each nutrient regime. Details of the extraction procedure have been given in a previous communication[5]. Total chlorophyll, chlorophyll a and chlorophyll b were determined by the method of Vernon[6]. Total protochlorophyll (assuming no conversion to chlorophyll), and the amount of protochlorophyll transformed to chlorophyll were estimated by the method of Smith and Benitez[7]. From the values thus obtained, the rates of transformation of protochlorophyll to chlorophyll were calculated.

Results and discussion. Figure 1 shows the time-course of formation of protochlorophyll in seedlings grown under potassium deficiency and full nutrient conditions. During the first 10 days of starvation, potassium deficiency did not affect the concentration of protochlorophyll in the maize seedlings. Beyond this point the deficiency resulted in a drastic decrease in the concentration of total protochlorophyll. A similar pattern of effect was observed for chlorophylls a and b.

Seedlings maintained under potassium deficiency showed initial rates of conversion of protochlorophyll to chlorophyll that were approximately equal to the rates recorded for control seedlings (figure 2). The rates increased under both nutrient regimes during the first 10 days of feeding with nutrient solutions and thereafter decreased. This decrease was more pronounced in the potassium-deficient seedlings than in the control seedlings.

It can thus be concluded that the decrease in chlorophyll formation during potassium deficiency is due, at least in part, to a decreased formation of protochlorophyll and a decreased rate of transformation of protochlorophyll to chlorophyll. Bogorad[8] suggested that in plants chlorosis is generally due to a block in porphyrin formation at a very early point in the biosynthetic chain. Potassium deficiency-induced chlorosis would seem to be due to an additional block later in the chain.

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Increased RNA synthesis during preconjugation and its effect on pair formation in Tetrahymena[1]

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Summary. Increased RNA synthesis, mainly mRNA, occurs in Tetrahymena very shortly before the pairing which takes place after conjugation has been induced. Specific inhibition of mRNA synthesis by cordycepin delays pairing.

The sexual phase in the ciliated protozoan Tetrahymena is an inducible developmental process. Formation of a cell pair between 2 mating-types requires several steps: 1. Deprivation of nutrients. 2. A specific cell-cell interaction. 3. Fusion of cell surfaces of both mating-types. The actual pairing is preceded by a process which makes possible the cellular recognition and attachment. This process involves at least 2 major stages: initiation and costimulation[2]. Initiation is induced by starvation in buffers such as 10 mM Tris or 50 mM Tricine for 2 or more h (depending on the mating types used at 28–30 °C) and is independent of the presence of the complementary mating-types[2,3]. After mixing mating types which were previously initiated, there is a lag period of 45–60 min until the first pairs of conjugates are formed. Obviously, therefore, during this lag period some form of communication is needed between cells of complementary mating types[3,4]. Since the aforementioned processes of initiation, costimulation and fusion occur sequentially[2], it is possible to examine each step separately. In order to understand more about the macromolecular events during costimulation, we have investigated the pattern of RNA synthesis in relation to this period of conjugation in Tetrahymena. In addition, the effect of cordycepin, an RNA synthesis inhibitor, was examined on RNA synthesis as well as on pair formation.

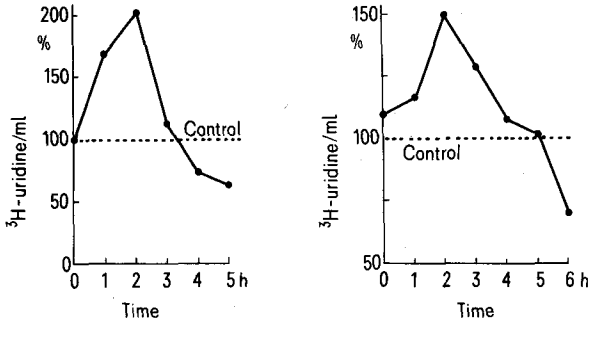

Fig. 1. RNA synthesis during costimulation and pair formation during the process of conjugation. After mixing starved cultures of mating types, 10 μC/ml ^{3}H-uridine was added at 1-h-intervals for 10 min. The radioactivity was determined by precipitation with cold 10% TCA. Solid line: RNA synthesis during costimulation and pair formation of conjugating Tetrahymena. The curves were calculated and drawn relative to the straight control line. Dotted straight line: RNA synthesis in starved controls. Each starved mating type alone served as a control. The dotted line represents the calculated mean of both mating types. Left curve: ^{3}H-uridine incorporation into phenol-chloroform extracted RNA. Right curve: ^{3}H-uridine incorporation into entire Tetrahymena cells.

