the histidine level is above 250 $\mu$g/ml. At 100 $\mu$g/ml, marked slowing of $His^-Tr^-_{his}$ growth appears. At 10 $\mu$g/ml the population size of $His^-Tr^-_{his}$ is stationary while $His^-Tr^+_{his}$ grows rapidly. Assay of the medium before and after incubation with $His^-Tr^-_{his}$ showed no destruction of histidine by the bacterial culture. Similar results were obtained with the proline and glycine mutants.

Two other mutants have also been isolated by the method described above—the first is a mutant of *E. coli* W which, unlike the phenylalanine auxotroph from which it was derived, requires very high supplements of phenylalanine for growth. This mutant, however, accumulated $^{14}C$-labeled phenylalanine as effectively as the wild-type, and the reason for the very high requirement for phenylalanine is not known. A second mutant (derived from an auxotroph of *E. coli* B requiring histidine, methionine and leucine) is defective in potassium transport, as shown by studies of growth in media containing low potassium ($10^{-4}$ *M*) and measurement of accumulation of $^{42}K$ (see ref. 12).

The specificity of the defect in these mutants has not yet been fully determined. Complete characterization awaits further isolation of transport-negative mutants.

This work was supported by U.S. Public Health Service Grants RG 6712, H-1498, Senior Research Fellowship SF-83 and the Muscular Dystrophy Associations of America, Inc. We are endebted to Misses S. P. GOOLSBY and D. H. ALIOTH and MRS. MICHÈLE M. SAINT-PAUL for laboratory assistance.

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Received March 23rd, 1960

*Biochim. Biophys. Acta*, 42 (1960) 535–538

## Intermittent ultrasonic disruption and localisation of enzymes in acetic acid bacteria

Ultramicroscopic particles, containing a great variety of enzymes, mostly oxidases, can be isolated from disrupted acetic acid bacteria[1–5]. Previously we brought evidence in favour of the view that these enzyme-bearing particles do not exist as such in the

Abbreviations: TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

cytoplasm of the acetic acid bacteria, but originate as artefacts from the cytoplasmic membrane[3,4]. This conclusion agrees with the experience of other authors on the origin of similar particles in bacteria as *e.g. Bacillus megaterium*[6], *Staphylococcus aureus*[7] and *Azotobacter vinelandii*[8,9].

In the present communication new evidence is supplied by means of a different technique, which supports the above conclusion. We used the method of MARR AND COTA-ROBLES[8], which is called here "intermittent ultrasonic disruption": at several definite time intervals, the ultrasonic treatment is stopped and samples are withdrawn for the analysis of the properties of the systems under investigations and the study of the kinetics of enzyme release; also, cell-fractions, obtained after a desired sonication time, may be purified and separately submitted to ultrasonic treatment. During sonication the viable cell count decreases faster than the turbidity of the suspension, because the turbidity is occasioned both with the intact cells and the broken-up cell-envelopes or "hulls". An enzyme linked to the hulls will be released from the latter during the ultrasonic disruption procedure at the same rate as the decrease in turbidity. On the other hand, a soluble enzyme, which is originally present in the cytoplasm, will be released into the supernatant at the same rate as the decrease of the viability curve.

