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Fundamental properties of uridine metabolism in *Closterium acerosum*

By

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Penetration of ^{3}H-uridine into *Closterium acerosum* cells and incorporation of it to their nucleic acids were investigated. Optimum pH for uridine uptake was 7.5, and optimum concentration for uridine uptake was 2 to 50 nmoles per 2000 cells per mℓ of reaction medium. Optimum temperature for uptake was 25°C and for incorporations was 25° to 30°C both in light and in dark. At optimum concentration of external uridine, ratio of incorporation into nucleic acid to total uptake was about 40 to 60 per cent in light and dark. About 98 per cent of uridine penetrated into cells was incorporated into nucleic acid. Degree of uridine uptake by the cells decreased during incubation for a long period in dark, and during mating process.

The present work is a part of studies of relationship between nucleic acid metabolism and cellular differentiation of algae Zygnemataceae. There are many significant changes in metabolism during mating process of *Clamydomonas reinhardi*[1], *Spirogyra* sp.[2~7], and *Closterium stsigosum*. *Closterium acerosum*, a large cell size alga, may be useful for the elucidation of the physiological and biochemical mechanism of cellular differentiation and of fertilization in plants, because its sexual cell division and zygote formation are controlled by environmental conditions. Of green algae, *Clamydomonas reinhardi*[8] and *Chlorella fusca*[9,10] were examined on some features of uptake and incorporation of radioactive uracil.

The primary purpose of this study was to investigate the fundamental properties of nucleic acid metabolism of *Closterium acerosum* through studies of uptake of ^{3}H-uridine by the cells and of incorporation of uridine into nucleic acid under several physiological conditions.

Materials and Methods

Plant materials and culture methods

Closterium acerosum cells were synchronously cultivated by the application of repeated sequences of light (16 hr) at 3,000 lux and dark (8 hr) under continous aeration at 25°C. Vegetative growth and mating media

were made according to T. ICHIMURA[11]. Maximum growth occured after about 15 days in 200 mℓ of growth medium. Cells divided at the mid-time of the dark period. Growth rate was determined by changes of OD at 678 nm in a 10 mm light path. When OD_{678} was 0.3, cell number was 2,000 per mℓ (equivalent to 0.181 mg of dry weight of cells).

For initiation of mating process, cells were harvested at a logarithmic growth stage, washed with sterile mating medium three times, transfered on agar-mating medium, and then cultivated at 200 lux for 7 days at 25°C. Thenceforth they were cultivated at 3,000 lux at 20°C. After 4 days, sexual cell division and zygote formation were observed. About 90% zygote formation was observed at a time of termination of mating culture.

Light source was white lamp (Hitachi Instrument Co., Sunline-ēs, SL, White). Specrophotometer was Hitachi Instrument Co., Type 139.

Standard condition for uptake and incorporation of ^{3}H-uridine

Cells were harvested at 8 hr of light period in the logarithmic growth stage, washed with three times, then suspended in reaction medium. Washing and reaction media were sterile culture and mating media. Cells were incubated with ^{3}H-uridine, 0.4 nmoles/2000 cells per 1 mℓ of reaction medium, at 25°C for 10 or 20 minutes in light, 3,000 lux, or in dark, unless otherwise described. Specific activity was defined as radioactivity, cpm/1000 cells/10 minutes.

Assay of uptake of ^{3}H-uridine

After the incubation with ^{3}H-uridine, cells in an aliquot (0.5 mℓ) of the reaction medium were quickly collected on a 24 mm glass-fiber filter (Whatman GF/C). The cells were washed with about 20 mℓ of cold washing medium and about 5 mℓ of cold 95% ethanol for less than 1 minute to remove excess radioactive uridine, then dried and counted. The radioactivity measured is total amounts of uridine taken by the cells (TU) which consists of intracellular uridine and its low and high molecular weight metabolites.

