II. MATERIALS AND METHODS

CHAPTER-II

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CELL-CULTURE:

The organism Streptococcus faecalis-Strain-R (ATCC-8043), used in this study was obtained from Division of Biochemistry, National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona-8 (India).

The organism was grown at 37°C on a medium having the following composition: D-glucose, 0.25 g; Bacto-tryptone, 1.7 g; sodium chloride, 0.5 g; Dipotassium hydrogen phosphate, 0.25 g; Yeast extract, 0.4 g and Agar powder, 3.0 g per 100 ml distilled water. The pH of the medium was adjusted to 7.4 and autoclaved at 15 lbs pressure for 20 minutes. The organism was maintained by subculture every fortnight.

CELL PREPARATION:

(a) Acetone Dried Cells:

The method followed was that of Gunsalus (1955). A thick aqueous cell suspension of S. faecalis-R was added slowly and with vigorous stirring to 10 volumes of anhydrous acetone - previously chilled to -20°C. After

brief stirring, the cells were allowed to settle down, the supernatant decanted and the residual solvent removed on a Buchner funnel using Whatman No.1 filter paper. The filter cake was washed twice with five volumes of chilled acetone and sucked dry applying gentle vacuum pressure. The dry cell cake was transferred alongwith the filter paper to a desiccator in the presence of liquid paraffin to absorb the excess solvent. This is then transferred to another desiccator and dried over silica gel. This dried cell cake was ground and preserved in a vacuum desiccator at 5 to 10°C.

(b) Slow Vacuum-Dried Cells:

The method followed was according to Gunsalus (1955) in which a thick aqueous cell slurry was transferred to the lid of a petri dish and placed in a desiccator over anhydrous calcium chloride. The desiccator was then subjected to vacuum, sealed and allowed to stand overnight. After 24 to 48 hours, the dried cells were removed, powdered and preserved in the desiccator.

CELL RESPIRATION:

Respiration of Streptococcus faecalis-R cells was followed in the conventional Warburg manometer at 37°C with air as the gas phase (Umbreit et al, 1957). Usually 0.5 ml of washed cell-suspension was used per flask.

The cell concentrations were determined by diluting 0.5 ml of a stock cell-suspension to 10 ml volume. The diluted suspension gave a reading of 150 in Klett Summerson Colourimeter using No.54 filter (540 nm).

DETERMINATION OF ENZYME ACTIVITIES IN S.FAECALIS-R EXTRACTS:

Enzyme Extracts:

All the enzyme activities were determined from the crude dialysed enzyme extracts. Enzyme extracts were prepared either from acetone dried or slow vacuum-dried cells of S.faecalis-R by mixing a weighed amount of dried cells (usually 10 mg/ml) in the desired buffer or water. Extraction was carried out at 37°C in a waterbath for 20 to 30 minutes by gentle stirring with a glass rod.

When slow vacuum-dried cells were used, the extraction was followed first by grinding the cells with buffer or water in a glass mortar and pestle and the extraction allowed to proceed at 37°C for 20 to 30 minutes.

When studying NADH-Oxidase or Diaphorase enzyme activities, the slow vacuum-dried cells were first ground with acid-washed glass powder in an icebath and then the extraction was carried out as before. The

cell-extract was cooled and the supernatant obtained after centrifugation at 3,500 rpm for 20 minutes was dialysed in the cold against distilled water or weak buffer, 5 X 10^{-3} M.

Usually 0.2 ml of the enzyme extract was used per 3 ml of reaction mixture. Conditions of extraction and the amount of extracts employed are given under Results, in each case of enzyme activity determined.

Following enzyme activities were determined:

(1) Fructose Diphosphate (FDP) Aldolase:

(EC.4.1.2.13; Fructose-1,6-diphosphate:D-glyceraldehyde-3-phosphate-lyase).

FDP aldolase assay was carried out by estimating the hydrazones according to the modified method of Sibley and Lehninger (1949; see also Beck, 1957) and also by determining the alkali labile phosphate of triose phosphate formed in the reaction (Herbert et al, 1940).

