THE EFFECT OF β-PROPIOLACTONE ON THE METABOLISM OF BLASTOMYCES DERMATITIDIS

GLEN R. GALE[1]

Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina

Received for publication October 17, 1952

Although a vast proportion of the bacterial infections of man has yielded to either one or a combination of several of the recent antibiotics, the systemic fungus infections, with few exceptions, have remained refractory to these agents. The treatment of systemic and pulmonary North American blastomycosis with inorganic iodides has been in use for many years and with some success. Of value also is the use of ultraviolet light for the cutaneous forms of the disease. More recently, certain aromatic diamidines have been employed with very beneficial results (Schoenback *et al.*, 1951).

The work of Bernheim (1942) was significant in that it was the first study of the oxidative metabolism of a pathogenic fungus. This type of investigation, while yielding knowledge concerning the life processes of the organism, also exposes several possible points of attack for a given antifungal substance. These factors are valuable in the designing of potential chemotherapeutic compounds.

Bernheim and Gale (1952) have reported an inhibition of the growth of *Blastomyces dermatitidis* by β-propiolactone,[2] a simple organic compound with a relatively low toxicity (Kelly and Hartman, 1951, 1952; Kelly, 1952). This compound previously had been shown to be effective against many viruses and bacteria (Hartman *et al.*, 1951).

The purpose of this work was to investigate the effects of this lactone on various phases of the metabolism of resting suspensions of *B. dermatitidis*.

MATERIALS AND METHODS

All manometric measurements were done in the Warburg apparatus at 37 C. Simple incubations were done in 50 ml Erlenmeyer flasks in a Warburg bath employing a wooden rack.

[1] Research Fellow of the United States Public Health Service.

[2] A product of the B. F. Goodrich Chemical Company.

The organism used was obtained from the Department of Medical Mycology at Duke Hospital. It was grown on Kelley's agar slants (1939) at 37 C. Suspensions were prepared by adding distilled water to the tubes and scraping with a blunt glass rod. This was transferred to a 20 by 100 mm centrifuge tube and spun at 2,000 rpm for 2 to 3 minutes. The fluid was decanted, more water added, the cells stirred, and transferred to a 6.5 ml Hopkins tube. This was centrifuged for 2 to 3 minutes. The cells then were taken up in M/20 phosphate buffer, pH 7.4, and centrifuged for 5 minutes. A suspension in buffer was prepared so that 0.05 ml of packed cells was contained in a total of 1.0 ml. One ml of this suspension was used in each Warburg vessel. When the cells were to be preincubated with either the drug or substrate, this was added to the main compartment of the vessel along with the cells. At the desired time, the substrate (or drug) was added from the side arm.

The chromatographic experiments were done according to the method of Berry *et al.* (1951), using Whatman no. 1 filter paper, a *n*-butanol-acetic acid-water solvent, and 0.2 per cent ninhydrin solution as indicator. To obtain a release of "free" amino acids from the interior of the cells, they were boiled, after the buffer had been decanted, with 0.2 N HCl in a water bath for ten minutes. An equal volume of 0.2 N NaOH was added then for neutralization. After centrifugation the supernatant was ready to be spotted. A salt concentration of 0.1 N did not cause undue streaking of the chromatograms.

Ammonia was determined by taking aliquots from the reaction vessels, diluting to 21.0 ml with water, adding 0.5 ml Nessler's solution (Vanselow, 1940), and determining the per cent transmission with an Evelyn colorimeter employing a 515 λ filter. It is recommended that the concentration of ammonium sulfate be not greater than 12 to 13 μg per ml (equivalent to about 3 μg NH_4 per ml) to obtain good results by this method. A series of known concentrations was

prepared simultaneously to obtain a standard curve. Upon standing, glucose gives a color and a fine precipitate with Nessler's reagent. Therefore, adequate controls were run and the necessary corrections made.

RESULTS

The oxidation of glucose and pyruvate was inhibited by the drug; the degree of inhibition was a function of the concentration (figure 1). The effect on the endogenous respiration of the cells was slight (figure 1). In order to determine whether or not the inhibition was competitive, increasing amounts of pyruvate were added to

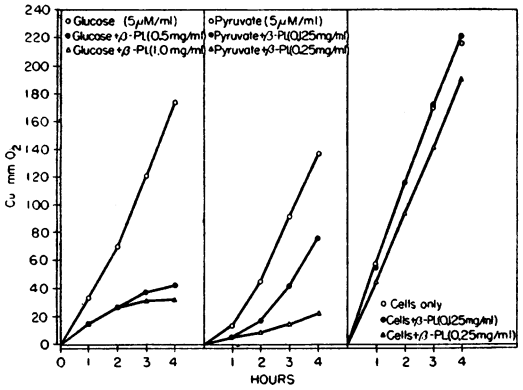

Figure 1. The effect of β-propiolactone (β-PL) on the oxidation of glucose and pyruvate by *Blastomyces dermatitidis* and on the endogenous respiration of the organism. The endogenous respiration has been subtracted from the oxidation curves.