Assay of incorporation of ^{3}H-uridine into macromolecular material

Another aliquot (0.5 mℓ) of the reaction medium was mixed with an equal volume of cold 10% TCA (containing 40 mg of unlabelled uridin/liter) and the cells were homogenized in a Teflon-glass homogenizer. After standing at 0–4°C for 2 hr, TCA-insoluble radioactive compounds were collected on a glass-fiber filter (Whatman GF/C), washed with about 20 mℓ of cold 5% TCA (containing 40 mg of unlabelled uridine/liter), then with

about 5 mℓ of cold 95% ethanol, dried and counted. The radioactivity measured is TCA-insoluble activity (TCAI) which consists of nucleic acid and protein. TU minus TCAI gives the radioactivity of TCA-soluble compounds (TCAS).

Assay of hot-TCA soluble radioactivity

After the incubation with ^{3}H-uridine, cells were homogenized in 5% TCA and heated at 90°C for 10 minutes. After cooling, the TCA-insoluble material was collected on a glass-fiber filter (Whatman GF/C), washed with cold 5% TCA, dried and counted. The radioactivity was the activity of hot-TCA-insoluble material (hot-TCAI). TCAI minus hot-TCAI gives the radioactivity of hot-TCA soluble material (hot-TCAS) which consists of nucleic acid.

Assay of alkaline-labile radioactivity

After the incubation with ^{3}H-uridine, cells were homogenized in 0.5 N KOH, and were heated at 30°C for 16 hr. After cooling, TCA was mixed to gives a final concentration of 5% at 0–4°C. TCA-insoluble material was collected on a glass-fiber filter (Whatman GF/C), washed, dried and counted. The radioactivity was the activity of alkaline-insoluble material (KOHI). The hot-TCAS minus KOHI gives the radioactivity of alkaline soluble (labile) material (KOHS), which consists of, mainly, RNA.

Whole experiments were repeated 2 or 3 lines of double or triple tests (deviation, less 5%).

Radioactivity was measured with a liquid scintillation counter (Beckman Instrument Co., Model CPM–100) in toluene-based scintillation liquid (4 g PPO and 0.4 g POPOP in 1 liter).

Chemicals

Tritium labelled uridine (5′-labelled, 5 Ci/mmole) was purchased from New England Nuclear Co.

Results

Optimum concentration of ^{3}H-uridine

Optimum concentration of ^{3}H-uridine for the uptake and incorporation was 2 nmoles per 2000 cells per 1 mℓ of reaction medium at 25°C, and half optimum concentration was 0.4 nmoles per 2000 cells (Fig. 1). ^{3}H-uridine was saturated in a range of 2 to 50 nmoles per 2000 cells. At an optimum concentration, the ratio of incorporation/uptake (TCAI/TU) was about 50% at a reaction time of 20 minutes, and at half optimum concentration, the

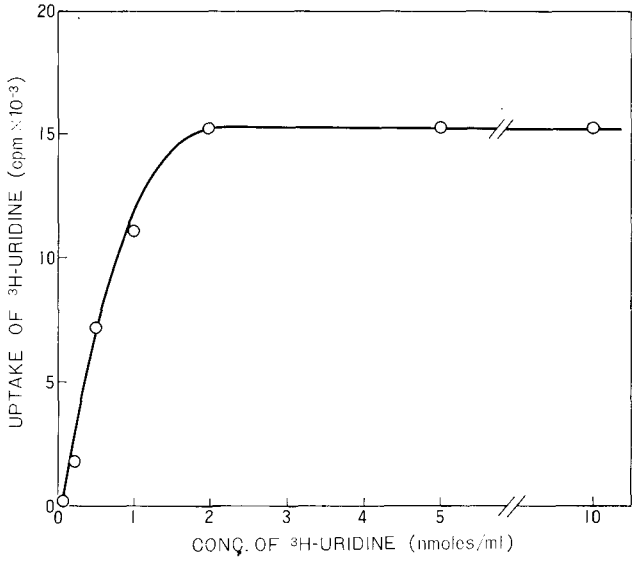

Fig. 1. Effects of concentration of ^{3}H-uridine on uptake. Cells were incubated with ^{3}H-uridine at 20°C for 20 minutes in light.