Materials and methods. Cell cultures. Tetrahymena pyriformis mating types WH_6 and WH_{52} of syngen I (obtained from the American Type Culture Collection) were maintained separately in 2% proteose peptone (Difco) at 28 °C. Cells used in the experiments were obtained by inoculating 100 ml of a medium containing 0.5% proteose peptone, 0.5% Bacto tryptone, 0.01% yeast extract, 0.1%

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sodium acetate and 0.1% K_2HPO_4 at pH 7.2. After 2 days of growth at 28 °C, the cells of each mating type were removed and utilized during their exponential growth phase.

Shiftdown. Cells were concentrated for shift-down by centrifugation with a GLC-2 (Sorvall) centrifuge at 1000 rpm for 1–2 min. Each mating type was washed separately 3 times in 0.05 M tricine buffer (pH 7.2) and resuspended to a concentration of $4–5 \times 10^5$ cells/ml, and maintained for about 24 h under starvation.

Conjugation. The mating reaction was induced by mixing 1 ml of an equal concentration of each mating type in 15-ml Erlenmeyer flasks which were then incubated at 30 °C in a water bath. Conjugating organisms were fixed by adding glutaraldehyde to them (final concentration: 1.5%). Samples were counted at 1-h-intervals with a Neubauer Hemacytometer and the percentage of conjugating cells was calculated. A minimum of 400 cells were counted in each sample.

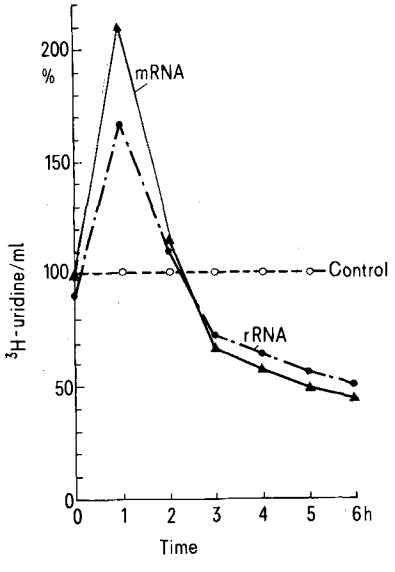

Fig. 2. Incorporation of ^{3}H-uridine into RNA during costimulation and pair formation of conjugating Tetrahymena. After phenol-chloroform extraction, mRNA was isolated on poly-U-sepharose columns. rRNA represents phenol-chloroform extracted RNA which could not be isolated on poly-U-sepharose.

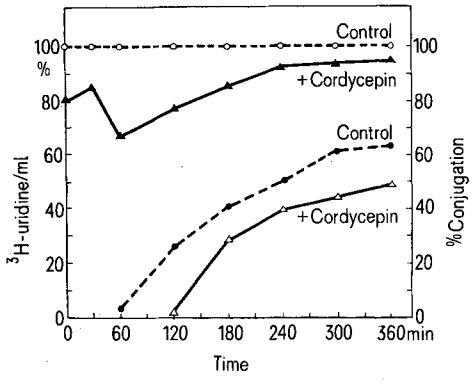

Fig. 3. The effect of cordycepin on the rate of pair formation and RNA synthesis in conjugating Tetrahymena. Cordycepin was added at the initial mixing time of the mating types. The 2 upper lines represent the inhibitory effects of cordycepin on RNA synthesis as compared to a control without cordycepin. The 2 lower lines represent the inhibitory effects of cordycepin on pair formation, as compared to a control to which no cordycepin was added. The rate of pair formation during conjugation was determined analyzing a minimum of 400 cells per count.

Cell labelling. Cells were labelled with 10 μC/ml of ^{3}H-uridine (specific activity 25 Ci/mmole, Amersham, England). RNA synthesis was measured by the incorporation of ^{3}H-uridine into whole cells, following precipitation with cold 10% TCA, or by the extraction of RNA with phenol-chloroform as described elsewhere[5].

Fractionation of RNA on poly-(U)-sepharose. Samples of phenochloroform extracted RNA were put on poly-(U)-sepharose columns and fractionated as described elsewhere[5]. The radioactivity of each fraction was determined in the TCA precipitant. Cordycepin (3′ deoxyadenosine) (Sigma Chemical Co.) was made into a stock solution of 500 μg/ml in buffer.

