated by shaking for 30 minutes, then 1 ml of 25% trichloroacetic acid was added and the precipitated material removed by centrifugation. Pyruvate accumulated in the supernatant was determined by the 2,4-dinitrophenylhydrazine-toluene extraction method (1).

RESULTS AND DISCUSSION

Previous work showed that starved mycobacteria responded to an addition of common sugars and polyols by continuously increasing rate of oxygen consumption. Chloramphenicol suppressed this effect leaving the basal level of the corresponding enzymes unchanged (4).

A similar behaviour has been obtained in the present work. As one can see in Fig. 1, the oxygen uptake resulting from xylitol addition proceeded in a way characteristic of induction (curve 2). At the same time, sample 1 containing both xylitol and chloramphenicol revealed a low respiration which proceeded at a constant rate owing to a constitutive level of xylitol dehydrogenase already present in non-induced cells.

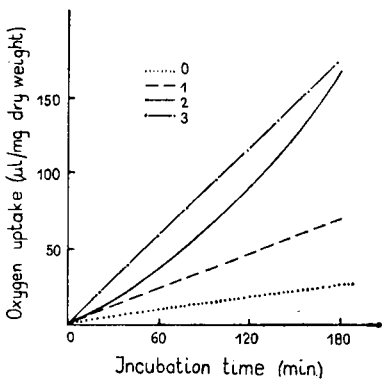

Fig. 1. Induction of xylitol dehydrogenase as observed manometrically at 37°C. Each Warburg vessel contained 100 μmoles of phosphate buffer, *pH* 7; 20 mg of wet weight cells; and 0.2 ml of 20% KOH in center well. 0 — endogenous respiration, 1 — basal response to xylitol (125 μmoles) in presence of chloramphenicol (250 μmoles), 2 — inducible response to xylitol (125 μmoles), 3 — non-inducible response to glucose as a reference substrate (125 μmoles). Prior to manometric determinations the bacteria were starved for 12 h by incubating cell pellicles on the surface of glucose-free medium (4, 5)

In further experiments the enzyme was induced by incubating starved and non-starved mycobacteria with xylitol, and then xylitol dehydrogenase activity was measured in cell-free extract. Table 1 shows that the original amount of the enzyme studied increased approx. 3- and 4-fold after 2 and 4 hours of shaking, respectively. In agreement with the former

Table 1. Xylitol dehydrogenase activity in cell-free extracts from *Mycobacterium* sp. 279, D-xylose mutant

Pretreatment of cells	Enzyme activity μmoles NADH/min/mg protein
Fresh	0.020
Starved*	0.023
Starved, induced** with xylitol for 2 h	0.064
Starved, induced with xylitol in presence of chloramphenicol (100 μg/ml) for 2 h	0.024
Starved, induced with xylitol for 4 h	0.080

* Starvation was performed as in Fig. 1.

** 0.5 g of packed bacteria were suspended in 50 ml of sterile 0.05 M phosphate buffer (*pH* 7) containing 0.5 g of xylitol and 0.5 ml of 1% Tween-80, and shaken for indicated times at 37°C. Thereafter, the cells were centrifuged, washed with cold water and disintegrated as described earlier (4, 5).

observation chloramphenicol suppressed the induction effect down to the basal level.

Fig. 2 shows the results of pyruvate determinations in mycobacteria with xylitol as the substrate and aresnite as the inhibitor of the Krebs cycle. As it was expected, considerably more pyruvate was formed in cells which had been preincubated with the inducer. The formation of the ketoacid was virtually proportional to time and cell density in the range tested. The highest concentration of pyruvate was attained using starved cells which had been exposed to xylitol for 2—3 hours (Fig. 3). The results were reproducible with either xylitol or xylose as evidenced by the fairly constant pyruvate/xylitol dehydrogenase activity ratio amounting to 2.80 ± 0.13 (Table 2).

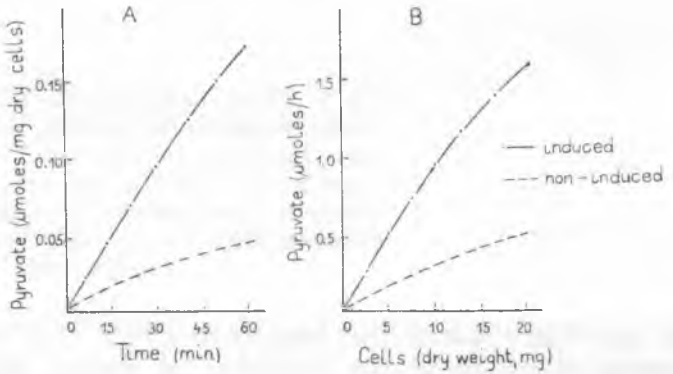

Fig. 2. Pyruvate formation in the presence of xylitol and arsenite. Analytical procedure as in Methods except for: time (A), cell density (B). Prior to pyruvate testing the cells were starved as in Fig. 1, and where indicated, induced for 1 h at 37°C by the underlayering technique (4, 5) with 1% D-xylose