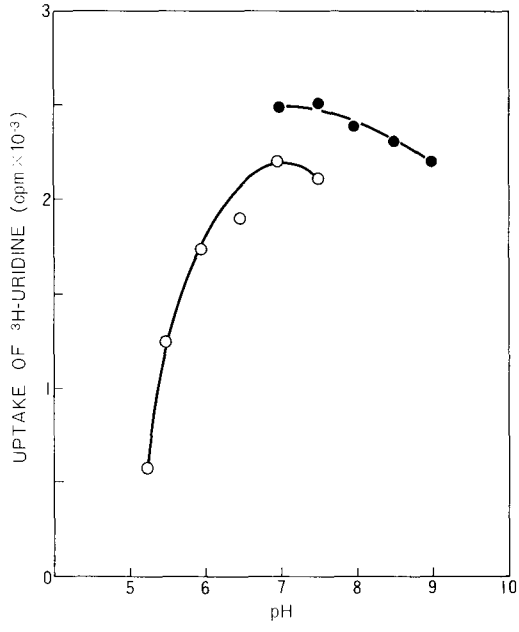

Fig. 2. Effects of pH on uptake of ^{3}H-uridine. Cells were incubated with ^{3}H-uridine in 0.02 M phosphate buffer (○——○) and 0.02 M Tris-HCl buffer (●——●) at 20°C for 10 minutes.

ratio was about 45%. These values were almost the same both in light and in dark.

In the presence of unlabelled uridine, labelling of cells decreased: the presence of molecular ratios of 10/1 and 100/1 inhibited the cell labelling by 60% and 90%, respectively, both in light and in dark. This inhibition may be dependent on a dilution effect of unlabelled uridine on labelled uridine.

Optimum pH

Optimum pH was 7.0–7.5 for uptake (Fig. 2) and for growth.

Optimum temperature

Optimum temperatures of uptake and incorporation were 25 °C and 25° to 30 °C, respectively, both in light and in dark (Fig. 3). Both activities were present at 0 °C and 5 °C. TCAI radioactivity decreased at a faster rate than that of TU at superoptimum temperature. No activities were seen at 50 °C.

Optimum light intensity

Uptake and incorporation increased gradually in a range of 200 to

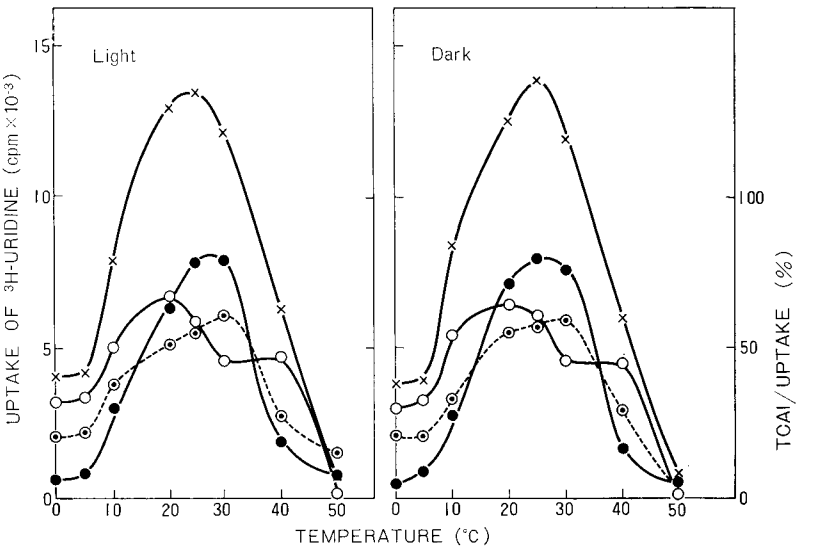

Fig. 3. Effects of temperature on uptake and incorporation of ^{3}H-uridine. After preincubation, cells were incubated with ^{3}H-uridine for 20 minutes. Total uptake (TU. ×——×), incorporation into TCA-insoluble (TCAI, ●——●), TCA-soluble (TCAS, ○——○), ratios of TCAI/TU (%) (◎——◎).

