MATERIAL AND METHODS

(a) Hydra culturing

A steady culture of *Hydra vulgaris* was maintained in the laboratory following the method described by Loomis and Lenhoff (1956). The larvae of brine shrimp (artemia) hatched in the laboratory were used as the food for the organisms. In order to obtain a homogenous culture, buds of equal age and size were separated from the mother culture and grown by feeding with equal number of nauplii larvae. Upon attaining sufficient health, hydras of equal age without bud, testis or ovary were selected and any organism bearing incipient bud or testis which was not detected earlier, if found in the preparations was discarded from the experimental series. For incorporation studies, single hydras have been used each time. The organisms used for experiments were starved for 24 hours. All the experiments were conducted at a constant temperature of 22°C ± 1°C.

(b) Labelling and extraction procedures

RNA and protein syntheses were measured by the incorporation of $\text{H-uridine (5 } \mu\text{Ci/ml) and } ^{14}\text{C-isoleucine (1 } \mu\text{Ci/ml) into TCA (trichloroacetic acid) precipitable material. Before amputations, the hydras were placed in the medium containing radioactive material. With the help of a microsyringe a}
Little of the radioactive medium was injected into the organisms also. The duration of labelling varied according to different experiments. Before extraction, the animals were washed twice in media lacking the radioisotope precursor and homogenized in an Elvehjem glass homogenizer (Duall 20) in 1 ml of 0.03 M Na₂HPO₄, 0.035 M Tris, 0.001 M EDTA, pH 7.6. The samples were chilled and 100 μg of unlabelled RNA was added as carrier. The homogenate was precipitated by addition of an equal volume of cold 10% TCA (in the case of proteins, 20% TCA was used). RNA was hydrolyzed in 2 ml of 0.5 N KOH at 37°C and after cooling the RNA was precipitated with 0.2 ml of 60% PCA (perchloric acid) at 0°C for 20 minutes. The precipitates were collected on Millipore filters, washed several times with 5 ml of cold 5% TCA and once with 5 ml of absolute ethanol-ether (3:1). The filters were dried at 60°C for 1 hour and then counted by a Packard Tri Carb liquid scintillation spectrometer in a toluene-based liquid scintillation fluid containing 4 g per litre PPO and 50 mg per litre POPOP. The radioactivity was expressed in terms of cpm (counts per minute) per hydra.

(c) Continuous labelling

In studies regarding RNA synthesis in normal non-regenerating hydras, organisms without bud, testis or ovary were placed in the medium containing radioactive material
immediately after feeding and without amputations. The labelling continued for a period of 96 hours after feeding and the rate of incorporation was measured with an interval of 1 hour. Single hydramas from the experimental series were taken each time and the total RNA was extracted as described previously.

In regeneration studies, the hydramas were amputated at (a) subhypostomal level and (b) basal disc level immediately after placing them in the radioactive media. The amputations were done in separate organisms. Special care was taken in all the amputations so that the cuts were made in extended condition and in transverse plane with a single stroke at particular regions by a sharp and pointed sterilized needle. The labelling continued till 36 hours after amputations and the incorporation into RNA and proteins were measured separately at 1 hour intervals. The radioactivity expressed in terms of cpm per hydra was plotted against regeneration time in hours.

(d) Pulse-labelling

In pulse-labelling studies, the regenerating organisms were given labels for one hour duration successively till 32 hours after the amputations were done. The organisms were separately vivisected for hypostome and basal disc in normal culture medium itself and following each hour’s
regeneration, they were transferred to the respective radioactive medium. RNA and proteins were extracted from separate hydras after one hour of labelling and the radioactive activity was determined as described previously.

