PREPARATION OF BACTERIAL ENZYMES BY CONTROLLED LYSIS

C. R. KRISHNA MURTI
Central Drug Research Institute, Lucknow (India)
(Received April 20th, 1960)

SUMMARY

1. Lysis of *Vibrio cholerae* and *Escherichia coli* under controlled conditions by Versene and lysozyme has been adapted to separate the cells into three major fractions.

2. Succinic oxidase, cytochrome oxidase and DPNH-cytochrome *c* reductase are located in the protoplast membrane or sediment.

3. TPN linked *iso*-citrate, G6P dehydrogenases, DPN-linked malic dehydrogenase, aldolase, aspartic acid deaminase, adenosine deaminase, histidase and *iso*-citritase activities are present in the lysates of the "protoplasts" obtained by this method.

INTRODUCTION

Extracts of bacteria for enzymic and antigenic analysis are usually prepared by rupturing the cells either by freezing and thawing, grinding with abrasives, extrusion under mechanical pressure or by exposure to high frequency sound waves.[1,2]. Intermixing of the constituents of the different anatomical structures of the cell such as the cell wall, the protoplast membrane and the cytoplasm is unavoidable in the use of these techniques. Preparations obtained by these procedures are, therefore, only of limited use in studies pertaining to localization or classification of enzymes and antigens on a morphological basis. Further, these drastic methods of cell disruption cause extensive damage to coupled enzyme systems[3]. A certain degree of denaturation of polymer constituents, resulting in the loss of their biological activity, is inevitable under these conditions.

The present study was undertaken with the object of devising a method free from the above limitations for the preparation of enzymically active cell-free extracts of bacteria. The results obtained with *Vibrio cholerae* and *Escherichia coli* indicate that controlled lysis by the combined action of metal complexing agents and lysozyme could be used to yield preparations suitable for localization and fractionation of enzymes.

MATERIALS AND METHODS

Organisms

Vibrio cholerae – Inaba-6-S^S and Inaba-6-S^R and *Escherichia coli* – M 133-3 were employed in this study.

Media

Nutrient agar (pH 7 for *E. coli* and pH 8 for *V. cholerae*) were used for the maintenance and propagation of the cultures.

Chemicals and reagents

Lysozyme (egg white, crystalline), diphospho- and triphospho-pyridine nucleotides (DPN and TPN) and the barium salts of glucose-6-phosphate (G6P) and hexose diphosphate (HDP) were procured from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U. S. A.; Versene from Eastman Organic Chemicals, Rochester, New York, U. S. A. and tris(hydroxymethyl)aminomethane (Light Chemical Company, Colnbrook, Bucks, England) was a gift from Dr. E. M. CROOK of the Biochemistry Department, University College, London. Dimethyl-d-*iso*-citrate lactone prepared originally by Dr. G. W. PUCHER from *Bryophyllum* leaves was kindly supplied by Dr. P. S. KRISHNAN, Professor of Biochemistry, Lucknow University, Lucknow, India. The ester was converted into the potassium salt of *iso*-citric acid by heating with a slight excess of 1 *N* potassium hydroxide in a water bath for 30 min and adjusting the pH of the resulting solution to 7 with 1 *N* hydrochloric acid. The barium salts of G6P and HDP were converted into their respective potassium salts by removal of barium as sulphate by the addition of the requisite amount of potassium sulphate.

Centrifugations were carried out in the Spinco ultracentrifuge Preparative Model L and optical density measurements were made in the Beckman spectrophotometer Model DU.

Enzyme assays were made by standard methods as indicated below:

Adenosine deaminase according to KORNBERG AND PRICER[4].

Aldolase according to SIBLEY AND LEHNINGER[5].

Aspartase according to IYER *et al*[6] or by following the increase in optical density at 300 mμ due to the formation of fumarate. This system was found to be valid for preparations free from fumarase.

DPNH-cytochrome reductase photometrically, using triphenyl tetrazolium chloride (TTC) as hydrogen acceptor[7]. The formazan was extracted and estimated according to SRIKANTAN AND KRISHNA MURTI[8].

Cytochrome oxidase manometrically using ascorbate as the reducing agent.

Histidase according to MEHLER AND TABOR[9].

G6P, *iso*-citrate and malic dehydrogenase spectrophotometrically at 25° following the increase in optical density at 340 mμ of reduced TPN and DPN. *Iso*-citritase according to OLSON[10]. Succinic oxidase manometrically. Protein concentration in extracts was estimated by the spectrophotometric method of WARBURG AND CHRISTIAN[11].