10,000 lux: at the high intensity of light, with a faster rate for longer reaction times than for shorter reaction times (Fig. 4 A). The ratios of incorporation/uptake (TCAI/TU) were lower in shorter reaction times than in longer reaction times: 20–25% in a range of 3,000 to 10,000 lux in 2

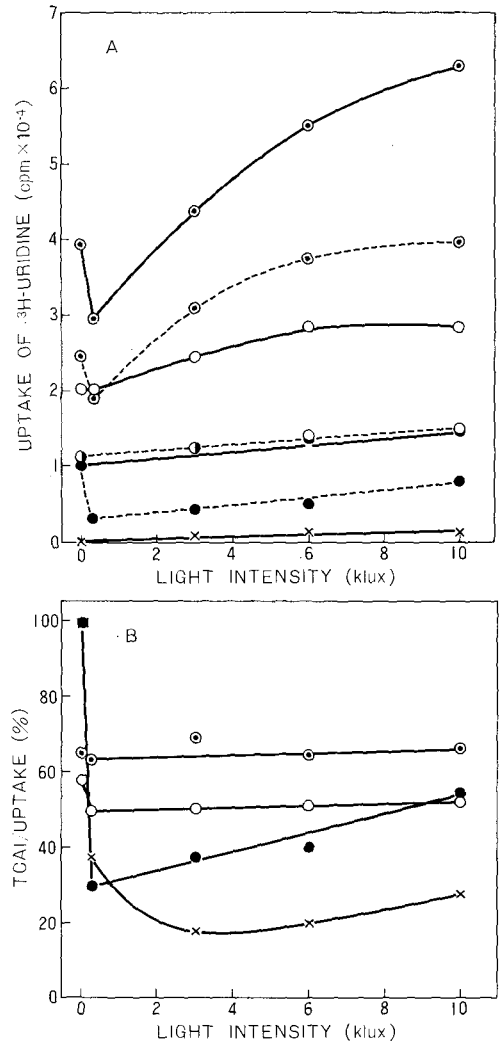

Fig. 4. Effects of light intensity on uptake and incorporation of ^{3}H-uridine. Cells were incubated with ^{3}H-uridine at 25°C. A, total uptake (TU, ——) and incorporation (TCAI, – – –) were measured at reaction time of 2 (×), 20 (●), 60 (○), and 120 minutes (◉). B, Ratios of TCAI/TU (%) were calculated at each of 2, 20, 60, and 120 minutes of reaction times.

minutes, 30 to 50% in a range of 200 to 10,000 lux in 20 minutes, and about 50 and 65% in a range of 200 to 10,000 lux in 60 and 120 minutes, respectively (Fig. 4 B).

Effects of reaction time

Time cources of uptake and incorporation were measured in light of 3,000 lux and in dark in a range of 0.5 to 60 minutes (Fig. 5). At an initial reaction time of 0 to 5 minutes, the activities were higher in darkness than in light, thereafter the activities increased gradually for 60 minutes with the same rate in light and in dark. The ratios of TCAI/TU were gradually increased from about 30% to 50% for 60 minutes.

Effects of preincubation for long periods in light and in dark

Cells were preincubated for 8, 24, or 72 hr in light (L-cells) and in dark (D-cells) at 25°C. Thereafter, the light conditions were exchanged from light to dark or dark to light. After 0, 10, 20, 30, or 60 minutes, uptake and incorporation of uridine were measured in light and in dark

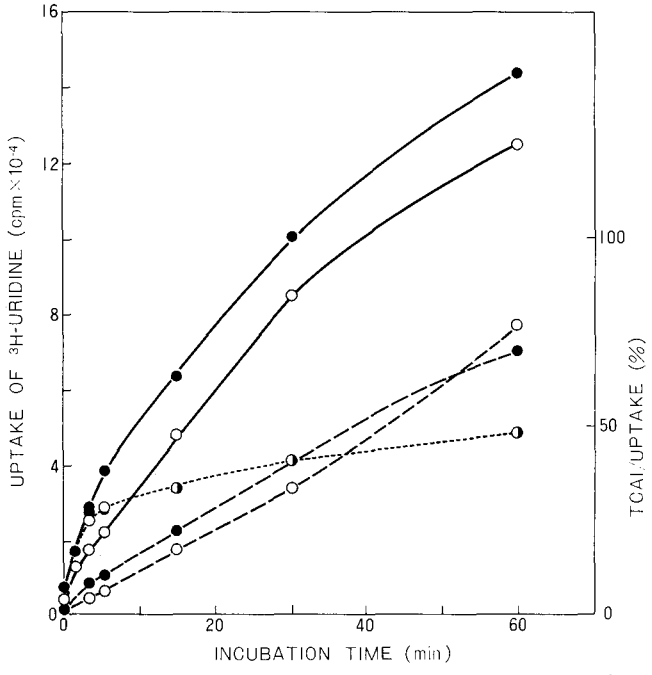

Fig. 5. Effects of reaction time on uptake and incorporation of ^{3}H-uridine. Cells were incubated with ^{3}H-uridine at 20°C. Total uptake (TU, ——) and incorporation (TCAI, – – –) were measured in light (○) and in dark (●).

