Enzymes Released from *Escherichia coli* with the Aid of a Servall Cell Fractionator

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ABSTRACT

DUERRE, JOHN A. (Rocky Mountain Laboratory, Hamilton, Mont.), AND EDGAR RIBI. Enzymes released from *Escherichia coli* with the aid of a Servall cell fractionator. Appl. Microbiol. **11**:467–471. 1963.—The release and stability of the enzymes *S*-adenosylhomocysteine nucleosidase, lysine decarboxylase, arginine decarboxylase, glutamic decarboxylase, formic hydrogenlyase, formic oxidase, and glucose oxidase from *Escherichia coli* during disruption of the organisms in a Servall-Ribi refrigerated cell fractionator were examined. With the possible exception of arginine decarboxylase, maximal activity was retained by all the enzymes reported here when the cell suspensions were processed at pressures necessary for rupture of all the organisms (15,000 to 25,000 psi). Considerable variation in the stability of different enzymes liberated by disruption at higher pressures (45,000 to 55,000 psi) was observed. It is reasonable to assume that mechanical forces rather than effects of temperature are responsible for inactivation of these enzymes.

One of the primary problems encountered in using high-pressure extrusion methods to disrupt microbial cells (French and Milner, 1955) has been the increase in temperature which occurs when the cell suspension passes through an orifice. Such increases in temperature caused considerable denaturation and aggregation of cellular proteins (Fraser, 1951; Ribi et al., 1959). This denaturation by heating has been circumvented previously by the application of the pressure cell principle to microbial suspensions in the frozen state (Hughes, 1951; Edebo, 1960). The present investigation was undertaken to determine the suitability of a high-pressure cell fractionator equipped with a refrigerated decompression valve for the preparation of enzymes.

MATERIALS AND METHODS

Culture. The organisms used throughout this study were *Escherichia coli* (wild type). Large quantities of cells were obtained by growing the organism in 1-liter amounts of a complex medium (Billen and Lichstein, 1951) contained in 2-liter Erlenmeyer flasks. After incubation at 30 C for 16 hr under stationary conditions, the cells from 50 flasks were pooled and harvested with a Servall continuous-flow centrifuge and washed twice with 0.05 M phosphate buffer (pH 7.0). Washed cells were suspended in 0.05 M phosphate buffer (pH 7.0) to a concentration of 250 mg of wet cells per ml, divided into two lots, and disrupted in a Servall-Ribi refrigerated cell fractionator. The cell fractionator is based on the release of a pressurized microbial suspension at a refrigerated orifice of a needle valve (Ribi et al., 1959; Perrine et al., 1962). When the cells emerge through the orifice, the cell walls are cracked and the cytoplasm is liberated as a result of rapid decompression and shear forces. The needle valve was cooled with a stream of prechilled (−50 C) nitrogen gas, and the temperature of the effluent was measured at a point 8 mm below the orifice. The temperature was measured with a thermistor thermometer with the probe inserted 8 mm below the orifice of the needle valve. The temperature of the effluent was regulated by varying the flow of prechilled nitrogen gas and effluent through the valve. Both lots were processed at various pressures ranging from 3000 to 55,000 psi. The temperature of the effluent of the first lot as it emerged from the orifice of the needle valve was maintained at about 0 C, whereas that of the second lot was kept at about 20 C. The effluents were centrifuged at 20,000 × *g* for 20 min, and the supernatant fluids were carefully removed and examined for content of protein, by the method of Lowry et al. (1951), and assayed for various enzymes.