(e) Inhibition studies with actinomycin D

In order to determine the specific concentration of actinomycin D which completely inhibits the RNA synthesis in hydra, different experimental conditions were set up. The organisms were pretreated with various concentrations of the drug and the incorporation of $^3$H-uridine into RNA was measured. Actinomycin D was used at three different concentrations namely 100, 60 and 30 μg/ml of culture medium. Before labelling the animals were given pretreatment with the different concentrations of the drug for 6 and 12 hours separately. Soon after the pretreatment, the hydras were placed in the medium containing radioactive material and the hypostome and basal disc were removed. The labelling was done continuously for 6 hours and the incorporation was measured at an interval of 1 hour.

In studies regarding the requirement of RNA synthesis for bud morphogenesis, nonbudding hydras were preliminarily treated with 60, 30 and 15 μg/ml of actinomycin D separately for 12 hour duration, prior to the formation of buds.
(Bud formation generally takes place 24 hours after feeding). The medium containing actinomycin D was replaced by normal culture medium when bud rudiments were observed in the experimental animals. During the final hour of actinomycin D treatment the radioactive precursor ($^3$H-uridine) was administered into the medium to give a final concentration of 5 μCi/ml. The labelling was done for one hour and the radioactivity was measured subsequently.

In a third set of experiments, actinomycin D pretreated hydras were used for amino acid incorporation studies. The organisms were pretreated with the specific concentration of the drug which would inhibit the RNA synthesis completely. They were then quickly transferred to the medium containing radioactive isoleucine and the amputations were done at hypostomal and basal disc level in separate organisms. Proteins were extracted after one hour of the labelling and the radioactivity was measured as described previously.

(f) Studies on half-life of messenger RNA

The approximate half-life of messenger RNA in regenerating hydras were calculated by the rate of decay of protein synthesis after the inhibition of new DNA-dependent RNA synthesis. (If there is any activation of preformed templates of messenger RNA, protein synthesis would
continue to take place in spite of inhibition of transcription). Before labelling, the hydrames were pretreated with the specific concentration of actinomycin D for the particular duration of time, so that all the new RNA syntheses were stopped. Immediately after the pretreatments, the organisms were transferred to the radioactive medium containing $^{14}$C-isoleucine and the hypostome and basal disc were removed. The incorporation was measured with an interval of 1 hour and was allowed to continue till the organisms lost their morphology and disintegrated in the medium. The synthesis was considered to be nil when the radioactivity was undetectable in the experimenta animals. The activity was measured in separate hydrames during hypostome and basal disc regeneration.

(g) **Inhibition studies with cycloheximide**

In order to determine the specific concentration of cycloheximide which would inhibit protein synthesis in hydrames, the organisms were given pretreatment with various concentration of the drug and the incorporation of $^{14}$C-isoleucin into TCA precipitable material was measured. Normal non-regenerating hydrames were placed in 3 different concentration of drug namely 30, 50 and 75 µg/ml for a period of 12 hour before labelling began. Soon after pretreatments the organisms were transferred to medium containing radioactiv
material and the amputations were done at subhypostomal and basal disc levels. The incorporation was measured during the first hour of differentiation and the percentage of inhibition was calculated subsequently.

(h) Inhibition studies with cordycepin

As in the actinomycin D experiments, cordycepin was added to the culture medium in various concentrations and the inhibitory effect on $^3H$-uridine incorporation into RNA was measured. The different concentrations used were 50, 75 and 100 $\mu$g/ml of culture medium. Non-regenerating hydas were given pretreatment with the different concentrations of the drug for 6 and 12 hours following which they were transferred to the radioactive medium. Amputation was done at the basal disc level and the rate of inhibition was measured from the incorporation values obtained during the 1st hour of regeneration. The drug was used without a preincubation period also.

In order to study whether this transcriptional inhibitor affects the in vivo translation in hydra, cordycepin pretreated hydas were used for amino acid incorporation studies. The organisms were given pretreatment with the optimum concentration of the drug to suppress RNA synthesis following which they were given the label. The pretreated hydas were placed in the $^{14}C$-isoleucine containing culture
medium and the basal disc was vivisected. The regeneration was allowed to continue for 32 hours. Proteins were extracted at regular intervals of 1 hour duration and the incorporation was measured as described previously.