two certain concentrations of the drug and the oxygen uptake measured. With concentrations of pyruvate from 5 to 50 μM per ml, 49 to 51 per cent inhibition of oxidation was observed at a β-propiolactone concentration of 0.125 mg per ml, and 76 to 85 per cent inhibition at 0.25 mg per ml β-propiolactone. The total amounts of oxygen consumed increased somewhat as the pyruvate concentration was increased, but the increases were proportional as indicated by the per cent inhibitions obtained. This indicates that the inhibition is noncompetitive.

It was then of interest to determine whether or not the previous addition of pyruvate would protect the cells from the effect of the drug. Therefore, the cells were preincubated 20 minutes with pyruvate (5 μM per ml) before the addition of the drug and the results compared with those obtained when the preincubation was with the drug, pyruvate being added at 20 minutes. At the end of 4 hours, the oxygen uptakes were: pyruvate (5 μM per ml) with no drug, 139 mm³; pyruvate added 20 min before β-propiolactone (0.125 mg per ml), 77 mm³; pyruvate added 20 min after β-propiolactone, 22 mm³. These figures have been corrected for the endogenous respiration.

Prolonging the preincubation with the drug before the addition of pyruvate did not alter markedly the per cent inhibition. Preincubation with 0.125 mg per ml for 20 min resulted in 62, 54, and 45 per cent inhibition at 2, 3, and 4 hours, respectively. Preincubation with the same amount

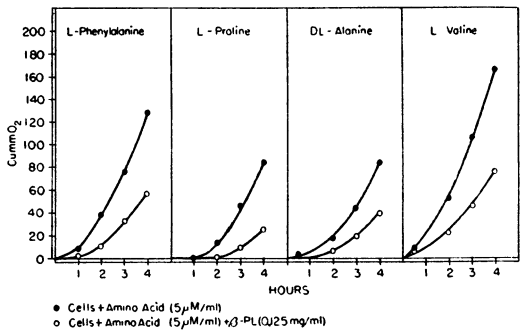

Figure 2. The effect of β-propiolactone (β-PL) on the oxidation of certain amino acids by *Blastomyces dermatitidis*. The endogenous respiration has been subtracted.

for 90 min gave 63, 55, and 42 per cent inhibition at the same time intervals. Similar results were obtained (80 to 85 per cent inhibition) with 0.250 mg per ml of β-propiolactone.

Bernheim (1942) found that certain amino acids stimulate the oxygen uptake of *B. dermatitidis* without themselves being utilized, and he postulated a "specific dynamic action" by which the amino acids in some way increase the rate of the inherent oxidative mechanisms within the cell. This was well substantiated by the findings that the theoretical amount of ammonia could not be recovered, and the fact that the stimulation was proportional to the number of organisms rather than to the concentration of substrate. Neither could definite end points be obtained with the acids. However, with the organism used in these experiments, evidence was obtained that amino acids were taken into the

cell and there utilized. Cells were incubated with glycine, DL-alanine, L-valine, DL-methionine, L-leucine, L-proline, L-phenylalanine, and L-tryptophan for 6½ hours. The supernatants and the cellular extracts after boiling with dilute HCl were chromatographed. Glycine was taken up completely from the fluid medium and utilized. All others were taken into the cell to various extents, with the exception of tryptophan. Analysis of lysed cells showed that the compounds were not simply taken in and incorporated into an amino acid "pool". They apparently had been used to form substances which did not give a color with the ninhydrin reagent. When β-propiolactone (0.25 mg per ml) was added during the incubation period, analysis of the supernatants showed that no transfer of amino acids across the cell wall occurred. Complete recovery of all the aforementioned compounds was obtained. As a check on this, analysis of the lysed cells showed no evidence of any of the added acids. The drug also inhibited the oxygen utilization due to the added compounds (figure 2).

TABLE 1

The inhibition by β-propiolactone of the utilization of ammonia during the oxidation of glucose and pyruvate and during the endogenous respiration of the cells. Concentration of glucose and pyruvate, 5 μM/ml; β-propiolactone, 0.25 mg/ml. Period of incubation, 6 hours.