Enzyme assays. *S*-adenosylhomocysteine nucleosidase was measured as previously described by Duerre (1962). The reaction mixture contained 50 μmoles of phosphate buffer (pH 6.5), 5 μmoles of *S*-adenosylhomocysteine, and 20 to 50 μliters of cell-free extract. After incubation for 30 min at 37 C, the reaction mixture was deproteinized by heat and analyzed for reducing sugar with Benedict's reagent. Activity of lysine, arginine, and glutamic decarboxylase was determined according to the Warburg technique by measuring CO_2 evolution at 37 C in the presence of air (Najjar and Fisher, 1954). The side arm contained an appropriate amount of cell-free extract (5 to 20 μliters); the main compartment contained 10 μmoles of substrate,

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100 μg of pyridoxal phosphate, and 100 μmoles of acetate buffer (pH 5.0) in water to give a total volume of 3.0 ml. Oxidation of formate and glucose was determined manometrically at 37 C in the presence of air (Asnis, Vely, and Glick, 1956). The main compartment contained 20 μmoles of substrate, 100 μmoles of phosphate buffer (pH 6.0), and water to give a total volume of 3.0 ml. An 0.2-ml amount of 20% KOH absorbed on filter paper was placed in the center well, and 0.1 to 0.5 ml of cell-free extract was placed in the side arm. Formic hydrogenlyase activity was determined by measuring the rate of formation of either H_2 plus CO_2 or H_2 (alkali in the center well) from formate under an atmosphere of nitrogen at 37 C (Peck and Gest, 1957). After equilibration, the extract was tipped into the main compartment, and the increase or decrease in pressure was noted at 5-min intervals for 1 hr.

RESULTS

Nature of breakage. Electron microscopy of the sediments (20,000 × *g*) of effluents released at various pressures revealed the presence of unbroken cells at pressures below 15,000 psi; at 15,000 psi only a few whole cells were in evidence, and at 25,000 psi all the cells were broken. The extracts prepared at 3000 to 7000 psi were extremely viscous, and this viscosity could be eliminated by the addition of deoxyribonuclease. A decrease in viscosity of the effluent at pressures above 15,000 psi indicated depolymerization of deoxyribonucleic acid (DNA). Most of the cell walls in the sediments from effluents prepared at 3000 to 25,000 psi were intact envelopes in which electron optically dense cytoplasmic material appeared to be trapped. As the pressure was increased above 25,000 psi, a gradual increase in fragmentation of these walls was observed with concomitant liberation of the trapped cytoplasmic constituents. When pressures in excess of 25,000 psi were used, an apparent loss of protein in the extracts owing to aggregation and sedimentation at 20,000 × *g* was noted (Fig. 1). The tendency to aggregate, which occurred when higher pressures (45,000 to 55,000 psi) were

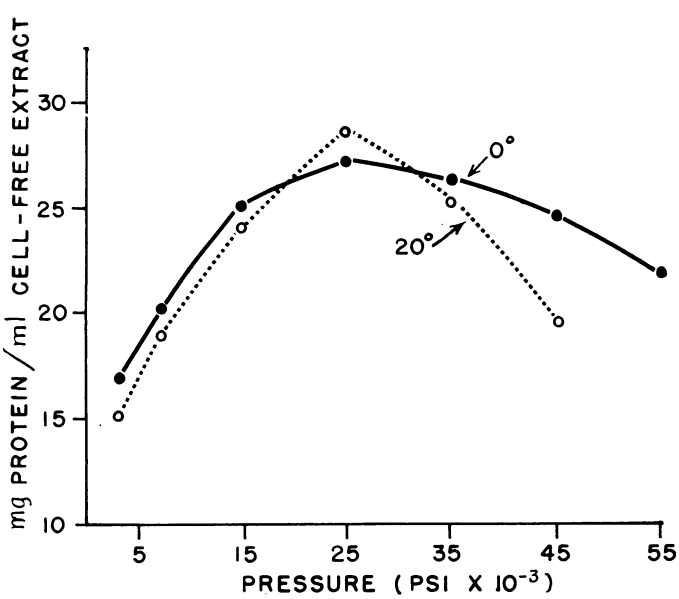

FIG. 1. *Protein retained in supernatant fluid after centrifugation of the disrupted material prepared from Escherichia coli at various pressures.*