for 10 minutes at 25°C: L-cells in light, Ll-cells; L-cells in dark, Ld-cells; D-cells in light, Dl-cells; D-cells in dark, Dd-cells (Table 1). The activity of L-cells was almost same in light and in dark (in dark, slightly higher than in light for short reaction time). The activity of D-cells (24 hr) was about one half of that of L-cells (24 hr) in dark and was almost same in light after 10 minutes.

Effects of culture age

Uptake and incorporation rates decreased gradually during aging of

TABLE 1. Effects of preincubation for long period in light and in dark on uptake and incorporation of ^{3}H-uridine

Preincubation for long period in (hr)	Exchange of light cond. Light (L) ⇌ Dark (D)	Time after exchange (min)	Activity (cpm/1000 cells/10 min) TU in Light	TU in Dark	TCAI in Light	TCAI in Dark
Light (8)	L→D	0	582[a)]	824	410	366
(L-cells)		60[b)]	868	912	614	620
		120[b)]	710	1032	603	568
(24)	L→D	0	741	1099		
(72)	L→D	0	931	934		
Dark (24)	D→L	0	1068	603[d)]	1034	460
(D-cells)		10[c)]	1425	1432	860	408
		60[c)]	1920		1213	
		120[c)]	1624	1767	913	874
(72)	D→L	0	1036	490[d)]		

Cells were preincubated without ^{3}H-uridine for 8, 24, and 72 hr at 25°C in light (L-cells) and in dark (D-cells). Thereafter, light condition was exchanged from light to dark (L→D) or dark to light (D→L). After 0, 10, 60, and 120 minutes of the exchange, cells were incubated with ^{3}H-uridine for 10 minutes at 25°C both in light and in dark. a) Ll-cells; b) Ld-cells; c) Dl-cells; d) Dd-cells.

TABLE 2. Effects of culture age on uptake and incorporation of ^{3}H-uridine

Culture age (Days)	Growth (Number of cells/mℓ)	Radioactivity (cpm/cell/10 min) TU	TCAI	Ratio (%) TCAI/TU
1	567	34.8	22.2	61
4	1500	18.8	11.8	59
7	1900	15.4	7.0	47
10	2667	11.4	6.3	56
14	3733	20.2	4.9	24

TU and TCAI radioactivities were almost same in light and in dark in the standard reaction condition.

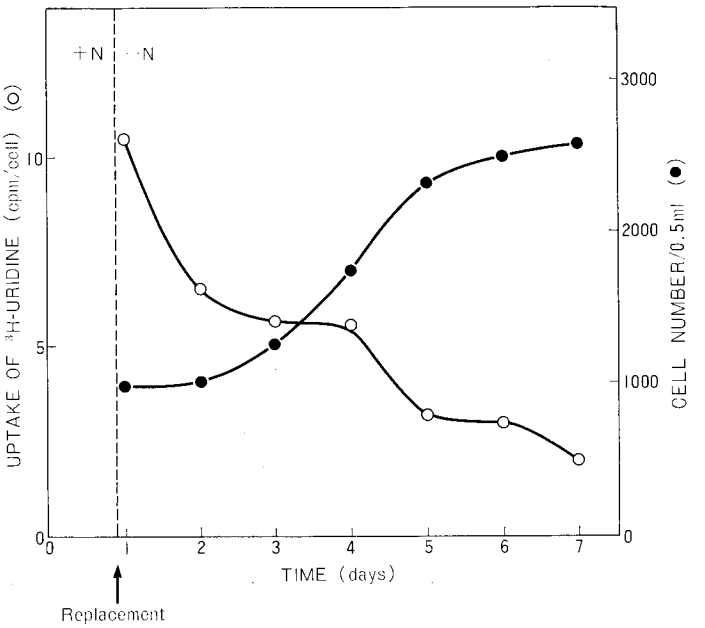

Fig. 6. Effects of mating process on uptake of ^{3}H-uridine. Cells were collected from agar mating cultures. Thereafter, cells were incubated with ^{3}H-uridine at 25°C for 30 minutes in dark. Cell numbers (●——●) and total uptake (TU, ○——○) were measured. Arrow indicates the time of transfer of cells from culture medium to agar mating medium.