Preparation of cells

Harvesting and preparation of the cells for lysis was carried out by a method routinely employed in this laboratory[12].

Controlled lysis of cells

Freshly harvested and washed cells were suspended in 0.85 % sodium chloride to an opacity value corresponding to 10–20 % light transmission (Red filter 650 mμ)

in the Lumetron colorimeter. The saline suspension of cells was added to a mixture of Tris–hydrochloric acid buffer (pH 8), versene, lysozyme and sucrose. In a typical experiment the constituents in a total volume of 600 ml were: 200 mg versene, 40 mg lysozyme, 100 ml of 0.1 *M* Tris–HCl buffer, 160 ml of 2 *M* sucrose and 100 ml of cell suspension. The flask containing the above reaction mixture was incubated at 38° with gentle shaking for 2–3 h. Within this period the majority of cells are usually found to assume spherical shapes when viewed through a high power microscope. The suspension was left overnight at 5–8°. The subsequent operations are summarized in Fig. 1. The term "protoplasts" is used here in the limited sense as applicable to Gram-negative organisms[13].

Extensive lysis occurred on suspending the "protoplasts" in chilled water yielding a very viscous suspension. High speed centrifugation of this suspension was facilitated by exposing the lysate for 2–5 min to sound waves (25 kc/sec with an output amperage of 2.5 generated in a Mullard Magnetostrictor Oscillator).

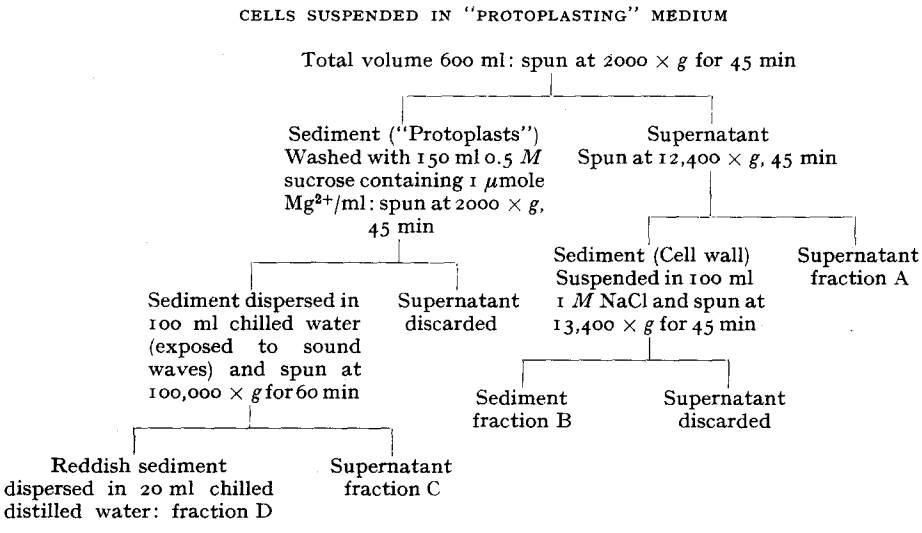

Fig. 1. A scheme for the fractionation of bacterial cells by lysis.

RESULTS

The four fractions (A, B, C and D) resulting from the controlled lysis of the cells were assayed for enzyme activity and their optical densities were read over the range 230 to 300 mμ after suitable dilution. Fraction A showed no absorption of u.v. light, fraction B exhibited a high absorption at 230 mμ and a sharp decline in absorption thereafter. Absorption curves of C and D were humped in the range 250–265 mμ characteristic of nucleoproteins. Fractions A and B were devoid of all the enzyme activities tested. Fraction D showed considerable activity with respect to cytochrome oxidase, succinic oxidase and DPNH cytochrome reductase but none of the other enzymes. Fraction C while not possessing the above activities was rich, however, in adenosine deaminase, aspartase, aldolase, histidase and the pyridine nucleotide linked dehydrogenases.

Enzyme activities of fraction D

Succinic oxidase and cytochrome oxidases: The results of the assay of these two enzymes are given in Table I. These observations support the earlier findings of SAGAR *et al.*[14] that the succinic oxidase activity of *V. cholerae* is localized mainly in the particulate fraction.