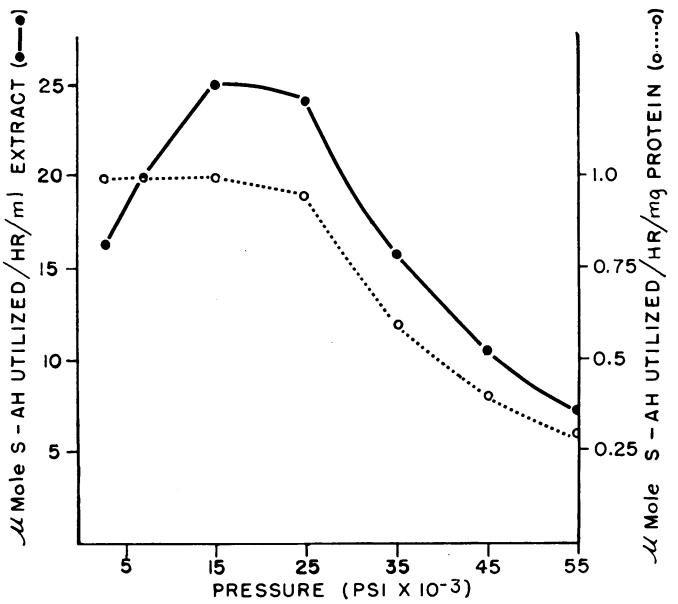

FIG. 2. *Stability of S-adenosylhomocysteine (S-AH) nucleosidase released by disrupting Escherichia coli at various pressures. The reaction mixture contained 50 μmoles of phosphate buffer (pH 6.5), 5 μmoles of S-adenosylhomocysteine, and 20 to 50 μliters of cell-free extract. After incubation for 30 min at 37 C, the reaction mixture was deproteinized by heat and analyzed for reducing sugar with Benedict's reagent.*

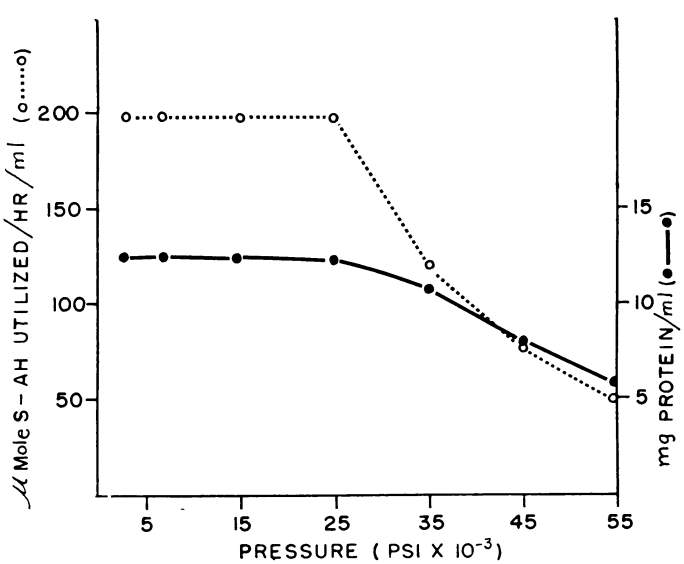

FIG. 3. *Effect of processing a partially purified S-adenosylhomocysteine nucleosidase in a cell fractionator. The partially purified enzyme (15 μmoles of S-adenosylhomocysteine utilized per hr per mg of protein) was subjected to various extraction pressures at 0 C, and the denatured protein was removed by centrifugation at 20,000 × g for 20 min. For conditions of assay, see Fig. 2.*

used, varied somewhat from experiment to experiment; however, loss at 20 C was always greater than at 0 C.

S-adenosylhomocysteine nucleosidase. When *E. coli* was subjected to disruption at various pressures at 0 C, release of *S*-adenosylhomocysteine nucleosidase was directly proportional to the release of protein from the cell (Fig. 2). Maximal enzyme release occurred when the cells were processed at pressures between 15,000 and 25,000 psi; at these pressures, essentially all the cells were ruptured. When pressures greater than 25,000 psi were used, enzyme activity gradually diminished, with only 28% recoverable upon extrusion at 55,000 psi. There was no measurable difference in nucleosidase activity per mg of protein released when the temperature of the orifice was increased to 20 C; however, total enzyme recovered was somewhat less (20%) since sedimentation of protein was greater (Fig. 1).