In preliminary dose tolerance tests, a dose of cordycepin as high as 100 μg/ml had no effect of the survival or morphology of the organisms even after 48 h of treatment. Since concentrations of 50 μg/ml and 100 μg/ml yielded the same effect, we chose to use the lower dose. Each of the starved mating type cultures, tested separately, served as a control.

Results. RNA synthesis during the induction of conjugation in Tetrahymena. When complementary mating types of starved Tetrahymena were mixed for the induction of conjugation, under our experimental conditions, there was a lag period of about 1.5 h until the first conjugating pairs were recorded. When RNA synthesis was measured from the initial mixing time of the mating types for a period of 4–5 h thereafter, an increase in the rate of RNA synthesis was observed. The maximal RNA synthesis stimulation was measured after 1–2 h from the initial mixing time (figures 1 and 2). At the end of this 2-h-period, the first conjugating pairs could be observed. 4–5 h after the initial mixing of the mating types, the rate of conjugation reached 50–60% of the total population and RNA synthesis decreased to the level of the control cultures (figure 1). The total amount of RNA, synthesized during the first 6 h after the initial mixing, was extracted and fractionated on poly-(U)-sepharose columns. The results showed that the syntheses of mRNA and rRNA were both stimulated, particularly the former. The result of these experiments are plotted in figure 2.

The inhibition of RNA synthesis by cordycepin. The above results suggest that RNA synthesis might be essential for the process of conjugation and especially during the initiation phase. Therefore, the inhibition of RNA synthesis during the first 2 h from the initial mixing time of the mating-types might also inhibit or delay conjugation. In order to investigate this point, a series of experiments was performed to determine the effect of cordycepin, a RNA synthesis inhibitor, on conjugation.

Figure 3 demonstrates the effect of 50 μg/ml cordycepin on the conjugation and RNA synthesis of Tetrahymena cultures when the inhibitor was added to the experimental cultures at the mixing time of the mating types. When the rate of conjugation was determined, there was a 1 h delay in the pairing process of conjugation following the cordycepin treatment. 4 h after adding the drug, there was no significant difference between cordycepin-treated and non-treated cultures in the amount of RNA synthesis. RNA synthesis measurements following cordycepin treatment revealed a decrease of 35–40% in ^{3}H-uridine incorporation after the first hour from the initial mixing time (figure 3). Our impression was that after 3–4 h the organisms recovered from the effect of the drug, and step-

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wise recovered from its inhibitory effect on RNA synthesis. In order to study the 'recovery' period more systematically, we performed additional experiments in which cordycepin was added at different intervals after the initial mixing time of the mating-types.

Results showed that the time of application of cordycepin did not effect the length of the lag period, which remained 1–1.5 h, but did effect the rate of conjugation. When cordycepin was added immediately after the initial mixing time of the mating-types, or within 1 h thereafter, there was a significant delay in the rate of pair formation. The addition of the drug later than 2 h from the initial mixing point of the mating-types had little effect on the rate of pair formation (figure 4). RNA synthesis in cells treated with cordycepin at the initial mixing time of the mating-types decreased by 60–80% after the first h. Application of cordycepin after 2–3 h from the initial mixing point of the mating-types decreased the level of RNA

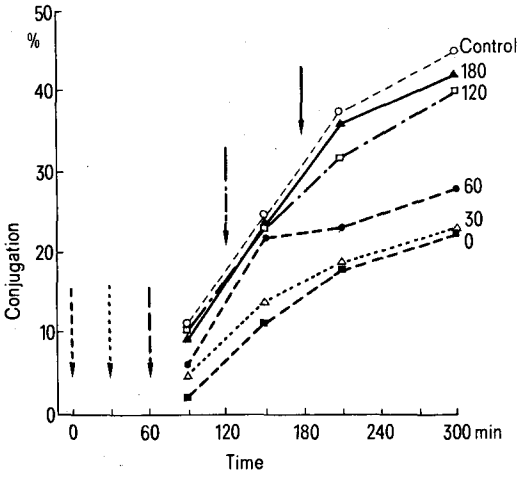

Fig. 4. The effect of cordycepin on pair formation in conjugating Tetrahymena. Cordycepin was added at 1-h-intervals, as indicated by arrows. The consequent pair formation was drawn with the same lines as its representing arrow.

