THE INHIBITION OF YEAST CARBOXYLASE BY HOMOLOGOUS ANTISERUM[1,2]

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In previous studies the inhibitory effect of immune factors on cellular metabolism (Sevag and Miller, 1948) and on the enzyme hexokinase isolated from yeast (Miller, Pasternak, and Sevag, 1949) was reported. Subsequently it was found also that antiserum prepared against the living whole yeast cells inhibited the activity of isolated yeast hexokinase. However, both antihexokinase and anti-whole-yeast sera failed to exercise an inhibitory effect on the fermentation of glucose by the whole yeast cells, a fact which could be interpreted to indicate a failure to inhibit hexokinase activity in the intact cells by various antisera or possible interference by the activities of other enzyme systems occurring simultaneously. For this reason the study of another yeast enzyme, namely carboxylase, which decarboxylates pyruvate and represents one of the terminal steps in the fermentation of glucose, was undertaken. Air-dried yeast decarboxylates pyruvate without any appreciable interference from other enzymes. It was hoped that any effect of anticarboxylase, anti-dried-yeast, and anti-whole-yeast sera would be free from interference when dried yeast cells or cresyl-violet-treated yeast cells were used as the source of carboxylase. This report presents the data obtained regarding these questions.

EXPERIMENTAL METHODS AND RESULTS

Carboxylase was isolated from fresh Fleischmann's bakers' yeast according to the method of Green, Herbert, and Subrahmanyan (1941). The isolated material was used for immunization and activity tests with various sera. The activity of the isolated material was determined manometrically by measuring the carbon dioxide evolved in the Barcroft-Warburg apparatus at 30 C during the reaction:

$$CH_3COCOOH \xrightarrow[pH\ 6 - 6.2]{carboxylase} CH_3CHO + CO_2$$

Preparation of anticarboxylase sera. Three different antisera were prepared by injection of rabbits with (1) isolated carboxylase dissolved in M/15 phosphate buffer, pH 7.2; (2) an extract of dried yeast cells (crude carboxylase); and (3) living whole yeast cells.

[1] This is the third in a series of studies on the effect of immune reactions on the metabolism of bacteria.

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Method 1. Using freshly isolated carboxylase, two rabbits were given 5 series of 3 injections at 1- to 3-day intervals with 1 to 3 weeks elapsing between each series. One rabbit received subcutaneously a total of 195 mg and the other 185 mg of protein.

Method 2. Batches of air-dried bakers' yeast were suspended in M/15 phosphate buffer, pH 7.2, incubated at 37 C for 1 hour, centrifuged, and the supernatant fluid used for injection. From 43 to 100 mg of the dried yeast were suspended in 5 to 10 ml of buffer, and each rabbit received an average of 3 ml of the supernatant fluid at each injection. A total of 15 injections were given, the first 6 subcutaneously, the others intravenously.

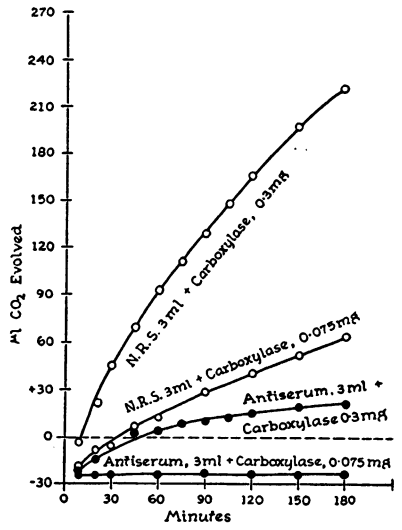

Figure 1. Inhibitory effect on carboxylase by homologous rabbit antiserum added at 0 hour.

Initial negative values were observed in all experiments when there was no immediate evolution of carbon dioxide. These negative values are probably due to the contraction of the volume soon after mixing the reagents.

Method 3. A culture of living bakers' yeast cells, isolated from the Fleischmann's yeast cake used for the isolation of carboxylase, was used for the immunization of two rabbits. Twenty-seven injections of saline-washed suspensions of standard turbidity, prepared from 18-hour cultures of yeast cells, were given at 1- to 3-day intervals, for a period of 3½ months. Seven doses, given subcutaneously, contained from 3.3 to 17 mg of yeast per dose. Twenty injections, given intravenously, varied from 1.47 to 4.6 mg of yeast per dose. An agglutination titer of 1:10,000 was obtained with the inactivated, untreated serum. The globulin fraction that was obtained by ammonium sulfate fractionation, after dialysis, had a titer of 1:20,000.

All three immune sera inhibited the activity of the isolated carboxylase (see figures 1 and 2). Immune serum prepared against hexokinase isolated from the

same strain of yeast had no effect on the purified carboxylase, thus showing specificity.

Elimination of the interference with the reaction by inactivated normal and immune sera. Inactivated (30 minutes' heating at 56 C) normal and immune sera decomposed pyruvic acid in the absence of added carboxylase. This difficulty was overcome by first precipitating the inactivated sera with 2.5 to 3 volumes of saturated ammonium sulfate, dissolving the precipitate in water, and dialyzing

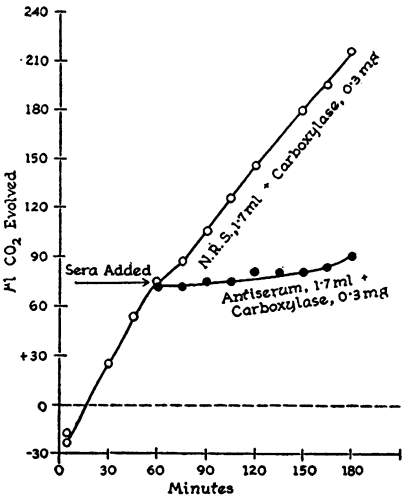

Figure 2. Inhibitory effect on carboxylase by homologous rabbit antiserum added after the reaction had proceeded for 60 minutes.