TABLE 1. *Release of amino acid decarboxylases from Escherichia coli by disruption at various pressures**

Disruption pressure at 0 C	Glutamic decarboxylase		Arginine decarboxylase		Lysine decarboxylase	
	CO_2 from extract†	CO_2 from protein‡	CO_2 from extract	CO_2 from protein	CO_2 from extract	CO_2 from protein
psi						
3000	480	31	420	27	316	20
7000	635	32	532	27	400	20
15,000	810	32	680	27	510	20
25,000	855	32	460	18	540	20
35,000	810	32	192	8	535	21
45,000	635	26	81	3	530	22
55,000	365	17	0	0	510	23

* Activity of lysine, arginine, and glutamic decarboxylase was determined manometrically by measuring CO_2 evolution at 37 C in presence of air. The side arm contained 5 to 20 μliters of cell-free extract; the main compartment contained 10 μmoles of substrate, 100 μg of pyridoxal phosphate, and 100 μmoles of acetate buffer (pH 5.0).

† Expressed as μliters of CO_2 per ml of extract per min.

‡ Expressed as μliters of CO_2 per mg of protein per min.

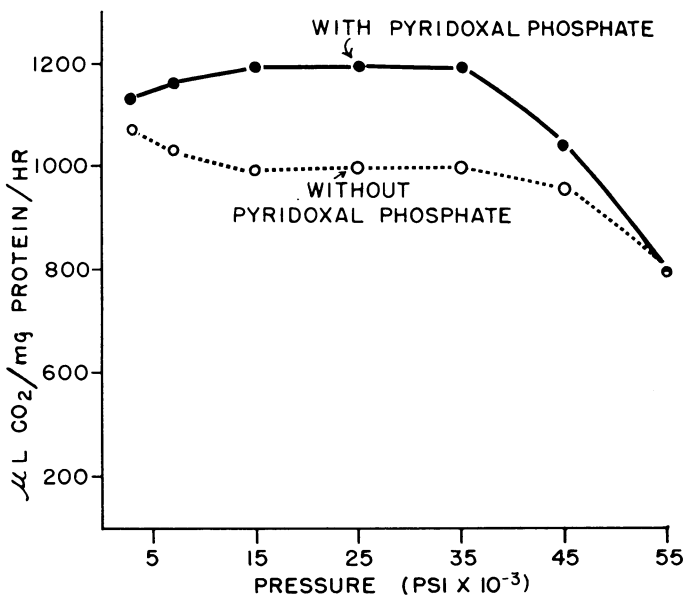

FIG. 4. *Coenzyme effect on glutamic decarboxylase in effluent prepared from Escherichia coli disrupted at various pressures. Activity of glutamic decarboxylase was determined manometrically by measuring CO_2 evolution at 37 C in presence of air. The side arm contained 5 to 20 μliters of cell-free extract; the main compartment contained 10 μmoles of potassium glutamate (pH 5.0) and 100 μmoles of acetate buffer (pH 5.0).*

To establish whether concomitant release and denaturation of *S*-adenosylhomocysteine nucleosidase had occurred at pressures between 15,000 and 25,000 psi, a partially purified enzyme, prepared according to methods described in an earlier communication (Duerre, 1962), was processed with the cell fractionator at various pressures at 0 C. Since inactivation of the enzyme was not observed at pressures less than 25,000 psi (Fig. 3), its simultaneous release and inactivation are considered improbable for this particular enzyme at these pressures. At pressures in excess of 25,000 psi, a gradual loss of enzyme activity occurred as well as marked protein denaturation. As with extracts prepared from whole cells (Fig. 2), enzyme activity decreased faster than could be accounted for by the loss of protein due to aggregation. It is conceivable that many of the remaining proteins may have greater stability than *S*-adenosylhomocysteine nucleosidase when released in the manner described.