Table 2. Comparison of pyruvate formation and xylitol dehydrogenase activity in starved mycobacteria submitted to induction under various conditions

Addition	Pyruvate (μmoles/h/mg wet cells) 1	Enzyme activity (μmoles NADH/min/mg protein) 2	Ratio 1:2
Nil	0.065	0.025	2.8
Xylitol	0.32	0.15	2.1
D-Xylose	0.39	0.17	2.3
L-Xylose	0.32	0.14	2.3
D-Xylose+chloramphenicol (100 μmoles/ml)	0.10	0.03	3.3
D-Xylose+arsenite (1 mM)	0.30	0.10	3.0
D-Xylose+arsenite (10 mM)	0.08	0.03	2.7

Three-day old cell pellicles were starved as in Fig. 1 and induced for 2 h using indicated substrates in citrate-salts medium. Thereafter, the bacteria were centrifuged, washed with cold water and used for analysis.

Longer induction times caused lower yields of pyruvate although xylitol dehydrogenase activity continued to increase, especially in non-starved cells. The fact that the process of enzyme synthesis was not parallel to the pyruvate formation beyond the first 2—3-hour induction period may be due to partial inactivation of the glycolytic pathway or to an alteration of its regulatory mechanism. The complication may render the pyruvate test less sensitive but does not invalidate its usefullness for screening purposes.

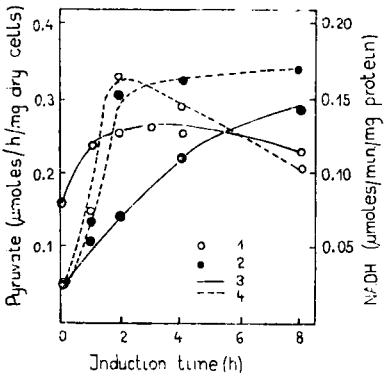

Fig. 3. Xylitol dehydrogenase activity and pyruvate formation in starved and non-starved cells. Induction was conducted for indicated times by the underlayering technique with 1% D-xylose; 1 — pyruvate, 2 — xylitol dehydrogenase activity, 3 — fresh (non-starved) cells, 4 — starved cells

It is of importance to note that arsenite at a concentration of 10 mM proved capable of inhibiting the induction of xylitol dehydrogenase (Table 2). For this reason arsenite must be added to cell suspension only after induction is terminated.

The main advantage of the pyruvate test lies in that it can be carried

out with as little as 25—50 mg of wet weight cells which constitutes an amount manifold lower than usually needed for preparation of cell-free extract.

The use of the test can be extended to some other enzymes involved in either pentose cycle or glycolysis provided their activities are not affected by arsenite. In fact, similar experiments performed with mannitol-, glycerol- and fructose-induced mycobacteria gave equally positive results. Other microorganisms could also be tested in this way. For example, when *Escherichia coli* was used as the test organism, the amount of pyruvate formed at the expense of glucose was 5 times that given by *Mycobacterium* sp. 279. Therefore, in this case the time of testing and the amount of bacteria could be reduced markedly.

Of course, obtention of reproducible results would require cell growth and other experimental conditions to be maintained as constant as possible.

REFERENCES

1. Friedemann T. E., Haugen G. E.: J. Biol. Chem. **147**, 415—423, 1943.
2. Głębicki T., Jałowiecka K., Sym E. A.: Gruźlica **18**, 413—421, 1950.
3. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. **193**, 265—275, 1951.
4. Szymona O., Szymona M.: Acta Microbiol. Polon. **20**, 125—133, 1971.
5. Szumiło T., Szymona M.: Acta Michrobiol. Polon. **20**, 113—123, 1971.
6. Umbreit W. W., Burris R. H., Stauffer J. F.: Manometric Techniques. Burgess Publishing Co., Minneapolis 1957.

Otrzymano 5 XI 1977.

STRESZCZENIE

Stosując mykobakteryjną dehydrogenazę ksylitolu jako enzym modelowy opracowano test do badania indukcji niektórych enzymów przemiany węglowodanowej. Test obejmuje: 1) eksponowanie komórek na induktor, 2) inkubację z substratem w obecności inhibitora cyklu Krebsa (10 mM arsenin) i 3) oznaczenie kwasu pirogronowego. W opisanych warunkach doświadczalnych ilość powstałego pirogronianu odpowiadała poziomowi aktywności badanej dehydrogenazy; fakt ten uzasadnia przydatność testu co najmniej do celów screeningowych.

РЕЗЮМЕ

Применяя микобактерийную дегидрогеназу ксилитола как модального энзима, обработано тест для исследования индукции некоторых энзимов углеводного обмена. Тест охватывает: 1) экспонирование клеток на индуктор; 2) инкубацию с субстратом в присутствии ингибитора цикла Кребса (10 mM арсенин); 3) определение пировиноградной кислоты. В представленных условиях исследования количество возникшего пиргромяна отвечало уровню активности исследованной дегидрогеназы. Этот факт свидетельствует о пригодности теста хотя бы в скрининговых опытах.