TABLE I

SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITIES OF FRACTION D

	Enzyme protein (mg/flask)	*μl oxygen uptake in 10 min*	
		Succinic oxidase	*Cytochrome oxidase*
Inaba-6-S[S]	12.8	14.8	26.8
Escherichia coli	8.4	17.2	64.0

Flask contents: succinic oxidase: 100 μmoles phosphate buffer (pH 8 for *V. cholerae* and pH 7.2 for *E. coli*); 10 μmoles Versene (pH 7.5), cytochrome *c* 100 μg; 50 μmoles potassium succinate (pH 7.5) in the side arm. 0.2 ml 10% KOH in the centre well with a square filter paper plug. Gas phase: air. Equilibrated for 15 min at 38°, succinate added, stopcocks closed and readings taken at 10 min intervals for 1 h. Cytochrome oxidase: 100 μmoles phosphate buffer (pH 8 for *V. cholerae* and pH 7.2 for *E. coli*); 33 μmoles sodium ascorbate (pH 7.5) in the side arm and 1 mg cytochrome *c*; total volume 3 ml. 0.2 ml 10% KOH in centre well with square filter paper, plug. Gas phase: air. Equilibrated for 15 min at 38°, ascorbate added, stopcocks closed and readings taken at 10 min intervals for 1 h.

DPNH-cytochrome c *reductase*: In a typical assay the reaction mixture contained 1 ml of sodium pyrophosphate buffer 0.1 *M*, pH 8, 0.5 ml of 0.5% TTC, 0.3 μmole DPNH and 8.0 mg enzyme protein in a total volume of 3.0 ml. The mixture was incubated aerobically at 38° for 30 min and reaction stopped by adding simultaneously 5 ml each of glacial acetic acid and toluene. The tubes were shaken and the layers allowed to separate. The toluene layer containing the formazan colour was pipetted out for optical density measurements. The optical density of the formazan at 495 mμ from the reaction tube containing DPNH, enzyme and TTC was 0.08. No colour was produced in the control tubes containing only DPNH and TTC or TTC and enzyme.

Enzyme activities of fraction C

Adenosine deaminase: Reaction mixtures consisting of 100 μmoles of Tris–HCl buffer (pH 8), 10 μmoles Mg^{++} as the chloride, 10 μmoles of adenosine and 0.2 ml of fraction C (750 μg protein) in a total volume of 2 ml were incubated at 38° for 30 min after which 2 ml 10% trichloroacetic acid were added. To the control tube, 10 μmoles of adenosine were added immediately after the addition of the trichloroacetic acid. The tubes were spun at 1800 *g* for 30 min and the optical densities of the supernatants read at 255 mμ after diluting 100 fold with distilled water. The O.D. values for the control and experimental tubes were 0.375 and 0.015 respectively indicating that over 95% of the adenosine had been deaminated by the enzyme in 30 min.

Aspartic acid deaminase: Reaction mixtures consisting of 100 μmoles of Tris–HCl buffer (pH 8), 10 μmoles of Mg^{2+} as the chloride, 20 μmoles of DL-aspartic acid (pH 7.5), 0.2 ml fraction C (750 μg protein) in a total volume of 2 ml were incubated at 38° for 60 min and ammonia determined in the filtrate obtained by deproteinization

with an equal volume of 10 % trichloroacetic acid. 58 μg of ammonia-N were liberated in the experimental tube accounting for about 40 % deamination of the substrate.

HDP-aldolase: Results of the aldolase assay of fraction C of *Vibrio cholerae* and *Escherichia coli* are given in Table II from which it can be seen that apart from possessing considerable HDP-alodolase activity, the extracts are apparently activated

TABLE II

HDP-ALDOLASE ACTIVITY OF FRACTION C OF *Vibrio cholerae* AND *Escherichia coli*

	Inaba-6-S^S	*Inaba-6-S^R*	*A. coli*
Without any activator	183	186	162
With 10 μmoles cystein-HCl	705	710	786

Results are expressed as Klett units (540 mμ) of the coloured dinitrophenyl hydrazones of the trioses formed in 1 ml filtrate. Reaction mixture: 100 μmoles Tris–HCl buffer pH 8.6; 140 μmoles hydrazine sulphate (pH 8.6); 6.5 μmoles HDP and 750, 720 and 1200 μg respectively of the enzyme protein in In-6-S^S, In-6-S^R and *E. coli* in a total volume 2.5 ml.

by cysteine. This is in conformity with observations on *V. cholerae*, *Pasteurella pestis*, and *Salmonella typhosa* reported earlier from these laboratories[15–17].

Histidase: Silica cuvettes (1 cm optical path) contained in a total volume of 3 ml, 100 μmoles phosphate buffer (pH 8), 5 μmoles reduced glutathione, 10 μmoles DL-histidine and 660 μg enzyme protein (Inaba-6-S^S). Increase in optical density at 277 mμ was followed against a reference cell containing all the reactants excepting histidine. The optical density reached a maximum value of 1.1 within this period. Specific activity of the extract was found to be 45 units/mg protein (1 unit of enzyme is the amount that causes an increase in O.D. at 277 mμ of 0.001/min[9]).