Prior to its addition, the substrate FDP was freed of Barium by treatment with sodium sulphate.

(2) Glyceraldehydephosphate (GAP) Dehydrogenase:

(EC.1.2.1.12; D-Glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating)).

The activity of GAP-dehydrogenase was carried out using slow vacuum-dried cell extracts by the method

of Krebs (1955) with FDP as the substrate in presence of arsenate and measuring the increase in O.D. at 340 nm due to the reduced NAD formed in the reaction.

(3) Enolase:

(EC.4.2.1.11; 2-phospho-D-glycerate hydro-lyase).

Enolase activity in cell extracts was followed spectrophotometrically at 240 nm (Warburg and Christian, 1941) using 3-phosphoglycerate (PGA) as substrate and acetone-dried cell extract. Wherever SRC-820 was present, the released phosphate from phosphoenopyruvate (PEP) by alkaline hypoiodide (Meyerhof and Oesper, 1949) was estimated by adapting the method of Simonsen et al (1947). In this method, the released phosphate is precipitated first as the ammonium magnesium phosphate complex, from which the phosphate is released by acid vanadate. The resulting yellow colour upon treatment with ammonium molybdate was estimated at 420 nm (Simonsen et al, 1947; Cf. Grisolia, 1962). Recourse to this method had to be taken instead of the direct spectrophotometric method since SRC-820, the inhibitor used in these studies gave a high O.D. and also interfered in the Fiske-SubbaRow method (Fiske and SubbaRow, 1925).

(4) Glucose-6-phosphate (G-6-P) Dehydrogenase:

(EC.1.1.1.49; D-glucose-6-phosphate: NADP oxidoreductase).

The assay of G-6-P-Dehydrogenase was carried out with slow vacuum-dried cell extracts by the method of Kornberg and Horecker (1955). Reaction was followed both spectrophotometrically (NADPH-formation) and colourimetrically using 2,6-dichlorophenolendophenol (DCIP) as the electron acceptor, according to the method of Mahler (1955).

(5) NADH-Oxidase and Diaphorase:

Activities of NADH-Oxidase and Diaphorase were determined spectrophotometrically using slow vacuum-dried cell extracts by the method of Ciotti and Kaplan (1957). 2,6-dichlorophenolendophenol dye was followed measuring at 600 nm.

The initial concentration of NADH was determined from its optical density at 340 nm.

ESTIMATION OF SRC-820:

A colourimetric method for the estimation of SRC-820 was devised. Preliminary acid hydrolysis of the compound SRC-820, followed by diazotization and coupling with β-naphthol was carried out. The yellow-orange colour of the diazoderivative was measured at

340 nm in 'UVISPEK' spectrophotometer. The details of this method are given in Chapter-III.

EXTRACTION OF SRC-820 FROM THE SERUM:

Known quantities of SRC-820 (20-80 µg) in a volume of 0.2 ml were added to 0.8 ml of normal human serum separately and the serum was extracted twice with chloroform (5 ml and 3 ml). The separated chloroform layer was mixed and then treated with 2 ml of 0.05 N sodium hydroxide and the chloroform layer siphoned off and dried over anhydrous sodium sulphate. A known aliquot (4 ml) of the chloroform layer was evaporated and subjected to acid hydrolysis, diazotization and coupling with β-naphthol as described in the Chapter-III. Suitable controls without added SRC-820, blanks and standards were run under identical conditions. Recoveries were then calculated.

Serum pH, after addition of known amounts of SRC-820 was adjusted to a range of values between 6 to 8 and extracted as described above. The influence of pH on the recovery of added SRC-820 was then determined.

IODOACETATE (Benzene treatment):

The stock aqueous solution of iodoacetate (10 µM/ml) was extracted once with an equal volume of benzene ('Analar' grade). On extraction, upper benzene

layer turned violet in colour, which was discarded. The lower colourless solution was kept in ice and used after neutralization and dilution.

