THE DEMONSTRATION OF RIBOSOME-BOUND PENICILLINASE IN BACILLUS CEREUS*

J. D. Duerksen and M. L. O'Connor+
Department of Microbiology,
University of Kansas School of Medicine
Kansas City 3, Kansas.

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Introduction: Recent evidence (e.g., Cowie et al., 1961 and Kihara et al., 1961) has indicated that in the process of enzyme synthesis, there is at any given time, a minute quantity of the newly formed enzyme still associated with the "active" ribosomes. A preliminary investigation on the Bacillus cereus penicillinase system (Duerksen, 1961) showed the probable existence of ribosome-bound penicillinase activity. The purpose of this paper is to describe the results of further experiments using improved techniques of penicillinase assay and cell rupture. These experiments have allowed us to demonstrate more conclusively the existence of ribosome-bound penicillinase and to follow the kinetics of the system subsequent to induction by penicillin.

Methods and Results: B. cereus 569/H (penicillinase formed constitutively) was grown according to the method of Pollock (1957) to a relatively low density of 0.04 to 0.1 mg dry weight/ml. The cells were harvested, washed, and resuspended to a concentration of 0.7 - 1.0 mg dry weight/ml in TS2M buffer (McQuillen et al., 1961), pH 7.2, containing 0.3 M surcrose and 50-100 μg lysozyme/ml. Incubation at 37°C results in greater than 95% "protoplast" formation in less than 60 minutes. The "protoplasts", pelleted by centrifugation, were osmotically

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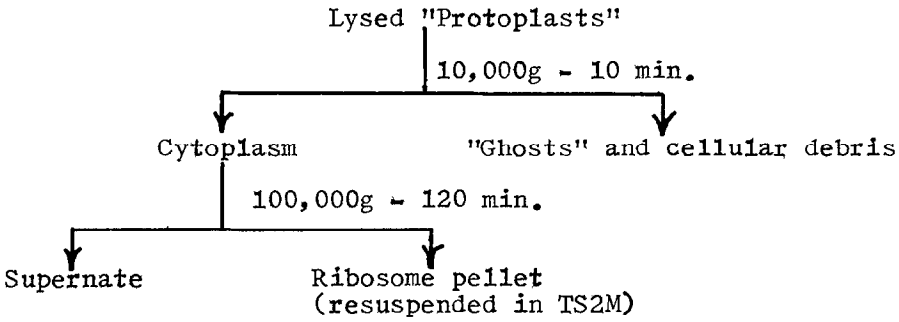
lysed by resuspension in TS2M at 4°C and fractionated as follows:



Sucrose density gradient (5 to 20% sucrose in TS2M) centrifugation of the ribosomal fraction was carried out in a Spinco swinging bucket rotor S W 25.1 at 24,000 rpm for 180 minutes. Ten-drop fractions were collected for measurement of light absorption at 260 mμ and penicillinase activity by a modification of Novick's (1962) iodine-decolorization method. The results are shown in figure 1.

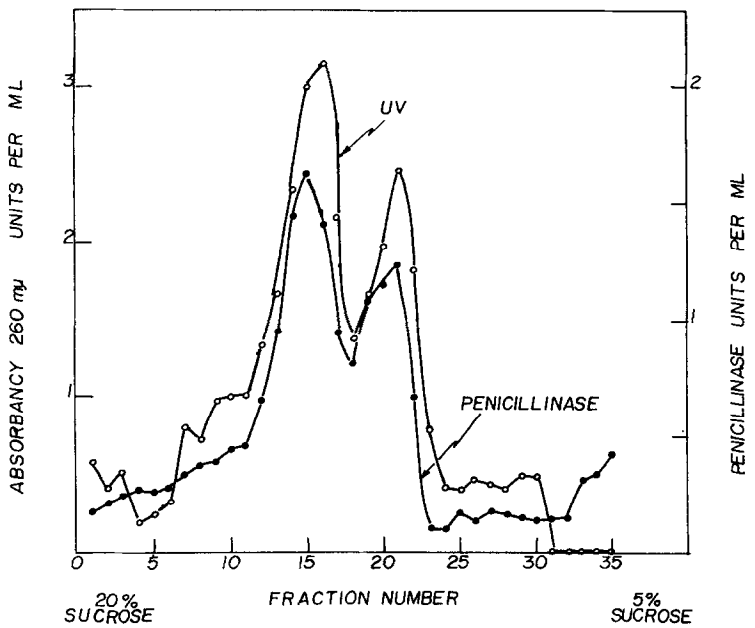


Figure 1. Sucrose density gradient centrifugation profile of a ribosome preparation obtained from B. cereus 569/H.

The magnesium concentration (0.01 M) and isolation procedure used result in the appearance of two ribosome species, 70S and 100S, in the gradient profile (Takai and Kondo, 1962). The coincidence of the ultraviolet absorption and the penicillinase activity peaks indicates that the enzyme activity is tightly bound to the ribosomes and that the fractions are relatively free of contaminating cytoplasmic membrane-bound activity. That the ribosome fraction is free of cytoplasmic membrane contamination is further demonstrated by the fact that no appreciable DPNH oxidase activity is found in this fraction, whereas, large amounts are found in the "ghost" fraction. In previous experiments using ribosome preparations obtained from cells broken in a modified French press, the sucrose density gradient profiles (ultraviolet and penicillinase) indicate possible cytoplasmic membrane contamination (Duerksen, 1961).