Iso-citritase: 3 ml of reaction mixture in silica cuvettes contained, in μmoles, cysteine-HCl 6; semicarbazide-HCl, 60; Mg^{2+} as the chloride 16; phosphate buffer (pH 8) 200; *iso*-citrate 10 and enzyme protein 280 μg. The increase in O.D. at 252 mμ was followed. The reaction started after a lag period of 6 min and the O.D. reached a value of 0.35 in 85 min giving a specific activity of 9 units (1 unit is the amount

TABLE III

DEHYDROGENASE ACTIVITIES OF FRACTION C

Organism	*Substrate (10 μmoles)*	*Enzyme protein in test system (μg)*	*DPN (μmoles)*	*TPN (μmoles)*	*Specific activity (units)*
In-6-S^S	Malate	750	0.3	—	130
Escherichia coli	Malate	1200	0.15	—	75
In-6-S^S	G6P	750	—	0.25	86
In-6-S^R	G6P	800	—	0.125	28
In-6-S^S	*Iso*-citrate	375	—	0.25	240
In-6-S^R	*Iso*-citrate	400	—	0.125	140

1 unit of enzyme is the amount that causes the increase in O.D. at 340 mμ of 0.001 in 1 min. Reaction mixture: 150 μmoles Tris–HCl buffer (pH 8 for *V. cholerae*; 7 for *E. coli*) substrate and enzyme as indicated in table; 10 μmoles of Mg^{2+} as the chloride in a total volume of 3 ml. Reaction started by the addition of the co-enzyme. The test system for malic dehydrogenase included 50 μmoles cyanide (pH 8) in addition to the above constituents.

of enzyme that causes an increase in O.D. at 252 mμ of 0.01 in the first 5 min after reaction started). Indirect evidence for the presence of this enzyme and hence the glyoxalate shunt in *V. cholerae* has been reported elsewhere on the basis of the isolation of glyoxalate as a dinitrophenylhydrazone identified chromatographically[18].

Dehydrogenases: Results of the assay of *iso*-citrate-TPN, G6P-TPN and malic acid-DPN dehydrogenases are summarized in Table III.

DISCUSSION

The method of lysis under controlled conditions as devised in this study makes it possible to effect, by a comparatively gentle technique, the separation of Gram-negative bacteria into three major components: *viz.*, a major part of the cell wall devoid of any enzyme activity, the "protoplast" membrane together with other 100,000 *g* sedimentable particles and a supernatant. Three of the pyridine nucleotide linked dehydrogenases, aspartase, adenosine deaminase, histidase and *iso*-citritase activities of *Vibrio cholerae* are present in the supernatant. Cytochrome oxidase, succinic oxidase and DPNH-cytochrome *c* reductase are, on the other hand, associated with the membrane fraction. Although only a few enzymes have been investigated in the present study, the distribution of their activities in the membrane fraction and the supernatant resembles broadly the distribution pattern reported for mitochondrial and supernatant fractions of rodent liver homogenates[19].

The membrane fraction obtained by controlled lysis is likely to retain part of the cell wall and also to be contaminated with cytoplasmic inclusions sedimentable at 100,000 *g*. By differential centrifugation this fraction can be resolved further into components of decreasing particle size. Similarly by the use of conventional techniques such as ammonium sulphate fractionation and calcium phosphate gel adsorption and elution or ion-exchange chromatography, the supernatant can be separated into individual enzymes.

Exploratory immunochemical analysis by gel diffusion technique (S.B. MISRA and D. L. SHRIVASTAVA, unpublished observations) has revealed that the cell wall fraction, the lysate and the lysate sediment give rise to precipitin lines with *Vibrio cholerae* anti-sera. While the cell wall fraction gives only one line, fractions C and D produce multiple lines. Also fraction D is found to give rise to a line corresponding to the one shown by the cell wall fraction. This is in conformity with the findings of other workers that the "protoplast" membrane of Gram-negative organisms retains part of the cell wall[20].