Amino acid decarboxylase. As in the case of *S*-adenosylhomocysteine nucleosidase, the release of amino acid decarboxylase enzymes from the cell was proportional to the release of protein (Table 1). Maximal enzyme release again occurred when the cells were processed at pressures between 15,000 and 25,000 psi. However, there was considerable variation in the stability of the various decarboxylase enzymes when higher pressures were employed. Arginine decarboxylase was sensitive to extrusion pressures greater than 15,000 psi, and the enzyme was completely destroyed when 55,000 psi were reached. Lysine decarboxylase was very stable, and little or no activity was lost at extraction pressures up to 55,000 psi. Glutamic decarboxylase was stable when the cells were broken at 35,000 psi, but 57% of the activity was destroyed at 55,000 psi. When the temperature at the orifice of the needle valve was increased to 20 C, glutamic and arginine decarboxylase activity decreased somewhat faster, but no change in lysine decarboxylase activity was observable.

The effect of increasing extraction pressure on the cofactor requirements was then examined. The requirement for exogenous pyridoxal phosphate was equivocal when extracts were prepared at 3000 psi (Fig. 4). However, when the extraction pressure was increased, a requirement became apparent and then leveled off at 15,000 psi. At pressures in excess of 35,000 psi where loss of enzyme activity was apparent (Table 1), the requirement for exogenous pyridoxal phosphate was reduced and no further addition was required with extracts prepared at 55,000 psi. Therefore, the cofactor appeared to be in sufficient

concentration to maintain maximal activity of the remaining enzyme when extracted at 55,000 psi.

Particulate enzymes. The release of formic hydrogenlyase, glucose oxidase, and formic oxidase could not be correlated with the release of protein from the cell. An increase in activity per mg of protein released was observed until extrusion pressures of 25,000 to 35,000 psi were used (Table 2). Inasmuch as formic oxidase (Asnis et al., 1956), glucose oxidase (Wood and Schwerdt, 1953), and formic hydrogenlyase (Peck and Gest,1957) appear to be particulate, this effect might be accounted for by sedimentation of the particles during centrifugation at 20,000 × *g*.

In attempts to clarify this matter, the whole disruption product prepared at 25,000 and 45,000 psi, where all the cells were ruptured, was assayed for various enzyme activities. With products prepared at 25,000 psi, considerable reduction in activity of the particulate enzymes occurred after centrifugation at 20,000 × *g*, whereas the soluble enzymes *S*-adenosylhomocysteine nucleosidase, lysine, and glutamic decarboxylase were fully recoverable in the supernatant fluid (Table 3). Solubilization of the particulate-bound enzymes by extraction at 25,000 psi was apparently negligible, since the particulate enzymes were recoverable in the sediment after centrifugation at 105,000 × *g* for 90 min, whereas the soluble enzymes remained in supernatant fluid. Sedimentation of the particulate enzymes at 20,000 × *g* was just as great with extracts prepared at 45,000 psi where most of the cell-wall envelopes were fragmented. Thus, under the experimental conditions used (Table 2), considerable amounts of the particulate enzymes were discarded with the sediment when the enzyme was prepared for assay.

TABLE 2. *Release of formic oxidase, glucose oxidase, and formic hydrogenlyase from Escherichia coli by disruption at various pressures*[a]

Disruption pressure	Formic oxidase[b]		Glucose oxidase[b]		Formic hydrogenlyase[c]	
	0 C[d]	20 C	0 C	20 C	0 C	20 C
psi						
3000	6	9	7	7	27	22
7000	20	22	14	12	58	52
15,000	39	40	17	17	88	83
25,000	48	45	20	19	88	79
35,000	51	33	20	20	51	30
45,000	36	15	20	19	23	10
55,000	17		17		2	

[a] Oxidation of formate and glucose was determined manometrically at 37 C in presence of air. The main compartment contained 20 μmoles of substrate and 100 μmoles of phosphate buffer (pH 6.0). In the center well was placed 0.2 ml of 20% KOH absorbed on filter paper; 0.1 to 0.5 ml of cell-free extract was placed in the side arm. Formic hydrogenlyase activity was determined by measuring the rate of either H_2 plus CO_2 or H_2 (alkaline in center well) from 20 μmoles of formate under an atmosphere of nitrogen at 37 C.

[b] Expressed as μliters of O_2 per mg of protein per hr.

[c] Expressed as μliters of H_2 per mg of protein per hr.

[d] Temperature at which cells were disrupted.