The experiment, the results of which are presented in figure 2, was conducted in the following manner: Double-armed Warburg flasks were used. Side arm 1 contained 0.8 ml N pyruvate, pH 6.1. Side arm 2 contained 1.7 ml serum. The main compartment of the flask contained 0.2 ml carboxylase (0.3 mg), and 1.8 ml normal serum (in order to obtain a uniform activity, 1.8 ml of normal rabbit serum [N.R.S.] was added to each flask). The pyruvate was added from side arm 1 after equilibration of the temperature was reached. The contents of side arm 2, immune or normal serum, were added after the reaction had been in progress for 60 minutes.

Initial negative values were observed in all experiments when there was no immediate evolution of carbon dioxide. These negative values are probably due to the contraction of the volume soon after mixing the reagents.

against 12 liters of 0.45 per cent sodium chloride over a period of 48 hours in the cold. The dialyzed clear solutions were concentrated to the original volume of the serum at room temperature by blowing air against the cellophane bag from an electric fan. The protein contents of these were determined by the biuret reaction, and comparable volumes were used in each reaction system. Hexokinase antiserum subjected to the same treatment retained its inhibitory action for isolated yeast hexokinase but had no action on purified carboxylase.

Effect of cresyl violet on the carboxylase activity of the whole yeast cells. Since *fresh whole yeast cells* exhibit no carboxylase activity anaerobically, an indirect

procedure was utilized to enable the cells to exercise their carboxylase activity. Fresh whole yeast cells were brought in direct contact with cresyl violet,[3] a basic oxazin dye, and the regular carboxylase system was set up. Usually 0.2 ml of an 18-hour-old yeast culture suspended in saline and ranging in turbidity from a 33 to a 53 Klett reading (filter no. 52; 1 to 1.5 mg dry weight) were treated with 0.3 ml of cresyl violet solution (0.21 mg) in the side arm of a Warburg flask, and the pyruvate and serum or buffer were put in the main compartment of the flask. In one of the experiments, after a 4-hour period, with 0.2 ml yeast cells (turbidity, a Klett reading of 50), the following results were obtained with the systems containing (a) normal serum, 331 μl of CO_2; (b) carboxylase antiserum, 333 μl of CO_2; (c) fresh whole yeast antiserum, 344 μl of CO_2; and (d) dried yeast antiserum, 333 μl of CO_2. Likewise no inhibitory effect was observed when from 2 to 3 mg of dried yeast cells[4] (in place of isolated carboxylase or cresyl-violet-treated yeast cells) suspended in 0.0067 M phosphate buffer of pH 6.2 were used instead of isolated carboxylase.

SUMMARY AND CONCLUSION

As previously reported (Miller, Pasternak, and Sevag, 1949), antiserum prepared against isolated yeast hexokinase completely inhibited the activity of purified yeast hexokinase. Later it was found that the same antiserum failed to inhibit the hexokinase activity of the fresh whole yeast cells. Likewise antiserum prepared against living whole yeast cells inhibited the activity of pure hexokinase but had no effect whatsoever on the hexokinase activity of the whole yeast cells. These results were confirmed in the carboxylase system as well.

Even though antiserum prepared against purified carboxylase, dried yeast cells, and living whole yeast cells inhibited the activity of the isolated carboxylase, none of these immune sera had any effect on the carboxylase activity of the dried yeast cells or the cresyl-violet-treated whole yeast cells. As with the oxidative enzymes in *Eberthella typhosa* and pneumococcus (Sevag and Miller,

[3] The authors are indebted to Prof. O. Meyerhof for the observation that the fresh whole yeast cells show carboxylase activity when acted upon by this dye.

[4] On the other hand, cresyl violet reduced the activity of isolated carboxylase and also the carboxylase activity of dried yeast cells. After a period of 2 hours, 0.6 mg of carboxylase preparation produced 286 μl of CO_2 from 0.6 ml of 1 N pyruvate in the presence of normal serum, whereas an identical system containing also 0.21 mg of cresyl violet produced only 114 μl of CO_2. This reduction in activity might be attributed to the tendency of the basic dyes to form complexes with the magnesium ion (Massart *et al.*, 1947*a*,*b*) of the enzyme, thus removing it from the field of reaction. In the case of wet yeast cells, the carboxylase activity which is exhibited only after they have been in contact with cresyl violet is reduced in the presence of added magnesium ion as a result of the removal of the dye. In the case of dried yeast cells, however, the addition of magnesium partially counteracts the suppressive effect of the dye. According to electron micrographs (ca. 50,000 magnification) kindly supplied by Dr. James Hillier of Radio Corporation of America, Princeton, N. J., the walls of yeast cells (*Hansenula anomala*) which were subjected to vacuum drying in the electron microscope appear to be undamaged.

In plate counts, an inoculum of 8.3×10^8 yeast cells, after having been in contact with cresyl violet for 10 minutes, yielded 1.15×10^2 colonies.

1948) and *Salmonella* (Harris, 1948), the yeast carboxylase and hexokinase appear to be intracellular. The homologous antibodies of these enzymes, because of their large molecular size, would appear to be incapable of penetrating the cell walls for a reaction with these intracellular enzymes.

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