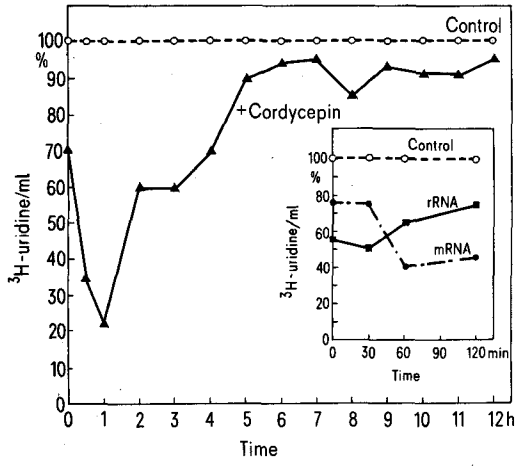

Fig. 5. The effect of cordycepin on RNA synthesis during 12 h of conjugation. The initial mixing time of the mating types is represented by the '0' point. Corycepin was added at 1-h-intervals. The incorporation of ^{3}H-uridine was measured by precipitation with cold 10% TCA. The inset represents the comparison of the effects of cordycepin on mRNA and rRNA synthesis during 2 h from the initial mixing point of the mating types. mRNA and rRNA were determined as described in figure 2.

synthesis by 40%. The addition of cordycepin later than 5 h after the mixing time of the mating-types decreased the RNA synthesis rate only by 10% or less (figure 5).

The effect of cordycepin on mRNA and rRNA synthesis was tested by the fractionation of such RNA on poly-(U)-sepharose. As demonstrated in the inset of figure 5, there was a slight difference in the effect of the drug between mRNA and rRNA synthesis; however its statistical significance was not tested.

Discussion. In this study we demonstrated the synthesis of increased amounts of mRNA during conjugation in Tetrahymena (figure 2) by a method which isolates poly-A fragments[5] of newly synthesized RNA. The isolation of poly-A-fragments by affinity chromatography on poly-U-sepharose has a powerful tool for mRNA isolation in eukaryores[6].

We believe that mRNA initiates specific protein synthesis during costimulation or pair formation in conjugating Tetrahymena. If such synthesis is inhibited by cordycepin, a delay in pair formation follows. However, if cordycepin is added following the completion of this mRNA synthesis, pair formation occurs in the normal fashion (figure 4). Frisch et al.[7] have recently demonstrated the inhibition of glycoprotein synthesis, the latter probably essential for conjugation, by tunicamycin, during costimulation and pair formation of Tetrahymena. The same investigators have shown the presence of specific proteins occurring during conjugation, by slab-gel electrophoretic combined with radioautographic methods (personal communication, A. Frisch). The linear sequence of events such as specific mRNA synthesis, as reported here, and specific protein synthesis during early stages of conjugation suggest a possible linkage between such synthetic processes. However, actual connection has not been demonstrated.

The requirement of protein synthesis for costimulation in conjugating Tetrahymena was reported before by P. J. Bruns and R. E. Palestine[8]. Tyler and Wolfe[9] showed that Actinomycin D inhibited 95% of RNA synthesis in Tetrahymena. A similar inhibition of protein synthesis was caused by cycloheximide[10]. The latter also inhibited completely the conjugation process in Tetrahymena. However Actinomycin D only inhibited conjugation if the drug was added at the initial mixing time of the mating types[10]. By adding the drug 1 h after the initial mixing time of the mating types, it did not block further conjugation. Upon these results Allewell et al. suggested 'that in Tetrahymena there is a need for RNA synthesis during costimulation of conjugation but not during pair formation'.

Such suggested RNA synthesis indeed materialized as demonstrated in this study. Figures 1 and 2 demonstrate the stimulation of RNA synthesis in Tetrahymena from the initial mixing time of the mating types for a period of 2–3 h. In an abstract McDonald[11] demonstrated continuous RNA synthesis during the conjugation of Tetrahymena. As we have not been able to obtain further information concerning this investigation, we are unable to explain the discrepancy between her results and ours. It is possible that the explanation lies in the different techniques employed. To our knowledge, information on RNA

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synthesis during conjugation in other ciliates is only available from the detailed studies by Sapra and Ammermann with Stylonychia[12,13]. These investigators measured the incorporation of RNA precursors into RNA of conjugating Stylonychia as well as RNA inhibition by Actinomycin D.