There was considerable difference in the stability of the three particulate enzymes when the cells were disrupted at high pressures. Formic hydrogenlyase was about 50% inactivated when the cells were extracted at 35,000 psi, and activity was completely destroyed at 55,000 psi (Table 2). Formic oxidase was also partially inactivated when the cell suspension was processed at pressures greater than 35,000 psi, whereas glucose oxidase remained stable. Formic hydrogenlyase and formic oxidase activity were inactivated somewhat faster when the temperature of the effluent at the orifice was allowed to increase to 20 C,

What remains to be explained is the much greater loss of particulate enzymes by centrifugation when extracts were prepared at low pressure (3,000 to 15,000 psi) rather than at high pressure. It is possible that many of these particles are trapped within the network of the DNA gel or within the cell wall envelope present when the cells were disrupted at low pressures, or both. Complete release of such particles would not be effected until depolymerization of the DNA or fragmentation of the walls, or both, occurred at higher pressures. Indeed, loss of particulate enzymes could be reduced by treatment of the disruption product with deoxyribonuclease and reduction of the centrifugal force.

TABLE 3. *Sedimentation of particulate enzymes by centrifugation of Escherichia coli disruption product prepared in a cell fractionator**

Enzyme	At 25,000 psi			At 45,000 psi		
	Whole extract	Supernatant†	Enzyme sedimented	Whole extract	Supernatant†	Enzyme sedimented
			%			%
Formic hydrogenlyase	4100	2600	37	500	270	47
Formic oxidase	1920	1350	30	1660	900	46
Glucose oxidase	700	234	67	610	206	67
Glutamic decarboxylase	85,000	90,000	0	40,000	36,000	10
Lysine decarboxylase	2000	2100	0	1600	1450	9
S-adenosylhomocysteine nucleosidase (μmoles per hr per ml)	25	25	0	11	10.5	4

* All values are listed as μliters per hr per ml of H_2, O_2, and CO_2 released or consumed using appropriate substrate, except for *S*-adenosylhomocysteine nucleosidase. All enzyme activities were assayed as outlined in tables and text.

† Supernatant fluid from 20,000 × *g* centrifugation.

whereas glucose oxidase activity remained essentially unchanged. With the possible exception of formic oxidase, the degree of inactivation remained unchanged when the whole disruption product was assayed for various enzyme activities (Table 3).

Discussion

It is apparent from electron microscopy that most of the *E. coli* cells were ruptured at 15,000 psi in a Servall refrigerated cell fractionator and disruption was complete at 25,000 psi. With perhaps the exception of arginine decarboxylase, all the enzymes examined seemed to retain maximal activity when released at pressures of 25,000 psi. At higher pressures (35,000 to 55,000 psi), extreme variation in the stability of the various enzymes was noted. Enzymes such as arginine decarboxylase and formic hydrogenlyase may be completely destroyed, whereas lysine decarboxylase and glucose oxidase remained active. It would appear that the stability of an enzyme during fractionation at high pressures depends to some extent on the structure of the particular enzyme. Such stability might also be greatly influenced by the proteins associated with the enzyme either in solution or in a particulate state. Variations in stability might also occur with similar enzymes prepared from different organisms.

Differences in lability to fractionation pressures of various enzymes might well be used in separating enzymes which carry out similar reactions as well as in studying substrate specificity. For example, the pressure-labile enzyme arginine decarboxylase could be completely destroyed by disruption of the cell at 55,000 psi, leaving behind the more stable lysine decarboxylase.

The extreme heat created by friction due to the rapid reversal of pressure and velocity of the microbial suspension at the orifice was minimized by introducing prechilled nitrogen (−50 C). The possibility exists that local heat inactivation of proteins may occur, but this effect would appear to be negligible since extreme variation in the stability of the thermolabile decarboxylases (Gale, 1940) is noted. Lysine decarboxylase, which is more sensitive to heat than glutamic decarboxylase (Najjar and Fisher, 1954), is denatured very little when released at high pressure, whereas arginine decarboxylase, which is also more sensitive to heat than glutamic decarboxylase, is denatured to a greater degree. It would appear that in these experiments mechanical forces were primarily responsible for the denaturation of protein.

Acknowledgments

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