V. EXPERIMENTAL

Materials

Animals – For urethane-protein interaction and cell concentration studies an inbred strain of adult Wistar albino rats were obtained from Nutritional Research Laboratory, Hyderabad. For mitochondrial swelling and enzyme studies an inbred strain of Wistar albino rats were purchased locally.

Rats susceptible to urethane carcinogenesis were obtained from Haffkin Institute, Bombay. Wistar albino rats bearing Yoshida Sarcoma (Ascites) cells were obtained from Cancer Research Institute, Bombay. All animals were fed ad libitum on Hindleaver Animal Feed.

Chemicals – Special chemicals obtained from commercial sources included:

- Urethane-(carbony 1-C\textsuperscript{14}) sp. activity, 31.4 mc/mM (Shwartz Bio Research, U.S.A.);
- L-Histidine-(ring-2-C\textsuperscript{14}) sp. activity, 35 mc/mM;
- L-Methionine-(methyl-C\textsuperscript{14}) sp. activity, 29.5 mc/mM;
- L-Threonine (l-C\textsuperscript{14}) sp. activity, 6.0 mc/mM;
- L-Phenylalanine (l-C\textsuperscript{14}) sp. activity, 170 mc/mM. (Radiochemical Centre, U.K.);
- Algal Protein Hydrolysate-UL-C\textsuperscript{14}, sp. activity, 54 mc/mAtom of carbon (Philips-Duphar, Holland);
- Cytochrome C and ATP (Biochemical Unit, V.P. Chest Inst., Delhi);
- Thioacetamide and Urethan, (E. Merck, Germany);
- Folin-Ciocalteu Reagent (BDH, India).
Methods

Isolation of Nuclei and Mitochondria from Lung, Liver and Kidney Tissues:

The animals were sacrificed by giving a blow on the head. The tissues were excised, pooled in cold 0.25 M sucrose and homogenized in a pre-cooled all glass or teflon pestle Potter-Elvehjem homogenizer. All manipulations were made at 4-5°C. The homogenate was collected and passed through a 200 mesh sieve to remove connective tissues etc. It was centrifuged at 600 x g in a MSE refrigerated centrifuge. Mitochondria and nuclei were isolated from supernatant and sediment respectively. In case of rat liver mitochondria Janetzki (Model K50) refrigerated centrifuge was used.

(i) Mitochondria: The supernatant was carefully collected and recentrifuged at 600 x g to eliminate any possible nuclei contamination. The resulting supernatant was centrifuged for 30 minutes at 10,000xg. The supernatant was rejected and the sediment containing mitochondria was washed twice with 0.25 M sucrose and finally taken in the required medium.

(ii) Nuclei: The pellet from first centrifugation at 600 x g, containing whole cells, cell debris and nuclei was dispersed mechanically at a very low speed in a teflon pestle homogenizer which contained 2.4 M sucrose in $1.5 \times 10^{-3}$ M CaCl$_2$. After centrifugation at 40,000 x g for
60 minutes in a SW 25.1 rotor of a Spinco (model L) Preparative Ultracentrifuge, the nuclei formed a thick pellet at the bottom of the centrifuge tube. The nuclei pellet was re-dispersed in 2.4 M sucrose containing 1.5 x 10^{-3} \text{M} \ \text{CaCl}_2 \text{ and again centrifuged at 40,000 x g for 60 minutes. To ascertain the purity of the nuclei preparation, a sample of the nuclei pellet was checked under a phase contrast microscope (x 80). If necessary it was resuspended and recentrifuged at 40,000 x g for 60 minutes.}

**Measurement of Swelling and Contraction of Mitochondria** - The swelling and contraction of rat liver mitochondria was measured according to the method of Lehninger (1959) by following the decrease and increase in absorbance at 520 nm respectively in a Bausch and Lomb Spectronic 20 spectrophotometer. For inorganic phosphate, thiocetamide and urethan induced swelling, the temperature was maintained at 20-22°C, whereas for spontaneous swelling experiments it was kept at 37°C. The protein content of mitochondria was estimated by Lowry (1951) method.

**Assay of Succinate-Cytochrome C Reductase** - Using succinic acid and cytochrome C as electron donor and acceptor respectively, mitochondrial succinate-cytochrome C reductase was assayed manometrically (Schneider and Potter 1943).