TABLE 3. Incorporation of ^{3}H-uridine into cellular fractions

Fraction	Activity (cpm/1000 cells/10 min)
Cells (TU)	18035
Cold TCA-insoluble (TCAI)	10705
Cold TCA-soluble (TCAS) (TU-TCAI)	7330
Hot TCA-insoluble (hot TCAI)	221
Hot TCA-soluble (hot TCAS) (TCAI-hot TCAI)	10384
Alkaline-insoluble (KOHI)	103
Alkaline-soluble (KOHS) (hot TCAS-KOHI)	10281

Radioactivities of TU, TCAI, hot TCAI, and KOHI were measured. Thereafter, radioactivities of TCAS, hot TCAS, and KOHS were culculated.

culture, and incorporation was more rapidly decreased than uptake (Table 2).

Changes in mating process

Uptake of ^{3}H-uridine decreased during sexual cell division and zygote formation (Fig. 6).

Chemical composition of TCA-insoluble materail

About 98% of radioactivity of cold TCA-insoluble material was hot TCA-soluble, and about 99% of hot TCA-soluble material was alkaline-soluble (labile) (Table 3).

Discussion

When the cells harvested at 8 hr of light period were used, a concentration for maximum uptake of ^{3}H-uridine was 2 nmoles per 2000 cells per 1 mℓ of reaction medium at 25°C for 20 minutes of reaction time (Fig. 1). Therefore, it seems that one cell is saturated with external uridine at a concentration of 1 pmole.

The ratio of incorporation/uptake (TCAI/TU) was about 50% in a range of 200 to 10,000 lux at a reaction time of 60 minutes and about 60% at a reaction time of 120 minutes (Fig. 4). This finding suggests that about one half of uridine penetrated into cells is used for synthesis of TCA-insoluble macromolecules.

About 98% of cold-TCA insoluble radioactivity was hot TCA soluble, and about 99% of hot TCA soluble radioactivity was alkaline soluble (Table 1). This data suggests that about 98% of radioactive macromolecules synthesized from uridine consists of RNA.

Optimum temperature for uptake of uridine was 25°C and for incorporation into acid-insoluble material was 25° to 30°C (Fig. 3). The activity of incorporation decreased at a faster rate than that of uptake at super-optimum temperature. Therefore, *in vivo* RNA synthesis from external uridine seems to be more sensitive to low and hight temperature than penetration of uridine into the cells.

TU and TCAI radioactivities were almost the same in light and in dark for 120 minutes of reaction times (Fig. 3, 5). When the cells were preincubated in dark (D-celld) or in Light (L-cells) for longer periods more than 24 hr, the activities of D-cells in dark were about one half of those of L-cells in dark (Table 1), and the activities of L-cells in dark were almost same in light. The activities of D-cells were stimulated by illumination within 10 minutes at the same level of L-cells in light. These findings

suggest that penetration of uridine and RNA synthesis are partially dependent on photochemical reaction and the activities are maintained for, at least, 120 minutes at a maximnm level in darkness, and that certain substance(s) requisite for maximum activities for penetration of uridine and RNA synthesis is formed in light.

TU and TCAI radioactivities decreased gradually during mating process (Fig. 6). Gametogenesis and zygote formation of *Closterium* were found to be dependent on light[11] in a nitrogen source-deficient mating medium (11, and personal communication). It was reported that RNA synthesis was modified during conjugation of *Spirogyra*[2,4,5,7], and during mating process of *Clamydomonas*[1]. These findings suggest that light is an important factor for induction of sexual cell division, gametogenesis, and for maturation of zygote, and that light has a role in regulation of permeability and *in vivo* RNA synthesis during the life cycle of algae.

At the present time, studies to elucidate the relationship between RNA metabolism and morphorogical changes in life cycle of *Closterium acerosum* are in progress.

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