For most of our experiments we used the same strain as previously, *Gluconobacter liquefaciens*. It was grown for 2 or 3 days at 30° on a solid medium containing 10% glucose, 1% yeast extract (Difco), 3% $CaCO_3$ and 2.5% agar. After harvesting, the bacteria were washed twice with 0.01 *M* phosphate buffer, pH 6.2, and finally suspended in a 0.02 *M* solution of the same buffer. The turbidity was adjusted to obtain a reading of approx. 200 in the Klett colorimeter with filter 66. The suspension was disrupted in the 10 kc, 250 W Raytheon Sonic Oscillator at 12° and in air. At the beginning of the sonication and for every 2 or 3 min up to 15 min, a sample was removed (called "total sonicate"), the turbidity measured in the Klett colorimeter with filter 66 and the activity of various enzymes determined as described below. These preparations were separated by centrifugation for 60 min at 6000 × *g* into a "sediment" and a supernatant, called "crude extract". The sediment was used for the estimation of the oxidase activity in the Warburg respirometer at 30°, the vessel containing 1.5 ml of enzyme preparation (the sediment suspended in the original volume of fresh 0.02 *M* phosphate buffer, pH 6.2), 20 μmoles substrate and 40 μmoles phosphate buffer, pH 6.2, in a final volume of 2 ml. Glucose-6-phosphate dehydrogenase activity was determined in the Beckman Spectrophotometer, model DU, in 3 ml containing 0.3 μmole TPN, 10 μmoles Na glucose 6-phosphate, 5 μmoles $MgSO_4$, 110 μmoles Tris–HCl buffer pH 8.5 and 1 ml of either total sonicate or crude extract. The viable cell count was determined by the method of MILES AND MISRA[10].

Several of the enzymes were inactivated to an appreciable extent by the ultrasonic disruption treatment (Fig. 1). These inactivations varied from nearly negligible for galactose oxidase to nearly complete for ethanol oxidase (after 12 min) and D-lactate oxidase (after 15 min).

The galactose oxidase behaved as expected for a "hull"-linked enzyme: its activity was retained on the sediment during sonication in the same way as the decrease in turbidity. For the other enzymes in Fig. 1 this clear-cut picture was complicated by the effect of inactivation. However, we could show that the inactivation occurred mainly on the small particles during sonication. Therefore, a sample

was withdrawn from the Raytheon after 4 min of sonic treatment. The crude extract was prepared, which was again sonicated for another 10 min. The 2-ketogluconooxidase of the "total sonicate" was inactivated to the extent of 2.6%/min, while the