In the examination of the kinetic increase, during induction, of ribosome bound penicillinase, B. cereus NRRL 569 (inducible strain) was grown as described above and induced at time zero by the addition of benzylpenicillin to a final concentration of 10 units/ml. At the time intervals indicated:

1. A small sample was taken into oxine-gelatin for total penicillinase activity measurement (Pollock, 1956), and

2. A large sample was taken to which chloramphenicol was added to a final concentration of 25 μg/ml to stop further penicillinase synthesis. Following centrifugation, an aliquot was taken into oxine-gelatin for measurement of exo-penicillinase activity. Ribosomes were obtained from the washed cells as described and purified by centrifugation through the usual sucrose density gradient in a S W 39 rotor at 37,000 rpm for 90 minutes. The zone equivalent to fraction 10 to 23 of figure

1 was dripped out into a single tube for measurement of 260 mμ absorption, penicillinase activity, and iodine sensitivity of the penicillinase activity. The latter was determined by means of a modification of the penicillinase assay procedure.

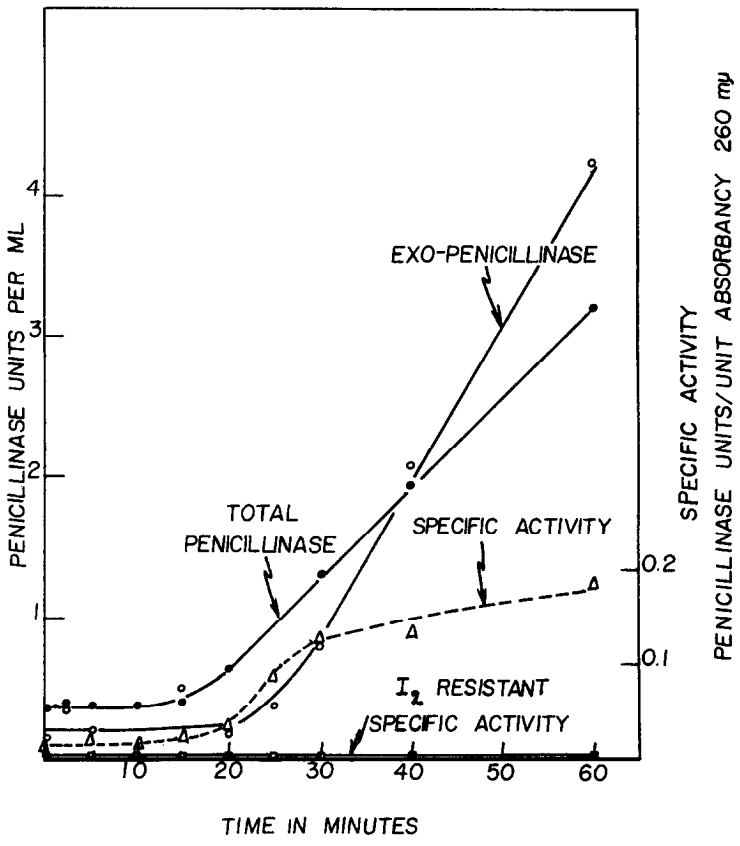


Figure 2. Kinetic increase of penicillinase activity in various fractions during induction of B. cereus 569.

The results are plotted in figure 2. Due to some difficulty inherent in the sampling technique, the values of the total and exo-penicillinase are not as meaningful as the time of change in the rate of appearance of each activity. The increase of total penicillinase and specific activity are essentially simultaneous and are followed by an increase in exo-penicillinase. The plat-

eauing of the specific activity after its initial rise at approximately 20 minutes is in contrast to earlier results with French press obtained preparations, in which the specific activity was always proportional to total penicillinase concentration of the culture (Duerksen, unpublished results). Iodine sensitivity determinations show that the ribosome-bound penicillinase is of the γ-type (Pollock, 1956 and Citri and Garber, 1958).

Discussion: The results presented here show that there is an extremely small amount of γ-penicillinase activity tightly bound to the ribosomes isolated from B. cereus. Furthermore, this activity rises sharply at the time the cells are changing over to the induced rate of penicillinase synthesis. From this data, it is also possible to estimate the number of ribosome-bound penicillinase molecules per cell using the following approximation: a) turnover number for exo-penicillinase at 25°C equals 10^5 molecules substrate hydrolyzed/minute/molecule of penicillinase (Pollock et al., 1956); b) one 260 mμ O. D. unit equals 1.4×10^{13} ribosomes (Cowie et al., 1961); and c) the number of ribosomes per cell equals 4×10^3 (Cowie et al., 1961).

These calculations give the numbers of 1 for the uninduced cell, 10 for the fully induced cell (60 minutes induction), and 15 for the constitutive cell. These results are not inconsistant with earlier isotope experiments (Pollock and Kramer, 1958) from which it was inferred that at most 400 molecules of high molecular weight penicillinase precursor per cell is present.

Unpublished results (Duerksen) using preparation from press broken cells give estimates of 2 and 175 ribosome-bound penicillinase molecules per uninduced and fully induced cell, respectively. The estimations obtained from the present experiments are close to the estimates of Cowie et al. (1961) for the number of ribosome-bound β-galactosidase molecules per Escherichia coli

cell, uninduced and induced. Kihara et al. (1961) obtained an estimate of 100 ribosome-bound β-glucosidase molecules per cell of a β-glucosidase constitutive synthesizing strain of Saccharomyces.

It must be pointed out, however, that the present experiments do not prove that the ribosome-bound penicillinase is the most recently synthesized penicillinase. This problem is now under investigation.

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