They concluded that: a) RNA synthesis during the first 5 h after pair formation was substantially lower in comparison to their vegetative state. b) 1 h later, i.e. between 5–6 h since pairing, they noticed a marked increase in RNA synthesis. c) 6 h since pairing and thereafter, little, if any, RNA synthesis could be observed. In addition the inhibition of RNA synthesis by Actinomycin D during 5–6 h since initial pairing, resulted in the blockage of further developmental processes in conjugation.

In the ciliates Stylonychia and Tetrahymena, a short duration of about 1 h of critical mRNA synthesis seems to govern the continuation of sexual reproduction. However this critical mRNA synthesis in both ciliates occurs at different periods of the conjugation process. In Tetrahymena such mRNA synthesis occurs during costimulation, i.e. before pairing; in Stylonychia it occurs after about 5 h postpairing. However these different timings of long acting mRNAs do not cause a principle difference in their mode of action.

In Stynlonychia as well as Tetrahymena, it seems that the 'sequence of events dependent upon presynthesized messengers include, in a strict chronological order, meiosis of the micronucleus, formation exchange, and fertilization of the gametic nuclei, and differentiation of the new macronucleus from the zygote'[13].

As previously mentioned, little has been published on RNA synthesis in other conjugating ciliates. Rao[14] and Berger[15] concluded that RNA synthesis occurs continuously during the course of macronuclear development in conjugating Euplotes and Paramecium, respectively. However Nobili[16] presumed that 'synthesis of probably specific RNA occurs any time sexually mature paramecia become reactive'. The fact that Tetrahymena cells appear to overcome the inhibitory effects of cordycepin after several hours following experimental treatment (figure 3) is mirrored by their reaction to certain antimitotic drugs. Such a phenomenon was shown by Wunderlich and Peyk[17] while testing the effect of colchicine and colcemid on vegetative division of Tetrahymena, and by Roberts and Orias[18] by testing protein synthesis inhibitors.

The systems involved in overcoming such inhibitory effects are as yet little understood. Perhaps Tetrahymena possesses an enzyme system capable of decomposition of these drugs, including cordycepin.

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Leucocyte invasion of the vaginal epithelium in the absence of bacteria in mice

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Summary. Influx of leucocytes in the vagina at metoestrus occurs in germfree mice and also in sterile isotransplants of vaginal tissue in conventional mice. In contrast to the situation in rats, bacteria thus do not seem to be required for the production of postovulatory leucotactic stimuli in the vagina.

Influx of leucocytes into the vaginal epithelium and lumen is a wellknown characteristic of the early postovulatory period (metoestrus) in small rodents. In rats, this influx appears to depend on the presence of bacteria in the vaginal lumen[3,4]. No data are available on the microbial-leucocytic relationship at metoestrus in the other rodents. However, the occurrence of a leucocytic vaginal exudate in germfree mice has been reported after repeated injections of progesterone[5]. This suggests that, in mice, leucocytic influx of the vagina at metoestrus does not depend on the presence of microorganisms in the vagina and that, thus, species differences exist in the mechanism underlying influx of leucocytes into the vaginal epithelium after ovulation. The data on mice, however, were obtained exclusively after injection of progesterone, and not during the normal ovarian cycle. We therefore examined the possible relationship between bacteria and leucocytes with the same methods as used in rats[3,4].

Materials and methods. Experiment 1. The numbers of aerobic and facultative anaerobic bacteria were determined in Swiss (9 animals) and ND_2/Rij (8 animals) cyclic mice. Vaginas with the cervix still attached were removed and placed in bottles with 10 ml saline and 3 g glassbeads (⌀ 3 mm) which were sterilized before use. The bacteria in the vaginas were suspended by shaking the bottles on a whirlmixer for 2 min. From the suspensions, 1:10 dilution series were made in nutrient broth, which were incubated for 48 h at 37 °C. The stages of the ovarian cycle, at which the vaginas were obtained, were approximated by examining the contents of the Fallopian tubes microscopically (fresh oocytes with surrounding cumulus-cells: day of ovulation; old oocytes without surrounding cumulus-cells: day after ovulation; no oocytes but clearly visible corpora lutea in the ovaries: all other stages of the ovarian cycle).

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