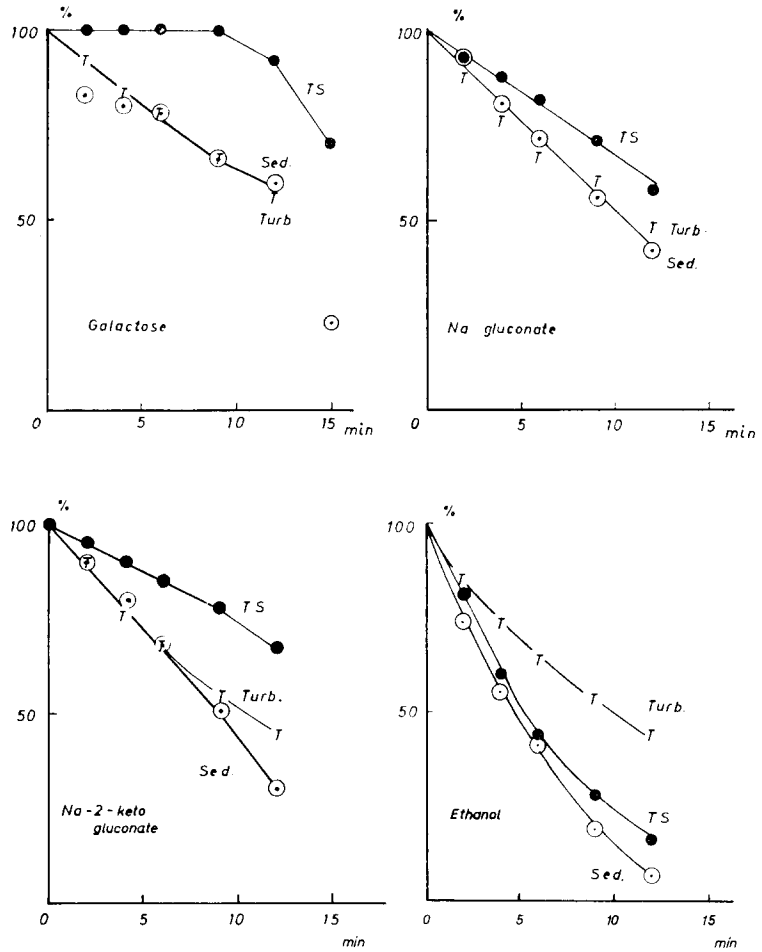


Fig. 1. Behaviour and localisation of various enzymes during the intermittent ultrasonic disruption of *Gluconobacter liquefaciens*. The time axis represents the duration of the ultrasonic treatment in minutes. The results at zero time represent the behaviour of the intact cells and are arbitrarily expressed as 100%. The activity of the oxidases for galactose, sodium gluconate, sodium 2-ketogluconate and ethanol were determined with the Warburg respirometer (see text). Curve T—T Turb., change of turbidity of the total sonicate; ●—● TS, activity of the enzyme in the total sonicate (inactivation curve); ⊙—⊙ Sed., activity of the enzyme in the sediment.

same enzyme in the "crude extract" was inactivated to the extent of 2.8%/min. It may thus be concluded that also in the latter cases, the activity is due to the enzymes linked to the "hulls". We found this to be the case for the oxidases of gluconate and 2-ketogluconate, for the enzyme systems for ethanol and D-lactate oxidation in *Gluconobacter liquefaciens* and for the glucose oxidase in *Acetobacter rancens*. When the activity of some of the enzyme systems was tested in the crude extract, it was found

to be very weak. All these enzymes were previously found to be linked to the ultramicroscopic particles and to the "ghost" fraction[3,4].

The viable count decreased much faster that the turbidity. After 15 min the latter had decreased by about 50 %, while more than 90 % of the original amount of cells were disrupted. The glucose 6-phosphate dehydrogenase activity was selected as a soluble enzyme because it was not noticeably inactivated by ultrasonic treatment up to 15 min. Nearly all the activity of the total sonicate was present in the crude extract. The enzyme was released from the sediment at the same rate as the killing of the bacteria, as expected for a soluble enzyme (Fig. 2). The soluble TPN-linked ethanol dehydrogenase was unsuitable for this purpose because of the pronounced inactivation; no activity remained after 12 min of sonic treatment.

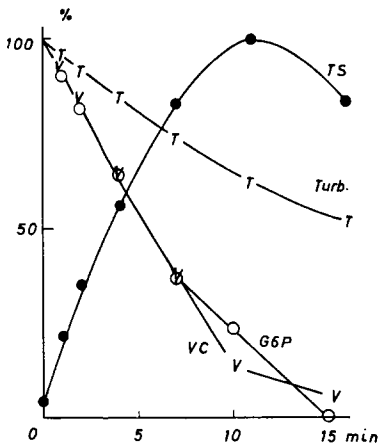


Fig. 2. The behaviour of a soluble enzyme (glucose 6-phosphate dehydrogenase) during the intermittent ultrasonic disruption of *Gluconobacter liquefaciens*. The time axis represents the duration of the ultrasonic treatment in min. The values of turbidity (curve T—T) and viable count (curve V—V) at zero time are the results obtained with intact cells and arbitrarily expressed as 100 %. The activity of the glucose 6-phosphate dehydrogenase in the crude extract (○—○ G6P) was maximal after 15 min and this value was taken as 100 %. The curve was inversed in the graph to show the congruence with the viable count. The activity o the dehydrogenase in the total sonicate (●—● TS) was maximal after 11 min and this value was taken as 100 %. Methods as described in the text.

These results bring additional evidence that the enzyme-bearing particles in the acetic acid bacteria are artefacts derived from the breakage of the outer cell envelope, most probably the cytoplasmic membrane. In these experiments, as in those described by MARR AND COTA-ROBLES[8], it appears likely that the cytoplasmic membrane remains tightly joined to the cell wall to form the "hulls" during breakage.

One of us (J.D.L.) is indebted to the Nationaal Fonds voor Wetenschappelijk Onderzoek and to the Sigma Xi Society for grants.

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Received April 7th, 1960