MEASUREMENT OF CELL GROWTH:

The growth of S.faecalis-R was measured turbidimetrically in Klett-Summerson photoelectric colourimeter using filter No.54. The growth was also measured by determination of acid production and for this, a known volume of broth culture was titrated against 0.01 N NaOH (Hayaishi, 1955).

Growth inhibition studies were carried out in five times diluted liquid medium. The required amounts of solid SRC-820 or solid methaqualone hydrochloride were added to the flasks containing diluted broth and then the pH was adjusted to 7.4 by means of weak NaOH before sterilization. The pH adjustment was required in the case of methaqualone only since its hydrochloride salt was used. The liquid medium containing the solid inhibitors was autoclaved at 15 lbs pressure for 20 minutes. After autoclaving, SRC-820 or methaqualone was found to dissolve in the medium. The highest possible amount of SRC-820 or methaqualone that could be solubilized in the medium by this method was found to be 30 µM/ml. Above this concentration, inhibitor (particularly SRC-820) used to reprecipitate out of the medium on cooling, and

such concentrations were not used in this study. UV spectrum of SRC-820 or methaqualone after autoclaving was found to remain unchanged. Control flasks did not contain the inhibitors.

Cells were allowed to grow at 37°C and the growth was followed upto 6 to 8 hours during which the growth attained - a stationary phase.

Six ml aliquots were withdrawn asceptically at different time intervals and the readings were taken against a water blank. The same aliquot was quickly titrated for the acid production by means of 0.01 N NaOH using phenolphthalein (1.0% W/V in 95% ethanol) as an indicator.

Zero time readings for both turbidity as well as acid production were taken and used for calculating the increase in turbidity or acid production.

PREPARATION OF REDUCED SRC-820:

Reduction of SRC-820 was achieved with nominal amounts (60 mg/ml) of lithium borohydride ($LiBH_4$) in tetrahydrofuran or anhydrous ether similar to the procedure of Brown and Smart (1969).

THIN LAYER CHROMATOGRAPHY (TLC):

TLC of acid hydrolysate of SRC-820 was carried out on a glass plate (20 X 20 cm) coated with silica gel G

(E.Merck) of 0.25 mm thickness. The plates were dried for 30 minutes at 110°C in a hot air oven. Plates were charged with ether extracts of acid hydrolysate (after neutralization) and they were allowed to run in a solvent system - n-butanol:glacial acetic acid:water:: 4:1:5 at room temperature (25°C).

In case of preparative TLC, the gel thickness was 0.5 mm and the two identical TLC plates were allowed to run simultaneously for the exact period of time.

In the preparative TLC experiments, each mg of SRC-820 was hydrolysed with 5 ml of 4.5 N sulphuric acid for 1 hour in a boiling waterbath and after cooling, this was first extracted five times with amyl alcohol, each time using a volume of 0.8 times that of the hydrolysate. The separated amyl alcohol layers were pooled and concentrated under reduced pressure at 50°C and used for charging the TLC plates as well as determining UV absorption studies. The residual aqueous layer was neutralized with solid sodium carbonate (Na_2CO_3) and extracted four times with solvent ether, each time using the 0.8 times that of the neutral aqueous phase. The ether layer was evaporated in a waterbath (40°C) to get the concentrated residue which was subjected to TLC.

Detection of the components were achieved by scanning the plates under ultraviolet light and/or spraying them with Dragendorff's reagent (Block et al, 1958).

Elution of the spots were accomplished with 95% ethanol. Equivalent amounts of uncharged silica gel treated in the identical way was used as the blank.

All reagents and chemicals used in these studies were of reagent grade, those used in culture medium were products of Difco Laboratories.

SRC-820, Methaqualone-HCl, Quinazoline-4-One, were obtained from Sarabhai Research Centre, Baroda (India).

3-PGA were obtained from Sigma.

G-6-P, FDP, NAD and NADP were the products of Biochemicals Unit, V.P.Chest Institute, Delhi-7 (India).

All solutions were brought to desired pH by addition of either dilute NaOH or HCl.