Although lysozyme was used by PENROSE AND QUASTEL[21] as early as 1930 to compare activities of certain enzymes of intact cells and extracts of *Micrococcus lysodeikticus*, techniques based on controlled lysis have not been employed hitherto for enzyme localization. Some attempts have, however, been made recently to locate in the lysates of *Escherichia coli* protoplasts[22], lysates of *Bacillus megatherium* protoplasts[23] and the 20,000 *g* sedimentable membrane fraction of *Alcaligenes faecalis*[24], the cellular activity that normally mediates the incorporation of labelled amino acids into structural proteins. Likewise over 90 % of the oxidative activity of *Staphylococcus aureus* is associated with the plasma membrane fraction[25]. STANIER[26], on the basis of WEIBULL's findings[27] suggests that the bacterial cytochrome system, along with other enzymes, may be a built in part of the cell membrane. The observations

tend to show that corresponding to the well-known differentiation of tissues with respect to function in higher organisms, discrete compartments of the bacterial cell are presumably equipped with enzymic mechanism to mediate specialized functions such as electron transport, oxidative phosphorylation or protein synthesis[28]. The importance of obtaining further insight into the structural organization of the cell from the point of view of drug action has been emphasised[29]. It is in this context that the practical value of the technique of controlled lysis as suggested in the present study assumes added significance.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. B. MUKERJI, Director and Dr. D. L. SHRIVASTAVA, Deputy Director of this Institute for their sustained interest in his work. His thanks are also due to ASHOK SEN GUPTA and SWARAN SINGH for able technical assistance.

REFERENCES

1 W. B. HUGO, *Bacteriol. Revs.*, 18 (1954) 87.
2 R. H. BURRIS, *Manometric Techniques*, Burgess Publishing Co., Minneapolis, U.S.A., 1958, p. 156.
3 C. G. NOZZOLILLO AND R. M. HOCHSTER, *Can. J. Microbiol.*, 5 (1959) 471.
4 A. KORNBERG AND W. E. PRICER, *J. Biol. Chem.*, 193 (1951) 481.
5 J. A. SIBLEY AND A. L. LEHNINGER, *J. Biol. Chem.*, 177 (1949) 859.
6 S. N. IYER, A. DUDANI, C. R. KRISHNA MURTI AND D. L. SHRIVASTAVA, *J. Sci. Ind. Research (India)*, 12B (1953) 316.
7 A. F. BRODIE AND J. S. GOTS, *Science*, 116 (1952) 588.
8 T. N. SRIKANTAN AND C. R. KRISHNA MURTI, *J. Sci. Ind. Research (India)*, 14C (1955) 295.
9 A. H. MEHLER AND H. TABOR, *J. Biol. Chem.*, 201 (1953) 775.
10 J. A. OLSON, *J. Biol. Chem.*, 234 (1959) 5.
11 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 303 (1939) 40.
12 C. R. KRISHNA MURTI AND ASHOK SEN GUPTA, *J. Sci. Ind. Research (India)*, 19C (1960) 83.
13 K. MCQUILLEN, *Proceedings of the IVth International Congress of Biochemistry*, Pergamon Press, London, 1959, p. 406.
14 P. SAGAR, S. C. AGARWALA AND D. L. SHRIVASTAVA, *Experientia*, 15 (1959) 220.
15 C. R. KRISHNA MURTI AND D. L. SHRIVASTAVA, *J. Sci. Ind. Research (India)*, 15C (1956) 125.
16 T. N. SRIKANTAN, C. R. KRISHNA MURTI AND D. L. SHRIVASTAVA, *India J. Med. Research*, 46 (1958) 1.
17 S. GHATAK AND D. L. SHRIVASTAVA, *Enzymologia*, 19 (1958) 237.
18 C. R. KRISHNA MURTI, *Biochem. J.*, 76 (1960) 362.
19 M. DIXON AND E. C. WEBB, *Enzymes*, Longman Green and Co., London, 1958, p. 630.
20 M. R. J. SALTON AND F. SHAFA, *Nature*, 181 (1958) 321.
21 M. PENROSE AND J. H. QUASTEL, *Proc. Roy. Soc. (London)*, 107B (1930) 168.
22 B. NISMAN, *Biochim. Biophys. Acta*, 32 (1959) 19.
23 J. A. V. BUTLER, A. R. CRATHORN AND G. D. HUNTER, *Biochem. J.*, 69 (1958) 544.
24 M. BELJANSKI AND S. OCHOA, *Proc. Nat. Acad. Sci. U.S.*, 44 (1958) 494.
25 P. MITCHELL AND J. MOYLE, *Biochem. J.*, 64 (1956) 19p.
26 R. Y. STANIER, *Cellular Metabolism and Infections*, New York, 1954, p. 23.
27 C. WEIBULL, *J. Bacteriol.*, 66 (1953) 688.
28 P. MITCHELL, *Biochem. Soc. Symposia (Cambridge, Engl.)*, 16 (1959) 73.
29 P. MITCHELL, *Symposium Soc. Gen. Microbiol.*, 8 (1958) 94.