**Preparation of Hepatic Cells in Suspension** - It was prepared according to the method of Jacob and Bhargava (1962). The method consists of perfusion
of the liver with 30-50 ml of cold 2.7 x $10^{-2}$ M sodium citrate in calcium-
free Lock's solution (Dawson et al., 1959), followed by dispersion in
0.25 M sucrose in a specially designed tissue disperser which is a modified
Potter-Elvehjem homogenizer having a soft rubber pestle. The dispersate
was filtered through a 200 mesh sieve and centrifuged in a MSE refrigerated
centrifuge for 10 minutes at 200 x g. The sediment of the single paren-
chymal cells was resuspended in cold Ca$^{++}$-free KRP buffer (Dawson et al., 1959
Preparation of Yoshida Sarcoma (ascites) Cells - Ascites cells drawn out
with the help of a syringe from the peritoneal cavity of rats were collected
in cold normal saline solution. They were spun down at a low speed in a
refrigerated centrifuge and supernatant containing blood cells was discarded.
The cells were repeatedly washed with cold normal saline till all the blood
cells were removed and a pure preparation of ascites cells was obtained.
These cells were once again washed with Ca$^{++}$-free KRP buffer and suspended
in the same medium.
Cell Count - The concentration (number of cells/ml) of parenchymal cells
and Yoshida sarcoma cells was determined on a haemacytometer (100 μ deep
having a graduated area of 9 mm$^2$ divided into 9 major squares each with an
area of 1 mm$^2$) under phase contrast microscope (x 80).
Isolation and Estimation of Protein and Total Free Amino Acids in Hepatic Cells in Suspension and Yoshida Sarcoma Cells - At the end of incubation period the samples were chilled in ice, centrifuged and the supernatant rejected. The pellet was repeatedly washed with cold Ca\textsuperscript{++}-free KRP buffer to remove all the radioactivity in the medium. The washings were monitored for their radioactivity till no counts were detected. For complete precipitation of proteins the cells were precipitated by 10\% TCA (w/v) and kept in cold for 20 minutes. The precipitate was centrifuged and the supernatant was kept for free amino acid estimation.

The precipitate was washed twice with 5\% TCA. These washings were combined with the first one for free amino acid estimation. The precipitate was then heated in 5\% TCA for 30 minutes at 90\(^\circ\)C to hydrolyse the nucleic acids and washed twice with 5\% TCA again. Lipids were removed by washing twice with 50\% alcohol, twice with 100\% alcohol, twice with alcohol-ether (3:1) and finally twice with ether. The protein residue obtained was used for estimation of radioactivity.

The pooled TCA washing collected earlier was shaken with ether till all TCA was removed as checked by pH paper. The aqueous solution which now contained only amino acids and other low molecular weight subst-
ances was passed through a column of Zeo-karb 225 resin (150-200 mesh, hydrogen form, 0.2 x 12 cm). Under these conditions only amino acids are known to be retained by the resin. The amino acids were eluted by 2 N aqueous ammonia. The eluent (10-20 ml) was evaporated to dryness under vacuum. The residue was dissolved in 1 ml of water and aliquotes from this were taken for the estimation of radioactivity and amino acids. This method is essentially the same as followed by Bhargawa et al (1959).

Estimation of Free Amino Acids and Protein - Free amino acids were estimated by Ninhydrin photometric method of Moor and Stein (1948) taking leucine as standard. Protein was estimated either by Lowry's (1951) method or expressed as dry weight.

Paper Chromatography of Amino Acids - Free amino acids obtained from hepatic cells were separated by two-dimensional chromatographic method using n-butanol-acetic acid-water (4:1:5) and phenol-ammonia (200:1). The spots were developed with Ninhydrin, cut into pieces and the amino acids eluted with 60% alcohol. Radioactivity was determined for each amino acid spot.

Radioactivity Assay - The total tissue proteins obtained as described above were plated as a slurry in 30% alcohol on 2 sq. cm. aluminium planchets. The planchets were dried under vacuum. The radioactivity was measured in a
Philips Geiger–Muller counter of a Tracerlab and window Geiger Muller counter. All samples were counted to a statistical significance of 5% as recommended by Calvin et al. (1949). Appropriate correction for self-absorption was made wherever necessary.

**Calculations of Dry Weight** - Dry weight of hepatic cells in suspension were calculated according to the unpublished results of Hussain, L.F., Gopinath B and Bhargava, P.M. where $1 \times 10^6$ hepatic cells of 4 month old rat weighing about 150-160 gms are equivalent to 0.82 mg dry weight. The dry weight of perfused and unperfused rat liver slices were calculated according to Ipe, P.T., Bhargava, P.M. and Taskar, A.D. (1965).