Particular attention has been given to basal disc regeneration only since cordycepin pretreated hydras regenerating for basal disc had been found to involve certain morphological abnormalities while differentiation was going on (Discussed vide infra).

(i) Analysis of proteins by polyacrylamide gel electrophoresis

Proteins from normal, regenerating and cordycepin treated hydras were isolated and analysed by polyacrylamide gel electrophoresis following the method described by Davis (1964). The stock buffer used was Tris/Glycine, pH 8.3. The stock buffer diluted ten times with distilled water was used as tray buffer.

In the first set of experiments, proteins were separated from normal non-regenerating hydras. 100 healthy hydras were taken from the homogeneous culture, washed several times with buffer and homogenized in 2 ml of Tris buffer at 4°C in an Elvehjem power-driven homogenizer. The homogenate was centrifuged twice at 4000 rpm for 20 minutes and the supernatant containing dissolved proteins was dialyzed overnight against tray buffer. A sample containing
approximately 100 µg of protein in a volume of 0.1 ml of 10 mM Tris/HCl, 5 mM 2-mercapto acetic acid, pH 8.0 was then mixed with 25 µ litres of 50% sucrose (w/v) and 5 µ litre of 0.1% bromophenol blue which was used as a marker. Using a micropipette with a suction tube and a mouthpiece the sample was applied to the top of the stacking gel carefully expressing the dense solution so as not to mix with the overlying reservoir buffer. When all the samples were applied, the electrophoresis was begun by applying a constant current of approximately 3.5 mA per gel with the anode at the bottom of the gels and the cathode at the top. Initially 2 mA current per tube was passed for 10 to 15 minutes for stacking the sample, after which it was raised to 3.5 mA per tube. Before electrophoresis 3 to 4 drops of 1% p-bromophenol blue was added to the buffer in the upper compartment as the tracking dye. The electrophoresis was terminated when the bromophenol blue marker came within ½ to 1 cm of the bottom of the gels. All the operations were carried out at 4°C.

Gels were removed in ice cold water with the help of a syringe and were kept immediately in cold 12% TCA for 1 hour to fix the proteins. They were then washed with distilled water and transferred to 1% aqueous coomassie blue for 2 hours for staining. Afterwards, the gels were destained for excess dye in a constant current of 7%
acetic acid till the bands were clearly visualized on the gels. The gels were finally scanned in a chromoscan (Joyce-Lobel 200) at 620 nm. In the same way proteins were analysed from hypostome and basal disc regenerating hydras after 24 hours of regeneration and in the case of cordycepin treated hydras, this was done after two intervals of basal disc regeneration, namely 12 and 24 hours.

(j) Isotopes and chemicals

The radioactive precursors, uridine, T(G), (Spec. Act. 2700 mCi/mmole) and L-isoleucine -C-14(U) (Spec. Act. 122 mCi/mmole) were purchased from Bhabha Atomic Research Centre, Bombay. Actinomycin D, cycloheximide, FFO, POPOP and db(dibutyryl) cAMP were obtained from Sigma Chemical Co., MO, USA and cordycepin as a gift. Millipore filters (type HAWP) were purchased from Millipore Corporation, MA, USA. Acrylamide, NN' Methylene Bis acrylamide, ammonium persulfate, NNNN' Tetramethylene diamine and Coomassie blue were from Bio-Rad Laboratories, CA., USA. Acrylamide and Bis acrylamide were purified by the method described by Loening (1967). A 70 g sample of acrylamide was dissolved in 1 litre of chloroform at 50°C. The solution was filtered hot without any suction. Crystals were obtained at -20°C and were recovered by filtration in a chilled filter funnel. The crystals were washed briefly with cold chloroform and dried. Bis-acryl-
amide was dissolved in acetone (approx. 10 g/l at 42-50°C) and filtered hot. The solution was slowly cooled to -20°C and the crystals were washed with cold acetone and recovered by filtration. The purified chemicals were stored at 4°C in a refrigerator.