	AMMONIA ADDED	AMMONIA RECOVERED	AMMONIA UTILIZED
	μg	μg	%
Cells + Glucose + Ammonia	272	81	70
Cells + Pyruvate + Ammonia	272	152	44
Cells + Ammonia	272	234	14
Cells + Glucose + Ammonia + β-Propiolactone	272	142	48
Cells + Pyruvate + Ammonia + β-Propiolactone	272	216	21
Cells + Ammonia + β-Propiolactone	272	272	0

Ammonium sulfate had no effect on either the rate of the endogenous respiration of the cells or the oxidation of glucose or pyruvate. However, ammonia was utilized during these processes, and β-propiolactone had an inhibitory effect on the utilization. Table 1 shows the extent to which the drug prevented the incorporation of ammonia into the cell.

It was thought that more conclusive evidence of the cell surface being the site of action of the drug could be obtained by lysis of the cells so as to retain at least partial enzymatic activity of the cellular material. Grinding in a Potter's homogenizer with Hyflo Super-cel (Johns-Manville) for 15 minutes according to the method of Dockstader and Halvorson (1950) failed to rupture the cells, as did eight rapid freezings and thawings with a dry ice-acetone mixture. No ghost cells were evident on microscopic examination. The former treatment resulted in a preparation with properties similar to those of intact cells; the latter procedure destroyed the ability of the suspension to either autorespire or oxidize pyruvate.

Succinate was not oxidized by the cells under the conditions of the experiments.

DISCUSSION

The available evidence leads to the conclusion that the cell surface or membrane is the principal site of action of β-propiolactone against resting cells of *B. dermatitidis*. This is supported by the facts that (1) in the presence of the drug, certain substances which normally are taken into the cell remain in the supernatant; (2) the inhibition of the oxidation of added pyruvate is non-competitive; (3) preincubation with pyruvate partially protects the pyruvate oxidase system from the deleterious effects of the drug; (4) the degree of inhibition of the pyruvate oxidation is not a function of the period of preincubation with the drug; and (5) the endogenous respiration of the cells, which is presumably at the expense of stored carbohydrate, and possibly amino acids within the cells, is not affected significantly by concentrations of the drug which produced marked inhibition of the oxygen uptake due to added substrates. The confirmatory experiment would be one in which the effect of the drug could be demonstrated on suspensions of cellular material in which surface phenomena would not be manifest. Unfortunately, the nature of the cell poses a problem in the preparation of such a suspension.

SUMMARY

The effect of β-propiolactone on the metabolism of *Blastomyces dermatitidis* was investigated.

This compound inhibited the oxidation of glucose, pyruvate, and certain amino acids. The inhibition of the pyruvate oxidation was apparently noncompetitive. The previous addition of pyruvate partially protected the pyruvate oxidase system from the drug. The period of preincubation with the compound was not, above a certain point, a determining factor in the degree of inhibition obtained.

The utilization of ammonia during the oxidation of glucose and pyruvate and during autorespiration was inhibited by β-propiolactone, as was the transfer of amino acids across the cell surface.

It was concluded that the cell surface or membrane is its principal site of action.

REFERENCES

BERNHEIM, F. 1942 The effect of various substances on the oxygen uptake of *Blastomyces dermatitidis*. J. Bact., **44**, 533–539.

BERNHEIM, F., AND GALE, G. R. 1952 Effect of β-propiolactone on metabolism of *Pseudomonas aeruginosa* and growth of certain fungi. Proc. Soc. Exptl. Biol. Med., **80**, 162–164.

BERRY, H. K., SUTTON, H. E., CAIN, L., AND BERRY, J. S. 1951 Development of paper chromatography for use in the study of metabolic patterns. Univ. Texas Publication, no. 5109, 22–55.

DOCKSTADER, W. B., AND HALVORSON, H. O. 1950 A study of grinding techniques for bacterial cells. Science, **112**, 618–620.

HARTMAN, F. W., PIEPES, S. L., AND WALLBANK, A. M. 1951 Virucidal and bactericidal properties of β-propiolactone. Federation Proc., **10**, 358.

KELLEY, W. H. 1939 A study of the cell and colony variations of *Blastomyces dermatitidis*. J. Infectious Diseases, **64**, 293–296.

KELLY, A. R. 1952 Effects of virucide beta-propiolactone and its degradation products on blood platelets. Federation Proc., **11**, 419.

KELLY, A. R., AND HARTMAN, F. W. 1951 Beta-propiolactone: its toxicity, degradation products and comparison with nitrogen mustard. Federation Proc., **10**, 361.

KELLY, A. R., AND HARTMAN, F. W. 1952 Biological effects of beta-propiolactone. Federation Proc., **11**, 419.

SCHOENBACK, E. B., MILLER, J. M., GINSBERG, M., AND LONG, P. H. 1951 Systemic blastomycosis treated with stilbamidine. A preliminary report. J. Am. Med. Assoc., **146**, 1317–1318.

VANSELOW, A. P. 1940 Preparation of Nessler's reagent. Ind. Eng. Chem., Anal. Ed., **12**, 516–517.